Bacteriophages of *Pseudomonas* bacteria: application in medicine and agriculture

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Bacteriophages represent small infectious agents that invade bacterial cells. Like other viruses, bacteriophage particle or virion consists of the nucleic acid (dsDNA, ssDNA, dsRNA or ssRNA) surrounded by protein or lipoprotein coat. The International Committee on the Taxonomy of Viruses (ICTV) traditionally uses virion morphology (tail type, polyhedral, filamentous and pleomorphic) and nucleic acid composition to classify isolated phages. The order *Caudovirales* that includes tailed, dsDNA phages with partial and limited hierarchical structure contains the vast majority of *Pseudomonas* phages (>97%). Only a small fraction (less than 3%) have a polyhedral, filamentous or pleomorphic morphology. Phage infection begins with adsorption to specific receptors on the surface of host cell. Rate of adsorption is determined by physical-chemical factors, host physiological state and cultural conditions. Then nucleic acid is injected into the cell. Depending on cycles of viral reproduction, either nucleic acid is used to synthesize viral components assembling into complete viruses, followed by lysis of the cell, or nucleic acid integrates with host DNA and stays in a dormant state as a prophage for extended periods of time. On the other hand, bacteria resort to various ways of protection against phages at different stages of infection. *Pseudomonas* species also possess some defence mechanisms. These mechanisms may include formation of biofilm, loss or mutation of receptors, presence of the CRISPR–Cas and restriction–modification systems. Bacterial strains often contain multiple antiphage barriers. In turn presence of these antiphage systems leads to selection of phages that have adapted to bacterial defence. Constantly increasing number of antibiotic-resistant bacteria forces to seek alternative antimicrobial treatments such as bacteriophages for use in medicine and agriculture. Phage applications set certain criteria determining practical interest in these viruses: 1) the chosen phages are truly virulent (killing bacteria under all conditions); 2) phages should display high efficiency against pathogenic bacteria; 3) bacteriophages must be safe for use, etc. Important technological properties of phages are represented by main following characteristics: 1) phages target only concrete species and strains and don't affect beneficial bacteria; 2) bacteriophages multiply at the infection site and maintain thereby high number of viral particles; 3) they can adapt to resistant bacteria, etc. As mentioned above, phages must provide safety that is sometimes hard to guarantee. It is not always possible to find appropriate non-pathogenic variants of bacteria for phage reproduction. Phages also may carry virulence factor or toxin genes and transfer such genes into clinical strains with the resulting pathogenicity. In medicine direct introduction of even highly purified phage particles into blood is risky. Therefore phage can be used only in cases providing direct contact between the virus and bacteria. Nevertheless, phage selection studies are going on. *P. aeruginosa* that causes disease in animals and humans is one of the most studied objects of phage therapy. The effectiveness of phage treatment for therapy of *P. aeruginosa* infections in mice, guinea pigs and dogs was demonstrated. Other experiments revealed that bacteriophages of *P. aeruginosa* can be used in medical treatment of burns, chronic otitis, keratitis, sepsis and bacterial lung infection of patients with cystic fibrosis. Researches concerning phage therapy of plants are relatively few, but studies in this field continue. Some treatments already have been developed such as AgriPhage for the control of pathogenic bacteria including *P. syringae* pv. *tomato*, which is the causative agent of bacterial speck on tomatoes. The review summarizes current knowledge concerning *Pseudomonas* phages, their application in medicine and agriculture.

Keywords: bacteriophages; *Pseudomonas*; phage infection; phage therapy

1. Introduction

Bacteriophages represent small infectious agents that invade bacterial cells and can be found everywhere disseminating their genome from one host cell to another. Phages display two phases in their life cycle: outside and within the cells. Unlike the cells, phages have no machinery for generating energy and no ribosomes for protein synthesis and therefore they can be reproduced only in host cells. Their ability to affect only specific bacteria makes phages an object for practical interest, especially during latest decades.

First antiseptic activity against many kinds of bacteria and against the cholera pathogen in particular was reported in 1896 by Hankin in the river water of India [1]. Unfortunately, his investigations were not resumed. The viruses of bacteria were discovered in 1915 and 1917 independently by Frederick Twort and Felix d'Herelle, respectively [2, 3]. Following these studies phages have been considered as promising antibacterial agents in medicine. However, lack of knowledge on basic phage biology and their molecular organization, confused therapeutic results, discovery and widespread recognition of broad-spectrum antibiotics in the late 1930s led to the decline of phage therapy in the West [4]. On the contrary, phage therapy continued to be studied and practiced in Eastern Europe and the Soviet Union. In the recent years keen interest in phages has revived due to emergence of multidrug resistant bacteria.

