Bacteriophages: therapeuticals and alternative applications

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1. Introduction

Bacteriophages as new antibiotics??

Bacteriophages ("eaters of bacteria") are viruses specific to bacteria and are one of the most abundant "life forms" on earth. There are probably no bacterial species without its specific bacteriophage(s). Bacteriophages are generally characterized by their strong host specificity; most infect not more then one species of bacteria, and the strong effects they have on their host upon infection. Lytic phages literally destroy their host upon infection (sometimes in less then 30 minutes) thereby releasing many progeny phages, each capable of infecting (and subsequently killing) a 'healthy' bacterium. Lysogenic phages hide themselves in the genome of the bacterium they infect and often transfer genetic material from other bacteria in the process.

Already soon after their discovery, now almost 90 years ago, the use of bacteriophages as therapeutic agents was promoted. However, because of limited success and the introduction of antibiotics in the 1940's their use was abandoned in the West.
In the former Soviet Union however, research on their application continued which resulted in a wide use as antibiotic agents. They were for example used successfully to disinfect wounds on the battle fields of the Second World War. However, for various reasons, not least the impermeability of the Russian language to Western researchers, former Soviet Union achievements and advances in the field remain relatively unknown in the West.

There is growing concern about the increasing antibiotics-resistance in many clinically important bacteria. This is one of the reasons for the growing (renewed) interest in the therapeutic use of bacteriophages in the West. Most specialists agree that their application holds promises, yet many questions remain. Issues like their specificity, possible immunogenic response to bacteriophages, bacterial resistance, the possibility of phages to carry harmful genes and the purity and safety of phage preparations need to be adequately addressed. Their safe and controlled use will require detailed information on the properties and the behavior of specific phage-bacterium systems. Important questions that remain are; what determines phage specificity and host range and how it can be influenced, the ability of the phages to adapt to changing conditions and environments, especially *in vivo*, and understanding the dynamics of phage infections. Will it one day be possible to tailor phages to specific bacterial diseases for which antibiotics no longer offer a reliable solution?

The 'Commissie Genetische Modificatie' (COGEM) specifically asked for insight into the (theoretical) possibilities for genetic modification of bacteriophages and the possible risks with regard to their host range and virulence.

Based on an extensive literature review from both Western as well as former Soviet Union resources we report here on:

- The history, developments and applications of bacteriophages as bacterial therapeutics both in the West as in the East European countries.
- Possible problems in their applications.
- The possibilities that might exist to influence or modify the therapeutic efficiency of bacteriophages by genetic modifications or conventional methods.
- The possible alternative applications of bacteriophages.

2. Taxonomy of bacteriophages

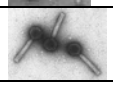
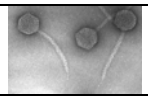
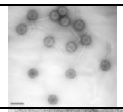
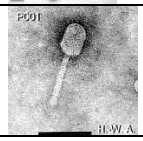
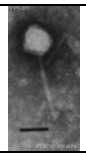
The latest edition (Fauquet *et al*., 2005) of the report of the International Committee on Taxonomy of Viruses (ICTV) lists bacteriophages as viruses of Eubacteria and Archaea. Bacteriophages are highly diverse and there are two main ways of differentiating between the different types:

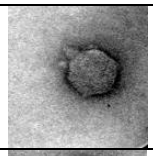
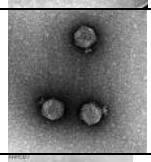
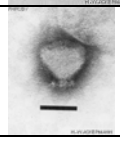
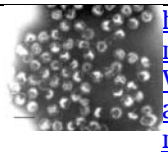
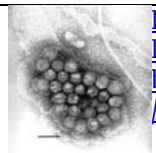
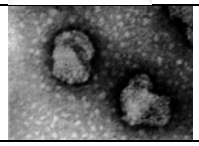
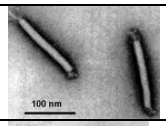
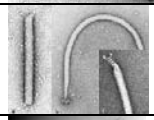
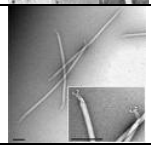
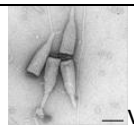
A: on the basis of their genetic material
B: on the basis of their life cycle

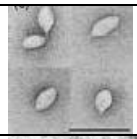
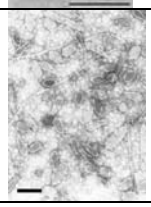
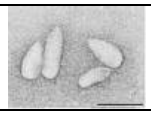
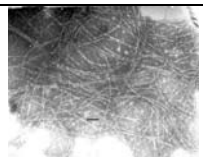
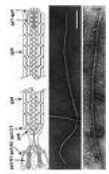
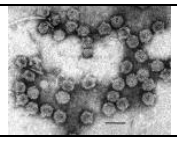
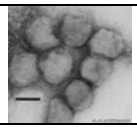
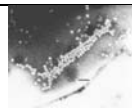
2.1 Differentiation of bacteriophages on the basis of genetic material

The main differentiation is on the basis of the type of genetic material i.e. DNA or RNA. Within the DNA and RNA phages, the distinction is further made between single-stranded (ss) DNA (or RNA) and double-stranded (ds) DNA (or RNA). Table 1 lists the taxonomic classification within the bacteriophages as it is recognized by the ICTV:

Table 1. Taxonomic classification of bacteriophages according to the 2005 report of the Int. Committee on the taxonomy of viruses

dsDNA				
Order Caudovirales				
Family	***genus***	***Morphology***	***Host***	***# Species[1]***
Myovirdae	*T4-like viruses*		Bacteria	8 (24)
	P1-like viruses		Bacteria	2 (8)
	P2-like viruses			2 (18)
	Mu-like viruses		Bacteria	1 (5)
	SP01-like viruses		Bacteria	1 (10)
	ΦH-like viruses		Archaea	1 (1)
				60 unassigned
Siphoviridae	*λ-like viruses*		Bacteria	3 (4)
	T1-like viruses		Bacteria	1 (10)
	T5-like viruses		Bacteria	2 (3)
	L5-like viruses		Bacteria	2 (4)
	c2-like viruses		Bacteria	2 (3)
	ΨM1-like viruses		Archaea	1 (2)
	ΦC31-like viruses		Bacteria	1 (7)