Genus *Pseudomonas* includes bacteria inhabiting a wide range of biological niches because they may readily adapt to diverse environmental conditions. Besides soil or water occupied by pseudomonades, this genus can also be represented

by pathogenic species of animals (*P. aeruginosa*, *P. plecoglossicida*) and plants (*P. syringae*). Large-scale application of antibiotics provoked appearance of multidrug resistant bacteria, including *Pseudomonas*. As a result pathogenic bacteria have turned into intractable object to remove. Introduction of bacteriophages seems an alternative way of fighting against pathogenic *Pseudomonas* without affecting beneficial bacteria and host organism. Phages can be used in clinical medicine and industrial agriculture.

Physiology of *Pseudomonas* bacteriophages, their ability to penetrate into the cell, bacterial defence mechanisms and phage strategies to avoid them, applications of phages in medicine and agriculture, criteria for their use will be discussed in this article.

2. Physiology of bacteriophages of *Pseudomonas* bacteria

Like other viruses, bacteriophage particle or virion consists of nucleic acid surrounded by protein or lipoprotein coat. The nucleic acid component comprises either RNA or DNA, but not both, with DNA being more common. It can be single-stranded or double-stranded. The latter type prevails in most phages. Nucleic acid also can be multisegmented, particularly tripartite, or monopartite for the vast majority of phage genomes. The outside portion of virion is capsid composed of multiple units (capsomeres). Some viruses contain capsid with outer lipid layer (enveloped phages) [5].

The International Committee on the Taxonomy of Viruses (ICTV) traditionally uses virion morphology (tail type, polyhedral, filamentous and pleomorphic) and nucleic acid composition to classify isolated phages. To date it has been examined 546 phages infecting the genus *Pseudomonas* among 6196 bacterial viruses. The order *Caudovirales* embraces tailed dsDNA phages with icosahedral heads or prolate capsids that have additional rows of subunits in their central parts. This order contains the vast majority of *Pseudomonas* phages (97.8%). Tailed phages are divided into three families depending on their tail morphology. Family *Myoviridae* have a long contractile tail, *Siphoviridae* have a long noncontractile tail and *Podoviridae* have a short noncontractile tail. Nearly half of *Caudovirales* is represented by family *Siphoviridae* (249 phages), the other half is represented by *Myoviridae* and *Podoviridae* (156 and 129 phages, respectively). The tail serves both as a signal transmitter and subsequently as a pipeline through which DNA is delivered into the host cell during infection. All types of tails have outer appendages attached to the distant end of the tail and often include a baseplate with several fibres and a tip or a needle showing specificity to the membrane receptors of the bacterium. The baseplate and tail fibres are involved in the irreversible attachment of the phage to the bacterial outer membrane.

A small fraction of *Pseudomonas* phages (2.2%) has a polyhedral, filamentous or pleomorphic morphology (PFP phages). PFP viruses are not grouped into order, like *Caudovirales*. Only 12 *Pseudomonas* viruses belong to PFP phages (3 phages of family *Cystoviridae*, 3 phages of family *Inoviridae*, 4 phages of family *Leviviridae* and 2 phages remain not identified). *Leviviridae* are unenveloped, spherical viruses containing single (+)RNA that acts as mRNA. *Cystoviridae* includes three molecules of dsRNA confined in icosahedral capsid with a lipid-containing envelope. *Inoviridae* phages are characterized by ssDNA and capsid that looks like a long, rigid or flexible helical tube [6-8].

Phages show varying susceptibility to a wide spectrum of chemical and physical agents. Bacteriophages are often sensitive to protein-denaturing agents such as urea and urethane, but the level of inactivation depends on both concentration and temperature. Enveloped viruses usually reveal susceptibility to detergents. Chelating agents also trigger effects in some phages correlated with cofactor requirements for adsorption. Mutagenic agents such as mustard gas, nitric oxide, and ultraviolet light can inactivate phage and induce the lytic cycle in many lysogens. On the other hand, phages usually retain stability at pH 5 to 8 [9].

Viruses infect host cells recognizing their specific receptors on the cell surface. For Gram-negative species these are specific lipopolysaccharide (LPS) components, outer membrane proteins, capsule and pili structures. The receptors are normal surface molecules involved in routine cellular functions, but molecular complex on the viral surface has a shape complementary to the shape of the outer part of the receptor, therefore phages take the opportunity to bind the receptor and get attached to the host cell surface. The phage genome will have to cross three major barriers in Gram-negative bacteria (the outer membrane, the peptidoglycan layer and the inner membrane). Further route depends on phage lifestyle (virulent or temperate). After adsorption to the surface of a host cell and injection of genome virulent phages change cell metabolism, forcing the host to replicate phage genome and lead to the transcribe/translate viral genes that encode the components of the phage particle. Virulent phages generally produce host-lethal proteins that can disrupt host replication, transcription, or translation, degrade the host genome, destroy or redirect certain host enzymes, modify the bacterial membrane. Then assembly of structural proteins takes place in the cytoplasm and phage genome is packaged in capsid by head-filling mechanism. At the final stage new phages are released by cell lysis. Degradation of bonds in the peptidoglycan matrix and destabilization of the inner membrane are carried out by lysin and holin, respectively. However, holin-independent lysin transport system and hybrid scheme between these two systems were described. *Pseudomonas* viruses φKZ, EL, φKMV, φ6 are examples of virulent phages [6, 9-11].