	N15-like viruses		Bacteria	1 (1)
				142 unassigned
Podoviridae	*T7-like viruses*		Bacteria	3 (16)
	P22-like viruses		Bacteria	1 (12)
	Φ29-like viruses		Bacteria	3 (10)
	N4-like viruses		Bacteria	1 (0)
				33 unassigned
Not assigned to Order				
Tectiviridae	*Tectivirus*	 http://www.ncbi.nlm.nih.gov/ICTVdb/WIntkey/Images/em_tecti.htm	Bacteria	4 (10)
Corticoviridae	*Corticovirus*	 http://www.ncbi.nlm.nih.gov/ICTVdb/WIntkey/Images/536-02.jpg	Bacteria	1 (0)
Plasma*viridae*	*Plasmavirus*		Bacteria	1 (7)
Lipotrixviridae	*Alphalipothrixvirus*		Archaea	1 (1)
	Betalipothrixvirus		Archaea	1 (3)
	Gammalipothrixvirus		Archaea	1 (1)
Rudiviridae	*Rudivirus*		Archaea	2 (0)

Fuselloviridae	*Fusellovirus*		Archaea	1 (4)
	Salterprovirus		Archaea	1 (1)
Guttaviridae	*Guttavirus*		Archaea	1 (0)
ssDNA:				
Inoviridae	*Inovirus*		Bacteria	36 (2)
	Plectrovirus		Bacteria	7 (5)
Microviridae	*Microvirus*		Bacteria	5 (24)
	Chlamydiamicrovirus		Bacteria	4 (0)
	Bdellomicrovirus		Bacteria	2 (7)
	Spiromicrovirus		Bacteria	1 (0)
dsRNA:				
Cystoviridae	*Cystovirus*		Bacteria	1 (8)
ssRNA:				
Leviviridae	*Levivirus*	 http://www.ncbi.nlm.nih.gov/ICTVdb/WIntkey/Images/089-30.jpg	Bacteria	1 (2)
	Allolevivirus		Bacteria	(0)
				33 unassigned

[1]: the number of bacteriophage species assigned to the genus by the ICTV. Between brackets the number of tentative species in that genus.

This classification indicates that a large number of different bacteriophages exist, distributed over many different families and genera. Each family (and often genus) has its own characteristics with regard to morphology, particle characteristics, genome organisation and expression, specificity for

particular bacteria groups or genera etc. Of many families phages have been described which were only partially characterized and currently have the status of tentative species or of which too little information is available to assign them to a particular genus. Examples of this are the lactococcal phages. These phages belong to the genus '*c2-like viruses*' (Family *Siphoviridae*) and are specific for lactococci. The ICTV reports states: "over 200 additional poorly characterized lactocollal phages have been reported that are morphologically indistinguishable from c2'.

A recent review originating from the Félix d'Herelle Reference Center for Bacterial Viruses, Quebec Canada (Ackermann, 2007), very nicely summarizes the latest survey of bacterial viruses. A main criterion in this survey was phage morphology and four types are distinguished:

- Tailed phages
- Polyhedral phages
- Filamentous pages
- Pleomorphic phages

The majority of phages is tailed (the three families within the Order *Caudovirales*) and they comprises > 96% of all phages described (5360 of 5500 examined). Only 3-4 % is represented by polyhedral, filamentous and pleomorphic phages.
Phages can also be grouped according to host genera. The bacterial genera in which the most bacteriophages were observed are Enterobacteria (>900), *Lactococcus* (700), *Bacillus* (380) and *Streptococcus* (290). Ackerman nicely lists a large number of bacteriophages observations in many different bacteria.

It is generally assumed that the number of bacteriophages is a very much larger then what is currently described and in the future not only many new species will be discovered but also new genera and families. Not only has a very limited number of bacterial phyla been investigated for the presence of phages, phages occur in very strange and scarcely investigated habitats like the deep sea, soils or hot springs. Also most phage descriptions originate from the Western World and former communist countries and no data are available from for instance Africa of Siberia.

2.2 Differentiation of bacteriophages on the basis of their life cycle

The differentiation between phages on the basis of their type of genetic material is a rather technical one. Another important differentiation can be made on the basis of the phage life cycle. Two main groups are distinguished there

- Lysogenic or temperate phages
- Lytic phages

2.2.1 Lysogenic phages

The most important feature of lysogenic phages is the integration of their genome in the bacterial genome after infection. This results in the so-called pro-phage stage. Genes that control the lytic pathway are repressed by protein repressors which bind to specific DNA-regions. This prevents transcription of genes whose products are involved in the lytic pathway. During this stage the bacteria grows and divides normally and the phage inside the bacteria can remain unnoticed. Upon activation of the pro-phage, in many cases for as yet unknown reasons, the bacteriophages' genetic material is transcribed and replicated inside the bacterium. This ultimately results in the formation of new bacteriophage particles which, once released form the bacterium, are capable of infecting new bacteria and integration of their genetic material into the bacterial genome, thus closing the circle.
Prophages are released by excision. Sometimes excision is not precise and bacterial genes adjacent to the prophage are packaged into a phage particle. These bacterial genes then can be transferred into other percipient bacteria and during, the formation of the prophage stage, integrated into the chromosome. This process is called transduction and has not only led to the exchange of bacterial DNA between bacteria but also to the transfer of bacterial DNA to the genome of certain phages. A nice example of this is the presence of so-called 'pathogenicity islands' in the genome of CTX Phi phage of the cholera bacterium (*Vibrio cholerae*) which contains a bacterial toxin encoding gene.

Although highly interesting, lysogenic phages are (as yet) unpredictable in their properties and behaviour. Transition from the pro-phage stage to the lytic phage is uncontrollable and the exchange of genetic material unpredictable. Lysogenic phages have been observed to transfer potentially harmful genes into their target bacteria (including genes encoding Shiga toxins of Shigatoxinogenic *E. coli* (Tóth *et al*., 2003) the cholera toxin of toxinogenic *Vibrio cholerae* (Campos *et al*., 2003) and various antibiotic resistance genes (Schmieger & Schicklmaier, 1999). Although many of the lysogenic phages are relatively well studied (e.g. Phage λ) and sometimes detailed knowledge is available about their genome and its regulation, because of their unpredictable behaviour, they are generally considered to be unsuitable of use in bacteriophage therapy. For this reason they will not be discussed in this report.

2.2.2 Lytic phages

Lytic phages infect their host, replicate and then kill their host by cell lysis, thereby releasing progeny viruses into the environment. Infection of the bacterial host is often based on specific recognition of particular components on the pili or cell wall of the bacterium which is likely to determine the specific recognition. After initial infection it can be a matter of hours before cell lysis occurs.
Lytic phages are theoretically ideally suited as an alternative antibiotic in phage therapy:

- Most bacteriophages are very specific for their host bacterium.
- Upon successful infection they replicate exponentially.
- They kill their hosts quickly (often in hours).
- Upon bacterial lysis a large number of progeny phages are released into the environment. Each of those phages is capable of infecting other target bacteria. As an ‘antibiotic’ bacteriophages are ‘self multiplying’.
- Bacteriophages depend on their hosts and will disappear with the destruction of their target bacterium leaving no residues; ‘antibiotic’ bacteriophages are ‘self eliminating’.

3. The history of bacteriophage therapy

The first observation of what can be considered now as the effects of bacteriophages dates back to 1896 when M Hankin (Hankin, 1896) described the bacteriocidal effect of filtered water from the Indian river Ganges on cholera bacteria (*Vibrio cholera).* From this observation he speculated that drinking river water might be effective in preventing the spread of cholera.

About 20 years later the first actual bacteriophages were described by the Englishman Edward Twort (Twort, 1915) and the French Canadian Felix d'Herelle (d'Hérelle, 1917). The latter generally became known as the discoverer of bacteriophages and he spent many years of research on the application of phages against animal and human diseases.

In the period following the initial discovery of bacteriophages it was soon realized that phages destroy their bacterial hosts yet are harmless to animals and humans. D'Herelle successfully tested this concept of phages as therapeutic agents with avian typhosis (*Salmonella gallinarum*) in chickens and *Shigella dysenteriae* in rabbits. Tests to protect waterbuffaloes in IndoChina against experimental inoculations with *Pasteuralla multocida* causing bovine hemorrhagic septicaemia were also successful. Following these results of therapeutic effectiveness d'Herelle proceeded with testing the *Shigella dysenteriae* phage, first on himself and family members and later with successful results on patients suffering from dysenteria.
D'Herelle's successful treatment of four patients suffering from bubonic plague attracted a lot of attention. As a result from this he was invited to India by the British government to work on phage therapy against plague. Later studies in India on the application of phage therapy against cholera provided encouraging results.

Following these early successes many phage therapy trials were reported and many of the major pharmaceutical companies sold phage preparations (e.g. Parke-Davis and Lilly in the United States). In the period 1917 – 1956 several hundreds of papers were published on medical applications of bacteriophages. A number of reviews give a good overview of these applications as well as a more detailed list of references to them (Carlton, 1999, Inal, 2003, Marks & Sharp, 2000, Sharp, 2001, Summers, 2001).

Generally the results for the clinical applications of bacteriophages were variable. The main reasons for this were:

- Most experiments were of poor scientific design and would never meet any of the modern standards set for medical experiments involving animals or humans.
- Most bacteriophage preparations contained very poorly or uncharacterised phages in unknown concentrations.
- No matching was performed between the phage and the target bacteria.
- Phage preparations were not cleaned and generally contained (half)dead bacteria and bacterial toxins.

In the 1930's even several dubious preparations were commercially marketed which claimed effectiveness against a number of inappropriate infections, some of which, such as herpes infections, eczema and urticaria, did not even have a bacterial aetiology.

In addition to the control of human and veterinary infections there was also an early interest in the use of bacteriophages for the control of plant diseases. Phages isolated from rotting cabbage were shown to be effective against bacteria that were also isolated from rotting cabbage (Mallmann & Hemstreet, 1924) and bacteriophages isolated from "*B. atrosepticus"* were shown to prevent the rotting of potato tubers (Kotila & Coons, 1925, Link, 1928).

In the 1930's the US Council on Pharmacy and Chemistry undertook an evaluation of phage therapy. The resulting report (Eaton & Bayne-Jones, 1934) was ambiguous, concluding that both positive and negative results were reported. Main concerns were the poor understanding of the biological nature of the bacteriophage and the observation that the lack of standardization of phage preparations and the lack of criteria for purity and potency made it impossible to compare most published studies.

Before the Second World War there had been conducted extensive studies on bacteriophages and their therapeutic uses. However the War and the introduction of antibiotics diverted attention away from this research. Antibiotics proved easy to produce and their relative broad spectrum of action and stability were considered significant advantages over bacteriophages.
In the Soviet Union however the work on bacteriophages and bacteriophage therapy continued. One important reason for this was military based. Phages were extensively used by the Soviets to treat the war-wounded. After the war this use continued, most likely for economic reasons however, possibly also for ideological reasons.

Felix d'Herelle apparently was a difficult man who was not very popular in the French scientific community. He was also a communist and after the October revolution in Russia he moved to Tbilisi in Georgia. In 1923 he founded, together with Giorgi Eliava, the Institute of Bacteriophage Microbiology and Virology. This institute performed extensive research on bacteriophages and established the world's largest collection of antibiotic resistant bacteria and matching bacteriophages. Within this institute, but also in other places in the Soviet Union, facilities were set up for the production of bacteriophages on an industrial scale.

In the former Soviet Union and its satellite states bacteriophages were widely and successfully applied against a large number of bacterial infections e.g.:

- *Streptococcus pyogenes, Staphylococcus aureus*
- *Pseudomonas aeruginosa, E. coli*
- *Proteus vulgaris / mirabilis*
- *Salmonella typhi / thypimurium*
- *Shigella dysenteriae / flexneri / sonnei*

Application was usually very practical as liquid, paste, pills (oral and rectal) and as bandages drenched in bacteriophage solution. Bacteriophages were even successfully applied by intramuscular or intravenous injections.

Many examples exist of successful applications of bacteriophages in Eastern Europe. Success rates were generally >90%. These were achieved with a very limited number of doses. Treatments with 2-4 applications were normally sufficient.