Temperate phages display two reproductive strategies when they infect a host cell. These phages can initiate above-mentioned lytic cycle causing death of the bacterial cell and release of new virus particles. In addition, temperate bacteriophage can initiate a lysogenic cycle. In this case phage genome either integrates into the host genome or resides as a plasmid in the bacterial cytoplasm resting in quiescent state called prophage. It remains in this condition

indefinitely replicating and transmitting to the daughter cells when the host is divided. Temperate phages can help protect their hosts from infection by other phages and can change properties of bacterial cells including restriction systems, resistance to antibiotics, pathogenicity, etc. Temperate phages, like virulent viruses, can carry few host-lethal proteins, but they must be kept under tight control during long-term lysogeny. It is provided by encoding repressor protein that regulates transcription and inhibits the lytic cycle. Phages F116, φCTX, D3112, B3 represent *Pseudomonas* temperate viruses.

Important feature of temperate phages is possibility to transfer host genes from one bacterial cell to another (transduction). It happens when the prophage DNA is excised mistakenly along with a piece of host DNA. There are two types of transduction: generalized and specialized. In these cases phage can carry either any bacterial sequence or particular piece of host DNA, respectively. Transduction plays an important role in bacterial evolution via its contribution in lateral gene transfer [6, 9].

3. Bacteriophage penetration and bacteriophage resistance (antiviral) mechanisms

Adsorption is the first stage of phage infection when virus recognizes specific structures on the surface of host cell. Adsorption usually includes two steps: reversible and irreversible binding with initial and secondary receptor molecules, respectively. As mentioned above, receptors of gram-negative bacteria are represented by LPS components, outer membrane proteins, capsule and pili structures. Proteins of outer membrane include structural proteins interacting with peptidoglycan layer, specific and non-specific porins forming membrane channels, enzymes, substrate receptors with high affinity and transport proteins responsible for secretion. LPS is a complex polymer made up by monosaccharides and fatty acids. It incorporates three parts: proximal hydrophobic “lipid A” region; long hydrophilic distal polysaccharide segment forming highly variable O-antigen; core polysaccharide region connecting these two parts. Rate of adsorption is determined by physical-chemical factors (pH, temperature, presence in the media of certain substances and ions such as tryptophan and divalent cations), host physiological state and cultural conditions. Penetration takes part after irreversible adsorption phase, but mechanisms of this process for *Pseudomonas* phages aren’t studied yet [9, 12].

Phages getting inside cell either reorganize host metabolism to produce new viral particles or turn into quiescent state that can be changed to lytic cycle. During coevolution bacteria have developed defence mechanisms against phage infection. Bacteria can resist to viruses at different stages of infection.

First defence mechanism is preventing phage adsorption. Adsorption to receptors is the initial step of phage infection and bacteria possess some ways to prevent this event. First, cell can develop resistance through loss or mutation of receptors [13]. However, such procedures don’t secure full protection against other phage types and can result in loss of some vital functions. Moreover, cell can produce extracellular polymers promoting bacterial survival in various ecological niches by shielding them from adverse conditions. These substances provide physical barrier between phages and their receptors. For example, *Pseudomonas* produces alginates surrounding cell and performing protective functions [9, 14].

Another defence mechanism found in *Pseudomonas* cells is restriction–modification systems (RMS). RMS protects the cell from invading DNA including viruses. *Pseudomonas* usually dispose of type II RMS. Type II enzymes are structurally and mechanistically simple. There are restriction endonuclease (dimer or monomer) that requires Mg^{2+} for activity and recognizes unmethylated DNA cleaved by enzyme within the site or only a few base pairs away and modification methylase that catalyzes transfer of a methyl group from AdoMet to one of the bases (A or C) on each strand of the site. Incoming phage DNA is usually cleaved before the methylation occurs, while host DNA remains protected. The more restriction sites are contained in phage genome, the more susceptible it is to restriction. [14-21].

The CRISPR-Cas systems also provide protection of bacteria from invasion by foreign nucleic acids such as plasmids and phages. These systems are divided into three main types and at least 11 subtypes (I-F system was revealed for *P. aeruginosa*). The CRISPR-Cas system includes two components: the CRISPR (clustered regularly interspaced short palindromic repeats) locus and the Cas (CRISPR-associated) proteins that function as the genetic memory and the catalytic core of the system, respectively. CRISPR locus contains repeats separated by variable sequences (spacers). These spacers are derived from invading nucleic acids. Both sequences are typically 25–40 nucleotides in length. Action of CRISPR-Cas system involves three stages: adaptation, expression and interference. At the first stage, short pieces of viral DNA are integrated into the CRISPR locus. New spacers are typically integrated at the leader-proximal end of the locus by Cas1 and Cas2 proteins. During the expression stage, pre-CRISPR RNA (pre-crRNA) from this locus is transcribed. Further, pre-crRNA is cleaved into separate short crRNAs by Cas endoribonucleases of type I. At the interference stage, crRNAs recognize and bind to complementary nucleic acid (double-stranded DNA for type I), whereupon Cas nuclease (type I) cleaves the target. The CRISPR-Cas system provides resistance to phages in the way similar to interference systems of eukaryotes. It was also shown that this system took part in regulation of swarming and biofilm formation in *P. aeruginosa* [22-25].

Bacteria have developed several mechanisms of phage resistance. However, a number of bacteria continue to succumb to phage infection despite advanced defence because phages also have evolved equally diversified strategies to overcome bacterial protection.

As mentioned above, bacteria may lack or mutate receptors preventing thereby phage adsorption. On the other hand, phages can adapt to new or modified receptors. Experiments revealed that rate of phage molecular evolution was by far higher when both bacterium *P. fluorescens* SBW25 and phage coevolved with each other. It was found that φ2 has numerous substitutions in the tail fibre gene involved in host adsorption [26, 27]. If bacterial receptor is masked by extracellular polymers, phage can hydrolyze them and gain access to the target. For example, *Pseudomonas* spp. phage F116 produces alginate lyase that reduces the viscosity of the alginate matrix, helping virus to infect host cells [28].

When phage genome enters into the cell, it can be confronted by intracellular antiviral defence. RMS cuts invading foreign DNA at specific recognition sites, but phage can produce methyltransferase (MTase) that modifies the recognition site and protects phage genome. It was shown that *Pseudomonas* phage B3 possesses DNA adenine MTase-encoding gene [29]. RMS also isn't able to recognize phage RNA genome.

Another intracellular antiphage mechanism is the CRISPR-Cas system. Phages can evade CRISPR interference via mutation caused by a single-nucleotide substitution. In addition, some viruses have anti-CRISPR genes. They were first discovered in *P. aeruginosa* PA14 lysogens. It was found that these genes inhibited the type I–F systems of other *P. aeruginosa* strains and failed to inhibit a type I–E CRISPR-Cas system of *E. coli* I–E as the most closely related system to type I–F. Products of studied genes didn't affect *cas* gene expression and the biogenesis of crRNAs. Hence it was suggested that proteins interfered at a step succeeding formation of the crRNA-Cas complex [30].

Thus *Pseudomonas* bacteria have developed various mechanisms to prevent phage infection (loss and mutation of receptors, biofilm generation, RMS, CRISPR-Cas system), while bacteriophages have adapted to bypass that defence (through mutations, acquisition of enzymes) and cause infection.

4. Criteria for selection of bacteriophages

Constantly increasing number of antibiotic-resistant bacteria urges to seek alternative antimicrobial treatments such as bacteriophages for use in medicine and agriculture as alternative to antibiotics. These applications demand formulation of criteria for selection of bacterial viruses for further use.

First, the chosen phages must be virulent killing bacteria under all conditions. Unlike virulent phages, temperate viruses can turn to prophage stage. At that stage they don't kill pathogenic bacteria and even prevent host from superinfection of the same viruses. They can remain in prophage phase indefinitely, so that pathogenic bacteria continue to exert negative effect on organism. These viruses also can carry virulence genes. It appears therefore that virulent phages are better choice in various applications. Second, phages must show high activity against certain harmful bacteria. Third, phage use must meet safety requirements. Fourth, viruses used in medicine should undergo functional tests in the organism, because immune system may destroy phages before they display their effect on bacteria. Fifth, bacterial viruses should be stable during storage and readily purified for further use. Sixth, phage biology should be well characterized prior to applications [31, 32]. These criteria allow to select suitable phages for medicine and agriculture.

5. Technological properties of bacteriophages

Phages are abundant microorganisms in various habitats. They infect and kill only bacteria without harm to other organisms, so these viruses can be used in medicine, food industry and agriculture. Phages possess several advantages favoring their successful applications in various areas.