Often mixes of bacteriophages were found to be the most effective. In some cases mixed applications with antibiotics were also reported as successful.

4. Applications of bacteriophage therapy

From the 1930's on, bacteriophages were widely and successfully used in Eastern Europe, both on a curative basis as well as on a prophylactic basis.

The success of the bacteriophage therapy system was based on careful selection of phages and on monitoring their effectiveness, often in individual patients.

- First the bacterium was isolated from a patient and a matching effective phage (or phages) was selected from a collection.
- The phage was produced and purified and applied to the patient.
- The effect of the treatment was monitored.
- If the bacterium was not completely killed a second round of phage selection, production and application would follow.

Numerous examples and reports of successful treatments can be found in (Russian) literature but almost all are (to Western standards) very poorly documented. Only a few studies have been published in peer reviewed journals. The Polish Institute of Immunology and Experimental therapy in Woclaw has conducted a large number of clinical trials of phage therapy against bacterial infections (Cislo *et al*., 1987, Kucharewicz-Krukowska & Slopek, 1987, Mulczyk & Slopek, 1974, Slopek *et al*., 1984, Slopek *et al*., 1983a, Slopek *et al*., 1983b, Slopek *et al*., 1985a, Slopek *et al*., 1985b, Slopek *et al*., 1985c, Slopek *et al*., 1987, Weber-Dabrowska *et al*., 1987, Weber-Dabrowska *et al*., 2000). These studies deal with over 550 patients in the age range from 1 week old to 86 years old. Most suffered from infections with antibiotic-resistant *E. coli, Klebsiella, Pseudomonas, Salmonella, Staphylococcus* and *Streptococcus*.
Sterile and standardized phage preparations were administered either via eye drops or orally before meals after neutralizing stomach acid with baking soda, gelatine or basic water. Treatments on average lasted for 5 weeks. Success rates were generally high (between 75 – 100%) but depended on the bacterium. Of the 550 patients, 518 suffered from antibiotic-resistant bacteria. For this group the success rate was 94%.

The following table (taken from Inal, 2003) summarizes a number of phage therapy studies in humans and animals.

Infection	**Causative agent**	**Summary**
Supprative skin infection	*E. coli, Klebsiella, Proteus, Pseudomonas, Staphylococcus*	Of 31 patients 23 cases at least had a marked improvement
Various	*E. coli, Klebsiella, Proteus, Pseudomonas, Staphylococcus*	Phage immunogenicity did not hinder therapy
Gastrointestinal, head, neck, skin	*E. coli, Klebsiella, Proteus, Salmonella, Staphylococcus*	506/550 patients (92%) successfully treated
Suppurative infection	*Staphylococcus* Gram-negative	Oral administration; 56 patients successfully treated, phage in blood (47/56) and urine (9/56)
Brucellosis	*Brucella abortus*	
Conjunctivitis, dermatitis, pharyngitis, rhinitis	*Enterococcus, E. coli, P. aeruginosa, Staphylococcus, Streptococcus*	1340 patients treated ; 360 with phage(86% clinical improvement), 404 with antibiotics (48% clinical improvement and 576 with combination (83% clinical improvement)
Infective childhood asthma		
Bovine udder infection		
Recurrent subphrenic abscess	*E. coli*	Patient left hospital after 33 days without any abscess
Cerebrospinal meningitis	*K. pneumoniae*	Following unsuccessful antibiotic therapy, newborn successfully treated with orally administered phage
Recurrent furunculosis	*Staphylococcus*	

Diarrhoea in calves	*E. coli*	*E.* coli causing diarrhoea eliminated from alimentary tract of piglets, calves and lambs
Septicaemia, meningitis in chicken and calves	*E. coli*	Septicaemia and meningitis-like infection in chickens due to *E. coli* K1+ cured with phage which multiplied in the blood

Before the Second World War, bacteriophages were studied and used in the West but when antibiotics became widely available, their use quickly diminished.
A limited number of studies are available especially on the effectiveness of bacteriophages in animal systems.

4.1. Phages in animal systems

Early studies on the efficacy of bacteriophages in controlling bacterial diseases in animals or animal models were carried out poorly and indicated that phages had little influence on the course of infection. A few studies showed some beneficial effects. With the advent of antibiotic therapy in the west, the idea of using phages became discredited after the Second World War (Barrow, 2001). In the 1980s, Williams Smith *et a.l* investigated phage therapy for pathogenic *Escherichia coli* in animals (Williams Smith & Huggins, 1983, Williams Smith *et al*., 1987a, Williams Smith *et al*., 1987b), the beginning of a renewed interest in phage therapy in Western countries. He showed that phage treatment of *E. coli* challenged mice was more efficient than treatments with several antibiotics.
Since then, many experiments were carried out in animal model systems in first place to investigate effectiveness of bacteriophages against bacterial diseases which are also a threat to humans, e.g. *Escherichia coli* in chickens and calves (Barrow *et al*., 1998), *Pseudomonas aeruginosa* in burn wounds of mice and guinea-pigs (Soothill, 1994), *Acinetobacter baumanii* (Soothill, 1992), *Klebsiella pneumoniae* (Bogovazova *et al*., 1992), *Enterococcus faecium* (Biswas *et al*., 2002), *Vibrio vulnificus* (Cerveny *et al*., 2002), *Salmonella* spp (Toro *et al*., 2005), and *Staphylococcus aureus (Matsuzaki et al., 2005)*.
In those animal model systems also application of phages could be studied. For oral application often a formulation is needed to overcome the low pH of the stomach, e.g. administration of phages with $CaCO_3$ (Williams Smith *et al*., 1987b). Most phages were more active at 37 ^{o}C than at 43 ^{o}C, although some were found that were active at both temperatures. Merrill and his co-workers showed that phage mutants could be selected from the parent phage that remain longer in the circulatory system (Merril *et al*., 1996) due to a change in the amino acid composition of the capsid protein (Vitiello *et al*., 2005).