Unlike antibiotics, phages show antagonistic action against specific bacteria. Their host range is generally narrower than that found in antibiotics. Phages normally lyse strains of one species or a subset of strains within a species without affecting normal bacterial flora. However, it is essential to set up collections of well-characterized phages for a wide range of pathogens and create express methods to reveal phage strain showing specify to particular infection. Antibiotics affect a wide range of bacterial strains, so a single antibiotic can often be used to treat diseases caused by various bacterial pathogens. Unfortunately, they can affect beneficial bacteria disturbing normal composition of microflora, which may lead to various side effects such as intestinal disorders, allergies, and secondary infections (e.g., yeast infections). Some antibiotics inhibit growth of bacteria rather than kill them.

Because of specificity, phages don't cause development of resistance to viruses in other bacterial species. Coexistence of bacteria and their viruses leads to appearance of above-mentioned defence mechanisms in bacterial cells, but phages also develop strategies allowing to overcome host cell resistance. Phages are found in various habitats, therefore it is easy to find new viruses when bacteria become resistant to them. Different phages can be mixed in cocktails to broaden their properties and increase antibacterial spectrum of activity. Use of antibiotics may result in selection of resistant mutants of many pathogenic bacterial species. Pathogen challenge demands constant development of new antibiotics against resistant bacteria that requires time and resources.

Phages meeting host cells multiply at the site of the infection until complete elimination of bacteria. It is sufficient to introduce one phage dose that will propagate exponentially in the presence of sensitive cells. Antibiotics do not necessarily concentrate at the site of infection and they must be supplied repeatedly.

Use of phages also has some disadvantages. Direct contact of the virus and bacteria must be provided which is hard to guarantee if phage host is intracellular parasite. In that case host is not available for interaction. Phages introducing into organisms also must be evaluated to survive the exposure to immune system. Constant use of phages in therapy of recurrent infections can lead to development of neutralizing antibodies against these phages. However, use of bacterial viruses in organism of immunocompromised patient could work. Another problem may appear to be closely related to phage resistance, but ways to solve this problem have been described above.

Use of phage in various applications demonstrates advantages and shortcomings as compared to other methods, like use of antibiotics. Bacterial viruses are attractive objects for medicine and agriculture. However, special problem is providing safety of bacteriophages [33, 34].

6. Safety of bacteriophages

Bacteriophages are the most numerous microorganisms found where their bacterial hosts exist. They inhabit salt and fresh water, soil, plants and animals. Phages may be recovered even from human organism. They are detected in gastrointestinal tract, skin and mouth, where they are harbored in saliva and dental plaques. Usually human organism shows good tolerance to those phages. Phages themselves don't influence macroorganisms in contrast to bacteria and are usually eliminated after certain time by immune system. From this viewpoint bacterial viruses are safe to use in therapy and agriculture. However, phage application for therapeutic purpose may cause side effects [33].

Phages may transfer various genes to host bacteria including virulence or toxic genes that can be acquired by bacterial populations and cause human diseases. For example, temperate phage φCTX transforms *P. aeruginosa* into producer of cytotoxin (CTX) by lysogenization. CTX is responsible for formation of transmembrane pores in the lipid bilayers [35, 36]. Apart from virulence, phages may carry genes that provide some features such as survival against adverse conditions. It was shown that bacterial viruses take part in transduction of antibiotic resistance in multiple-antibiotic resistant strains of *P. aeruginosa* [37, 38]. Temperate phages can remain in host cell for a long time in prophage stage without immediately killing the bacteria. Bacterial lysogens tend to be resistant to the phage types that have lysogenically infected them. It is important therefore to use virulent phages rather than temperate.

Another problem is release of bacteria-encoded toxins in the result of lysis of pathogenic host cell. Release of these toxins can lead to undesired side effects. However, similar complications also may be observed during antibiotic therapy. At the end of the lytic cycle, holin destabilizes inner membrane resulting in access of the lysin to the peptidoglycan. Lysin degrades rigid layer leading to disintegration of the cell envelope and lysis with release of cell wall components. Problem can be solved by the construction of phage variants with defective lysin genes. The bacteria are killed by endolysin-deficient phage, but cell envelope remains intact. Cellular disintegration also can be avoided by using of non-lytic phage encoding bactericidal proteins. An export protein gene of the *P. aeruginosa* filamentous phage Pf3 was replaced with a restriction endonuclease BglIIR gene. This rendered Pf3 variant (Pf3R) causes nonrepairable breaks in double-stranded chromosomal DNA of *P. aeruginosa* PAO1, while phage could be stably propagated in a host strain expressing the BglII methylase gene. Structural integrity of the cells was retained upon killing. When PAO1 was infected with Pf3R, endotoxin levels in the supernatant increased no more than five- and seven-fold by 90 and 240 min, respectively, as compared with 32- and 60-fold endotoxin levels following infection with phage Pt1. More than 70% of the mice treated with Pf3R survived the 7-day observation period, whereas only 20% of the mice treated with Pt1 lived past the second day [39]. Use of these methods allows to solve endotoxin problem, but this modification stops exponential growth of phages.