4.2. Phages in aquatic systems

Actual application of phages to cure animal diseases are found for fish, e.g. *Lactococcus garvieae* and *Pseudomonas plecoglossicida* (Nakai & Se, 2002, Nakai *et al*., 1999, Park *et al*., 2000), *Aeromonas hydrophila* and *Edwardsiella tarda* (Hsu *et al*., 2000). Karunasagar (Karunasagar *et al*., 2007)) showed that two bacteriophages were very effective in reducing *Vibrio harveyi* populations in shrimp hatcheries. A treatment with $2x10^6$ pfu ml^{-1} resulted in over 85% survival of shrimp (*Penaeus monodon*) larvae. The authors conclude that bacteriophages could be an effective alternative to antibiotics in shrimp hatcheries.
Actually, application of phages to control fish pathogens turned out to be very successful. The phages involved are perfectly suited to the environment as they are usually found in the water. The advantage of using therapeutic phages in a water medium is the close and continuous contact that can be achieved between the host, whether fish, crustacean or even mollusk, and phage (Inal, 2003).

4.3. Phages and food

Food quality can be severely affected by bacterial infections. These spoilage infections may occur during pre- and post harvest stages of food production and storage. However some components of the microbiological flora in foods are beneficial from an ecological perspective. Therefore the indiscriminate elimination of all microflora is not desirable. One approach with proven efficacy is the preferential manipulation of the microbiological flora of foods using biopreservation with bacteriocinogenic lactic acid bacteria (LAB). Many dairy products like cheese are the proof of the success of this approach. However, LAB can themselves be infected with bacteriophages resulting in poor or insufficient preservation. The detrimental effects of bacteriophages on the dairy industry have initiated intensive research on the bacteriophages of LAB.
Another strategy to manipulate or modify the microbiological flora of food is the use of bacteriophages. Perishable refrigerated foods such as red meats, poultry and produce can harbor relative large proportions of bacteria (2 to 6 log CFU/gr). An extensive survey of refrigerated foods (Whitman & Marshall, 1971) showed 38 phage-host systems in about 50% of the retail foods examined, including ground beef, pork sausage, chicken and raw skimmed milk. Phages have been applied to control the growth of pathogens such as *Listeria monocytogenes* (LISTEX™ P100, EBI Food Safety, Wageningen, The Netherlands), *Salmonella*, and *Campylobacter jejuni* in a variety of refrigerated foods such as fruit, dairy products, poultry, and red meats. Phage control of spoilage bacteria (e.g., *Pseudomonas* spp. and *Brochothrix thermosphacta*) in raw chilled meats can result in a significant extension of storage life. Phage biocontrol strategies for food preservation have the advantages of being self-perpetuating, highly discriminatory, natural, and cost-effective. A review by Kennedy and Bitton (Kennedy & Bitton, 1987) summarizes phage-host interaction in food. A review by Greer (Greer, 2005) nicely summarizes the use of bacteriophages against food borne bacteria in pre harvest as well as in post harvest control of foods both of plant and animal origin.

Below we will give some examples of the use of bacteriophages in our food chain.

4.3.1. Dairy products

In the dairy industry phages are used to control and eliminate harmful bacteria.
Bacteriophage P100 is specific for *L. monocytogenes* and was thoroughly studied on genome sequence, bioinformatic analyses, oral toxicity study, and application (Carlton *et al*., 2005). This phage is now commercially available (LISTEX™ P100, EBI Food Safety, Wageningen, The Netherlands), and is the first phage product for food that is FDA-approved as GRAS (Generally Recognized as Safe) and applied in the production of all food products.
The ability of phage SJ2 to control *Salmonella* Enteritidis during manufacturing, ripening, and storage of Cheddar cheese produced from raw and pasteurized milk was investigated. It was concluded that the addition of phage may be a useful adjunct to reduce the ability of *Salmonella* to survive in Cheddar cheese made from both raw and pasteurized milk (Modi *et al*., 2001).
Phages are not only tools to get rid of pathogenic bacteria, they can also be threat for beneficial bacteria, such as *Lactobacillus* and *Lactococcus*
Strains of lactic acid bacteria (LAB) including *Lactobaccilus*, *Lactococcus* and *Streptococcus* are commonly used in industrial fermentations for the production of food products such as cheeses, buttermilk, sauerkraut, and yoghurt. It has long been recognised that bacteriophages infecting LAB strains can cause serious problems in dairy fermentations. Already in 1936 the contamination of dairy starter cultures of lactic streptococci with bacteriophages was recognized as a significant challenge to the dairy industry (Whitehead & Cox, 1936). Investigations in milk factories revealed the presence of high numbers of bacteriophage strains (Madera *et al*., 2004) These phage infections result in significant economic losses and therefore research has been focused on elucidating the mechanisms by which these bacteriophages proliferate.

Both classical and molecular approaches have been used to improve strains of bacteria involved in yoghurt and cheese production and to develop starter cultures resistant to bacteriophage and bacteriocin attack (Coffey *et al*., 1994). In recent years, significant advances in molecular biological research tools, genomics and bioinformatics has allowed researchers to gain an insight into the genetic processes underlying many steps in LAB bacteriophage lifecycles. The knowledge gained from this research has in turn enabled the development of novel genetic tools utilizing phage

genes, promotors, and DNA fragments for use in LAB strains (McGrath *et al*., 2002, Sturino & Klaenhammer, 2004b).

4.3.2. Meat and poultry

Retail beef steaks, inoculated with graded amounts of a *Pseudomonas* spp. and homologous phage were studied under different temperatures. Phages were capable to reduce the number of spoilage bacteria and the potential of phage to increase the case life of retail beef steaks was unaffected by temperature within the range of 1 – 10 ^{o}C but was significantly influenced by the interactive effects of initial bacterial density and phage concentration (Greer, 1988).
However, application of a bacteriophage pool to rib eye steaks under retail conditions showed that this was not efficient to control beef spoilage (Greer & Dilts, 1990).

Another strategy is that of pre-harvest control: reduce food-borne pathogens in animals prior to slaughter through the use of bacteriophages. Reducing the numbers of pathogens entering the slaughter plant should reduce the exposure of pathogens to consumers (Callaway *et al*., 2004).
In the United States a bacteriophage was approved for hide washing (OmniLytics) in order to kill *E. coli* O157:H7 on the hides of live animals just before they are slaughtered.
Also the reduction of *Campylobacter jejuni* (Atterbury *et al*., 2003) and *Salmonella* and *Campylobacter* (Goode *et al*., 2003) on chicken skin by application of lytic bacteriophages was reported.