In order to gain phages with maximal activity against pathogenic strains, sensitive strains should be used. It is not always possible to find appropriate non-pathogenic sensitive variants, so clinical (pathogenic) isolates are used to receive necessary phages. These isolates usually contain virulence genes that can be transferred into other cells, hence it is better to use only well-studied bacterial hosts that don't carry dangerous genes. It is also desirable to seek strains sensitive to different phages. Besides, it is necessary to provide good purification to avoid above-mentioned endotoxins generated during phage production. Use of purified phages doesn't elicit noticeable side effects in animals or humans [33, 40].

Observations strongly suggest that phage application may provide safety for therapy. However, in order to evade side effects, it should provide good purification to exclude exotoxins and pathogenic bacteria from phage preparations. In addition, well-studied strains that don't carry virulence or toxic genes should be used and virulent phages are preferred to use [33].

7. Medical and therapeutic application of *Pseudomonas* bacteriophages

The genus *Pseudomonas* represents a wide group of bacteria inhabiting diverse niches such as terrestrial and marine environments as well as organisms of animals and plants. Their enormous capacity to survive in various conditions is provided by the ability to adapt to challenging environments, degrade and synthesize a variety of low-molecular-weight compounds and biopolymers.

P. aeruginosa is a member of the genus that is known for its potential pathogenicity for animals, including humans. This bacterium is an opportunistic pathogen causing various types of infection (e.g., skin, eyes, ears, respiratory tract, urinary tract, gut-derived sepsis) mainly in immunocompromised patients such as patients suffering from AIDS, cancer, burn wounds and cystic fibrosis. *P. aeruginosa* infections are usually difficult to eradicate. That pathogen is characterized by ability to adapt to diverse environments. This bacterium can grow both aerobically and anaerobically using NO_3 or arginine as respiratory electron acceptors, over a wide range of temperatures, from common ambient temperatures up to 42^{o}C, utilizing many different metabolic compounds. *P. aeruginosa* is able to produce a wide variety of virulence factors such as phospholipase C, elastase, protease, siderophore, DNAse, pyocyanin, exotoxins, etc. The most remarkable feature of this bacterium is its multi-drug resistance. *P. aeruginosa* may acquire resistance through mutation or mobile genetic elements such as plasmids. Multi-drug resistant strains pose a problem closely related to antibiotic therapy. *P. aeruginosa* is frequently found to be resistant to many antibiotics administered in hospitals, so new methods are required to eradicate pathogens, e.g. phage therapy [41].

Cystic fibrosis (CF) is a hereditary disorder that usually affects the lungs and digestive system. A mutation in the gene coding for the CF transmembrane regulator results in the disturbance of cell membrane permeability for ions and accumulation of fluid in lungs, which creates conditions for growth of bacteria, including *P. aeruginosa*. This bacterium causes chronic inflammation leading to destruction of the lungs. Coupled to resistance to many antibiotics, *P. aeruginosa* is able to form biofilms in the lungs of CF patients complicating elimination of the bacterium. It was shown in experiments with mice that phages φMR299-2 and φNH-4 effectively removed bacteria from lungs in six hours. Phage mix suspension (1:1) was given after two hours following infection. The mixture of these phages was able to clear 24-h biofilms of *P. aeruginosa* NH57388A (mucoid) and MR299 (nonmucoid) strains growing on a cystic fibrosis bronchial epithelial CFBE41o- cell line [42]. Similar results in experiments with mice were obtained for phage PAK-P1 that was also supplied after two hours of infection. However, at 24 hours 75% of mice were still alive in the groups treated 4 or 6 h after infection. At 72 hours 75% and 25% survival rates were revealed for the 4 and 6 h groups, respectively. Additionally, these experiments showed that phages were able to prevent lung infection and provide 100% protection when treatment occurred 24 h before bacterial infection [43]. Efficacy of phage therapy for CF patients was reported in clinical studies of the former Soviet Union [44]. Recent investigations have demonstrated successful treatment of *P. aeruginosa* lung infection in a seven-year-old patient when Pyophage was used [45].