Oral bacteriophage therapy was used for reducing *Campylobacter jejuni* colonization of broiler chickens. The phage treatment resulted in *campylobacter* counts falling between 0.5 and 5 $\log^{10}$ CFU/g of cecal contents compared to untreated controls over a 5-day period post administration. These reductions were dependent on the phage-*Campylobacter* combination, the dose of phage applied, and the time elapsed after administration. *Campylobacters* resistant to bacteriophage infection were recovered from phage-treated chickens at a frequency of less than 4%. These resistant types were compromised in their ability to colonize experimental chickens and rapidly reverted to a phage-sensitive phenotype *in vivo* (Loc Carrillo *et al*., 2005). Also in The Netherlands it was concluded that phage treatment is a promising alternative for reducing *C. jejuni* colonization in broilers (Wagenaar *et al*., 2005). The concentration of *Salmonella* Enteritidis PT4 in broilers could be reduced by oral treatment with a mixture of bacteriophages and therefore the contamination of poultry products by this food-borne pathogen can be reduced (Fiorentin *et al*., 2005)

Removal of pathogens from the intestinal microflora using bacteriophages (or predatory bacteria such as *Bdellovibrio* spp.) has had mixed successes. A mechanism that could explain these findings is based on competitive hindrance by non-prey, or decoy species. It is shown that this hindrance tends to damp out predator-prey oscillations, and therefore reduces the probability of prey extinction. This theory, put forward by Wilkinson (2001), explains that an ecosystem becomes more stable by the presence of decoys, so that the oscillatory behavior needed to drive the prey to (near) extinction is reduced. Only when the prey (or host) is abundant compared to other species can the predators, such as lytic phages, reach the same levels at steady state as in the absence of decoys (Wilkinson, 2001).

4.3.3. Sea food

Another application of bacteriophages was found in sea-food. Oysters infected with *Vibrio vulnificus* can present a serious health risk for diabetic, immunocompromised, and iron-deficient individuals. Two natural oyster-associated components were used to fight this bacterium: pooled *V. vulnificus*-specific bacteriophage and an extract of the eastern oyster (*Crassostrea virginica*) that contains a component bactericidal for *V. vulnificus*. Although these components could reduce *V. vulnificus* numbers alone, the simultaneous use of both components was more successful (Pelon *et al*., 2005).

4.3.4. Fruits and vegetables

Fresh-cut fruits and vegetables raise food safety concerns, because exposed tissue may be colonized more easily by pathogenic bacteria than intact produce. This is due to the higher

availability of nutrients on cut surfaces and the greater potential for contaminations because of the increased amount of handling. A mixture of four distinct *Salmonella* Enteritidis phages gave a significant reduction in the number of Salmonella recovered from artificially inoculated melon slices under refrigeration and abusive temperatures (Leverentz *et al*., 2001). Remarkably, none of the surviving bacteria were phage resistant. The same workers treated *L. monocytogenes* populations on slices of apple and honeydew melons by spraying or pipetting *L. monocytogenes*-specific phages. Treatments reduced bacterial numbers, but the effectiveness depended on the initial concentration of *L. monocytogenes* (Leverentz *et al*., 2003). It should be noted that the phage spray application could be optimized by adjusting concentration and timing (Leverentz *et al*., 2004). In contrast to these successful results, the numbers of *Salmonella* Enteritidis and *L. monocytogenes* on artificially inoculated apples slices were unaffected by phage treatment. This was attributed to the more acid environment of the apple in comparison to the melon slices. Phages could not persist on the apple slices and were not longer detectable 24 hours after application (Leverentz *et al*., 2001).

Sprout contamination has caused numerous food borne disease outbreaks. For example, in 2000, a *Salmonella* Enteritidis outbreak in The Netherlands was linked to eating contaminated bean sprouts (Van Duynhoven *et al*., 2002). A number of seed-sanitizing methods, including chlorine treatments, have been used in sprout production to enhance food safety (Beuchat *et al*., 2001). In sprout production, soaking seeds in water for up to 12h causes seeds to swell and hulls to soften, facilitating sprout growth. Research to applying phages in hydroponic systems, during the soaking process, may help in developing alternative, organic methods for ensuring sprout safety. Pao *et al*, (Pao *et al*., 2004) investigated the use of bacteriophages to control *Salmonella* in experimentally contaminated sprout seeds. Suppression of *Salmonella* growth was found after applying Phage-A on mustard seeds and a mixture of Phage-A and Phage-B in soaking water of broccoli seeds. Host specificity observed in this study stresses the importance of developing phage mixtures that can control a broad range of potential contaminants.

4.3.5. Natural phage defense mechanisms

Many LAB possess natural phage defense mechanisms. The most extensively studies are those of the *Lactococci*. In the 1980s it was discovered that many of these mechanisms resided on naturally occurring plasmids (Klaenhammer & Sanozky, 1985, McKay & Baldwin, 1984). Nowadays four main groups of natural defense systems are recognized on the basis of their mode of action:

- adsorption interference, which prevents the adsorption of phage particles to the cell surface.
- DNA injection blocking, which allows phages to attach to the cell surface but prevents DNA penetration into the cell cytoplasm
- Restriction/modification, causing intracellular degradation of incoming DNA molecules
- Abortive infection which encompasses a range of mechanisms any of which at any time interferes with successful phage development.

Over the years, the use of natural phage-defense systems proved to be invaluable for the protection of strains that are expected to perform consistently and over extended time frames within industrial applications. For more detailed reviews on these mechanisms see (Allison & Klaenhammer, 1998, Daly *et al*., 1996, Dinsmore & Klaenhammer, 1995, Forde & Fitzgerald, 1999, Garvey *et al*., 1996, Hill, 1993, Sturino & Klaenhammer, 2004b).