P. aeruginosa is one of the main pathogens that colonize skin injuries and cause wound infections resulting in bacteremia, septic shock and high rates of mortality and morbidity [46, 47]. Phage PA709 on human skin derived from surgery and then artificially contaminated with *P. aeruginosa* 709 clearly reduced the number of viable bacteria by 4 logs upon 2 hours of incubation. However, after this period, 2 logs of bacteria still remained viable, but overall population of bacteria might be decreased to the level sufficient to be eliminated by the host immune system [48]. High efficiency of phage therapy was achieved in the treatment of suppurative skin infections caused by *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Proteus* and *Escherichia* [49]. In other experiment mice were compromised by a burn wound injury, then subjected to a fatal infection with *P. aeruginosa*. Cocktail consisting of three different phages was administered intraperitoneally (IP), intramuscularly (IM) or subcutaneously (SC) immediately after the bacterial exposure. The results revealed that a single dose of phage cocktail could decrease the mortality of thermally injured and infected mice. The rate of mortality was reduced to 12% when the phages were delivered IP, compared with 72% mortality rate for IM, 78% for SC and 94% for untreated mice [50]. However, similar experiments with bacteriophages Pa29, Pa30, Pa31, Pa33 and Pa34 didn't secure protection from bacteria in infected mice [51]. Experiment with guinea pigs demonstrated that phages could be used prophylactically to prevent destruction of skin grafts. Six of seven test animals with grafts infected with *P. aeruginosa* and treated with a purified phageBS24 experienced successful outcomes compared with seven control animals [52].

Phages can be used in treatment of eye infection. *P. aeruginosa* is an important cause of infectious keratitis, especially in contact lens wearers. Keratitis progresses rapidly and it is characterized by infiltration of inflammatory cells and tissue degradation, which may result in corneal perforation [53, 54]. Experiment showed that a single dose of bacteriophage KPP12 in the form of eye-drops efficiently eliminated *P. aeruginosa* from the infected cornea of mice. In control animals the ring abscess was observed on day 1 after infection, and opacities had spread across the entire cornea by day 3. Most of the corneas were perforated on day 5. Mice treated with single dose of KPP12 showed only slight or focal corneal opacities on day 1 gradually fading by day 5. Histopathological examination on day 5 after infection revealed that stromal structure of cornea was destroyed and that very few stromal collagen fibrils remained at the center of the cornea while phage-treated mice showed almost normal corneal structure. These results indicate that phage eye-drops may be used for the treatment of infectious keratitis [55].

Another application for *P. aeruginosa* phages is treatment of ear infection such as chronic otitis. Use of cocktail of six phages in treatment of ten dogs suffering from chronic otitis caused by *P. aeruginosa* revealed that mean bacterial count fell by 67% at 48 h following phage inoculation, whereas phage density increased approximately 100-fold [56]. Clinical trials in patients with chronic otitis also showed efficacy and safety of phage application for biological therapy [57]. The results prove solid potential of use of bacterial viruses in effective treatment of otitis.

Phage therapy may be used in treatment of sepsis. Mice with gut-derived sepsis caused by *P. aeruginosa* were fed phage KPP10 orally either 1 day before (group 1), 1 day after (group 2), or 6 days after (group 3) the oral inoculation of

pathogenic bacteria. Survival of the mice in group 2 was significantly higher than that of the control group (66.7% and 0%, respectively), but there was only a minor improvement in the survival rate among the mice in group 3 (10%) and no improvement among the mice in group 1 as compared to the control mice. Additionally, mice treated with phage had lower numbers of viable *P. aeruginosa* cells in their blood, liver, and spleen than in the blood and organs of untreated animals [58]. In other experiments mice were injected via either the IM or IP route with different doses of phages MPK1 and MPK6 (2×10^6 and 2×10^7 plaque forming units or PFU in 100 µl phosphate-buffered saline) for treatment of PAO1-induced peritonitis-sepsis. The 2×10^7 PFU dose of both phages significantly protected the infected mice as compared to the untreated mice, however, 2×10^6 PFU dose of MPK6 didn't provide significant protection by the IM route. IP administration displayed better efficacy than IM administration for both phages. It is most likely that IP administration can deliver the phages more directly or effectively to the infection site. It was also shown that MPK1 treatment could reduce the bacterial burden by about 2 to 3 logs in lung, spleen, and liver of live mice at 24 h after infection, while MPK6 slightly less reduces bacterial load (by about 2 logs) in treated mice [59]. In another experiment a single IP injection of 3×10^9 PFU of the phage CSV-31 was sufficient to rescue 100% of the septicemic mice after 45 minutes of bacterial infection. Approximately 50% of animals can be rescued by a single injection, when treatment was delayed to the point where all animals were moribund [60].

Experiments with animals and clinical trials show elevated potential of bacterial viruses in phage therapy. At the moment some medicines such as Pyophage, Phagobioderm, Intesti including phages of *P. aeruginosa* are produced. However, it will take time for phage therapy to become a common or preferred method of treating infections caused by pathogenic bacteria.

8. The use of *Pseudomonas* phages in agriculture

Researches and applications of *Pseudomonas* phages for plant and animal treatment in agriculture are not so widely practiced as use of phages against *P. aeruginosa* in medicine. Most studies with animals are carried out for medical and therapeutic purposes.