Genetically engineered phage defense mechanisms
Especially the complete or partial sequencing of a number of LAB phage genomes and their analysis and comparison has enabled the development of a number of ‘genetically engineered’ resistance systems. These systems are not based on the genetic engineering of phages themselves but rather on the genetic modification of specific LAB plasmid with specific phage genes or sequences (McGrath *et al*., 2004, McGrath *et al*., 2002, Sturino & Klaenhammer, 2004b, Sturino & Klaenhammer, 2006).
The most important mechanisms are:

- Phage encoded resistance (Per)

Lactococcal phage *origin of replication* (ori) sequences are supplied *in trans* on a plasmid vector. The resulting resistance phenotype is characteristic of an abortive infection mechanism. The

number of bacteriophages genomes replicated is reduced and plasmid–associated replication factors catalyze an increase in plasmid copy-number. This strategy was successfully employed against p335-type phages φ50 (Hill *et al*., 1990), φ31 (O'Sullivan *et al*., 1993) as well as different *S. thermophillus* phages (Stanley *et al*., 2000).

- Antisense RNA

Antisense technology has been tested against target genes from different *S. thermophilus* and lactococcal phages. However results have been variable. Generally early-expressed genes involved in genome replication have been more effective targets than genes expressed later in the lytic cycle. Sturino and Klaenhammer (2004a) list a number of successful and unsuccessful RNA antisense phage defense strategies.

- Bacteriophage triggered defense

In bacteriophage triggered defense systems the expression of a toxic gene product is put under the control of a phage-inducible promotor. Expression of such a system located on a high-copy plasmid upon bacteriophage infection will lead to the death of the bacterium, aborting the phage infection. The combination of a φ31 phage-inducible promoter with the *Lla*I restriction modification system from a lactococcal plasmid resulted in the death of the host cell harboring this plasmid upon infection with φ31 (Djordjevic & Klaenhammer, 1997).

- Subunit poisoning

The expression *in trans* of mutant proteins suppressed the function of native, multimeric proteins in a dominant negative fashion in *S. thermophilus* (Sturino & Klaenhammer, 2004a). Multiple alignments of the putative primase, a component of the phage Sfu21 type genome replication module encoded by the *S. thermophilus* phage κ3, were used to identify invariant and highly conserved amino acids within a phage ATPase/helicase domain. Expression *in trans* from a high-copy number plasmid of several different mutant proteins appeared to completely inhibit phage replication.

- Host factor elimination

Certain bacterial host factors have been shown to be essential for phage replication (Lucchini *et al*., 2000). Specifically blocking these factors (i.e genes) through mutagenesis was shown to confer phage resistance. Mutagenesis of one particular open reading frame (*orf 394*) of *S. thermophilus* conferred complete resistance to 15 *S. thermophilus* phages.

- Superinfection exclusion and immunity

The increasing amount of information on bacterial genomes has shown that approximately half contain prophage associated sequences (Lawrence *et al*., 2001). Generally only a very small portion of these sequences are transcribed in the lysogenic state and it is assumed that maintaining this extra genetic information should decrease the bacteriophage's fitness. However, it is also known that some prophage associated sequences can provide advantages to the lysogen by encoding one or more factors that increase the fitness of the bacterium. In *S. thermophilus* two genes, the superinfection-immunity gene and the superinfection-exclusion gene, give a selective advantage by providing protection to subsequent bacteriophage infection. Expression from a high-copy number plasmid of gene *orf203* in *S. thermophilus* conferred protection to 12 of 21 phages tested (Bruttin *et al*., 2002), presumably by blocking phage genome injection ('superinfection exclusion'). Expression from a recombinant plasmid of the CI-like repressor protein of phage Sfi21 (encoded by *orf127*) prevented the multiplication of the homologous temperate phage (Bruttin *et al*., 1997), presumably by repression of the lytic functions of the superinfecting phage ('superinfection immunity').
Very recently an apparently new basic resistance mechanism of Bacteria and Archae against bacteriophages was described (Barrangou *et al*., 2007). Earlier, detailed genomic comparisons of closely related *S. thermophilus* strains had revealed that the genetic polymorphism primarily occurs at hypervariable loci, such as the *eps* and *rps* operons, as well as in two clustered regularly interspaced short palindromic repeats (CRISPR) loci. These CRISPR loci typically consist of several non-contiguous direct repeats separated by stretches of variable spacers and are often adjacent to *cas* genes (CRISPR-associated genes). Remarkably *in silico* analyses of the CRISPR spacer sequences has shown sequence homology with bacteriophage and plasmid sequences and it is speculated that CRISPR and *cas* genes provide immunity against foreign genetic elements like

bacteriophages through a mechanism based on RNA interference (Makarova *et al*., 2006). Detailed comparisons between several *S. thermophilus* strains including some phage-resistant, revealed differences at the CRISPR1 locus. Additional spacer sequences only present in phage resistant strains suggested a correlation with differences in phage sensitivity. Experiments showed that indeed CRISPR loci are altered during the natural generation of phage-resistant mutants. Additional inserted spacers had sequence similarities with random sequences throughout the genomes of the phages used in the challenge. Phage resistance was linked to the CRISPR spacer content i.e. the level of sequence similarity between the spacer and the phages containing the particular spacer sequence. These results suggest that prokaryotes have evolved a nucleic-acid based "immunity" system in which CRISPR spacer (and *cas* genes) provide resistance to bacteriophages.

4.4. Phage therapy for bacterial diseases of plants

Plant pathogenic bacteria cause several plant diseases in crops. Usually, chemicals are sprayed to fight these diseases. In 1934, Massey suggested that phage was a prime factor in limiting severity of bacterial blight disease in field-grown cotton, when the incidence of bacterial blight was reduced in fields that had been flooded by the Nile river (Massey, 1934). Early workers had promising results when treating seeds with phage isolated from plant material to fight Stewart's wilt disease (*Pantoea stewartii*) in corn (Thomas, 1935). However, when the use of bacteriophages to control bacterial diseases in plants was explored (Okabe & Goto, 1963), the conclusion was, as Goto wrote later (Goto, 1992), that they were not successful in controlling the bacterial diseases in the field.

Phage therapy was attempted to control bacterial leaf spot in e.g. mungbeans (Borah *et al*., 2000), *Xanthomonas pruni* associated bacterial spot of peaches (Civerolo & Kiel, 1969), *X. campestris* of peach trees (Randhawa & Civerolo, 1984), fire blight and soft rot caused by *Erwinia* (Eayre *et al*., 1990, Erskine, 1973), fire blight of pear and apple (Gill *et al*., 2003, Schnabel *et al*., 1998) and bacterial blight (*X. campestris* pv. *Pelargonii*) of Geranium (Flaherty *et al*., 2001).