P. plecoglossicida is known to cause bacterial hemorrhagic ascites disease in ayu fish (*Plecoglossus altivelis*) which is the most popular freshwater species for culture and sport fishing in Japan. Fish that were orally inoculated with *P. plecoglossicida* and then received feed without phage began to die 7 days after the bacterial infection and the cumulative mortality in 2 weeks was 65.0%. In contrast, fish that received feed with phages PPpW-3 and PPpW-4 died later, and the average cumulative mortality was 22.5%. Cells of pathogen were always detected in the kidneys of control fish and quickly disappeared from the phage-treated fish. It was also shown that bacterial growth in freshwater was lower in the presence of phage, and the number of PFU increased rapidly, suggesting that the phage could be used prophylactically to prevent transmission of the pathogen [61]. Other experiments revealed that mortality rates of fish receiving PPpW-3, PPpW-4, PPpW-3/W-4, and the control fish receiving no phages were 53.3, 40.0, 20.0 and 93.3%, respectively [62]. These results indicate large potential of phages in control of the disease caused by *P. plecoglossicida*.

Control of bacterial diseases is problematic because of lack of effective bactericides, high pathogen variability, rapid population build-up under optimal conditions, high mutation rates resulting in development of mechanisms of resistance to various conditions [63]. Use of phages can solve this problem. However, reports on use of *Pseudomonas* phages in plant treatment are few as compared with studies of other plant pathogens.

In 1989 a US patent was granted for the phage-based method of eliminating *P. syringae* from contaminated bean culls, and reducing the severity of disease symptoms in bean leaves experimentally infected with these bacteria [64]. Phage therapy has been used successfully against bacterial blotch of mushrooms caused by *P. tolaasii* [65]. Some products already have been developed such as AgriPhage for the control of pathogenic bacteria including *P. syringae* pv. tomato, which is the causative agent of bacterial speck on tomatoes. It was shown that recent studies have been focused on search of phages active against *P. syringae* pv. actinidiae that causes bacterial canker of kiwifruit (*Actinidia* sp) [66].

9. Conclusion

The genus *Pseudomonas* includes widespread bacterial species capable to colonize diverse niches. Pathogens of animals and plants were revealed among these species. For a long time antibiotics have been used in treatment of diseases caused by bacteria. However, in recent years multidrug-resistant *Pseudomonas* strains were detected. They pose a grave challenge in treatment of bacterial infections and in this respect phages may offer excellent solution of the problem.

Major viruses of *Pseudomonas* and other genera are represented by tailed dsDNA phages of order *Caudovirales* and few viruses belong to PFP phages. LPS components, outer membrane proteins, capsule and pili are usually entry ports for phage infection. Coexistence of *Pseudomonas* species and their phages provides for development of bacterial antiviral defence systems. *Pseudomonas* obtains outer- and intracellular mechanisms allowing to overcome phage infection. These are loss and mutation of receptor molecules and formation of biofilm preventing phage adsorption on host cell surface; there are RMS and CRISPR-Cas systems aimed at breaking down foreign nucleic acids. On the other

hand, viruses also develop strategies helping phage to evade bacterial defence mechanisms and infect host cell. Phages can adapt to new and modified receptors, hydrolyze biofilm, take substitutions in genome, produce protein inhibiting bacterial defence systems.

Ability to adapt to and ignore bacterial resistance, high specificity of action against certain species and strains of bacteria without harming beneficial microflora and host organism, fast propagation at the site of infection and maintaining a significant number of viral particles secure competitive superiority of phages in various spheres. However, it is necessary to ensure safety of bacteriophages to evade side effects. It would be better therefore to use well-studied variants not carrying dangerous genes and able to kill bacteria with high efficiency.

Species of the genus *Pseudomonas*, especially *P. aeruginosa*, are known for their ability to cause various diseases. *P. aeruginosa* is an opportunistic pathogen difficult to control. Nevertheless, some experiments with animals and clinical trials revealed high therapeutic efficiency of bacterial viruses. Introduction of phages allows to decrease adverse effects of bacterial pathogens, prevent spreading of bacteria and reduce the number of viable cells. Encouraging results have been attained in treatment of lung infections such as cystic fibrosis, skin and wound infections, keratitis, chronic otitis, sepsis and bacteremia. Production of some medicines against diseases caused by bacterial pathogens is established. Phage therapy isn't widely distributed in contrast to antibiotics, yet, it is an extremely promising trend with a vast progress potential.

Agricultural applications of *Pseudomonas* phages are limited in comparison with medicine, although research in this direction continues. Successful use of phages in treatment and prevention of hemorrhagic ascites caused by *P. plecoglossicida* was described. Some studies are centered on phage control of plant bacterial diseases.

Phages are able to prevent and suppress certain bacterial pathogens. Thus use of phages opens wide frontiers in various areas, although so far further research and development is indispensable. Providing for higher efficiency and safety of bacterial viruses will allow to replace antibiotics and avoid the related side effects. From now on practical significance of phage therapy will tend to expand.

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