One of the main problems in spraying bacteriophages on plants to cure them from bacterial diseases is to keep phages infective in the phyllosphere, the aerial plant structures. The phyllosphere is a rather hostile environment for viruses, due to the exposure to ultra violet light, intensive visible light and desiccation (Gill & Abedon, 2003). Also temperature and exposure to copper bactericides influence the survival of the phages (Iriarte *et al*., 2007). Recent work has been done on the management of bacterial spot (*X. campestris* pv. *Vesicatoria*) on tomato by bacteriophages (Flaherty *et al*., 2000). Protective formulations were developed to increase the longevity of these phages on plant surfaces (e.g. casecrete or skim milk) (Balogh *et al*., 2003). Bacteriophage preparations against bacterial spot in tomato and pepper are commercially available now (Agriphage, Omnilytics Inc. Salt Lake City, US) and were tested in a management system of tomato bacterial spot in the field. Application of host-specific bacteriophages was effective against the bacterial spot disease, providing better disease control than chemical treatments (copper-mancozeb) or untreated control. When results of the disease severity assessments or harvested yield from the bacteriophage-treated plots were grouped and compared with the results of the corresponding nonbacteriophage group, the former provided significantly better disease control and yield of total marketable fruit (Obradovic *et al*., 2004).

For a recent overview of the use of bacteriophages for plant disease control see (Jones *et al*., 2007).

In the former Soviet Union the application of bacteriophages against plant bacterial diseases has been fairly well studied (see Appendix). In a recent study at the Taras Shevchenko Kyiv National University (V. Polischuk, personal communication) four separate bacteriophages were tested to control *Pseudomonas syringae* on sugar beets plants in experimental field plots. Plants obtained from sterile seeds that had been soaked in phage suspension for 3-4 hours were transferred to the field and treated a second time by spaying with a phage suspension 30 days after planting. Plots treated with phages clearly scored better with respect to germination efficiency, average root mass, average weight and sugar yield and average sugar content in comparison to untreated control plots.

The following table (taken from Greer, 2005) summarizes a number of uses of bacteriophages to control bacterial pathogens preharvest.

Food production system	**Disease/symptom**	**Bacteriophage host strain**
Cultivated mushrooms	Bacterial blotch	*Pseudomonas tolaasii*
Tomatoes	Bacterial spot	*Xanthomonas campestris* pv. *Vesicatoria*
Apples	Fire blight	*Erwinia amylovora*
Stone fruits	*Prunus* bacterial spot	*X. campestris* pv. *Pruni*
Sprouts	Seed contamination	*Salmonella* Enteritidis
Fish	Red fin disease hemorrhagic ascites	*Aeromonas hydrophila* *Pseudomonas plecoglossicida*
Chickens	Cecal *Salmonella* lethal infection respiratory infection growth depression	*Salmonella* Enteritidis *Salmonella* Typhimurium *Escherichia coli* *Streptococcus faecium*
Beef cattle	Bacterial shedding	*E. coli* O157:H7
Calves, piglets lambs	Diarhea, lethal infection	Enterpathogenic *E. coli*
Sheep	Bacteria in rumen, faeces, colon	*E. coli* O157:H7
Dairy cattle	Mastitis	*Staphylococcus aureus*
Pigs	Tonsil and cecal *Salmonella*	*Salmonella* Typhimurium

The following table (taken from Greer, 2005) summarizes a number of uses of bacteriophages to control bacteria in food.

Food	**Bacteriophage host strain**
Melon and apple slices	*Listeria monocytogenes* *Salmonella* Enteritidis
Milk	*Staphylococcus aureus* *Pseudomonas fragi*
Cheese	*Salmonella* Enteritidis
Chicken skin	*Campylobacter jejuni* *Salmonella* Enteritidis
Retail chicken	*Salmonella* Typhimurium DT104
Chicken frankfurters	*Salmonella* Typhimurium DT104
Beef steaks	*Pseudomonas* spp. (spoilage control) *Escherichia coli* O157:H7
Vacuum packed beef	*L. monocytogenes*
Pork fat	*Brochotrix thermosphacta* (spoilage control)

5. Possible problems in the applications of phages

5.1 Bacteriophage specificity

Most antibiotics in use today are broad-spectrum and kill many different bacteria. The disadvantage of this is that also beneficial bacteria are killed, especially bacteria that reside and act in the intestinal tract. Bacteriophages, due to their often extreme host specificity do not have this disadvantage.

For therapeutical applications the high host specificity of phages can also be a disadvantage since the correct combination of host and phage needs to be determined and, if necessary, adjusted. Mutations or other changes in the target bacterium population could lead to the loss of the specific recognition/interaction between the phage and the bacterium. (Re)selection of phages from a collection or the use of cocktails would then be necessary to ensure efficiency of the bacteriophage treatment.
In many studies however, loss of bacteriophage specificity has rarely been observed. Upon infection of the bacterium, lysis generally occurs very fast, sometimes even within 30 minutes. The exponential amplification of the phage results in a very rapid decline of the target bacterial population. This leaves very little room for adaptation of the bacterium. Clinical bacteriophage therapy generally lasts only for a limited time. In some cases some bacteria survived the initial bacteriophage treatment (Williams Smith & Huggins, 1982). Phage treatment of *E. coli* strain MW infected mice resulted in lower death rates, but a few phage resistant MW bacteria were isolated from the phage treated mice. These mutants were essentially non-toxic because of their inability to invade tissues and greatly reduced virulence. If resistant bacteria occur, a simple remedy is the re-isolation of a new phage from an existing library using the surviving bacterium.

5.2 Bacteriophage immunogenicity

Bacteriophages can evoke an immunological reaction in mammalian bodies and they will eventually be degraded in liver and spleen. Practical experience however, shows that this effect sometimes does occur but usually too late to have serious negative effects on the treatment. Kucharewicz-Krukowska and Slopek (Kucharewicz-Krukowska & Slopek, 1987) analyzed the immunogenicity of anti-staphylococcal phage, comparing titers of haemagglutinating and neutralizing antibodies before and after phage therapy for 57 patients. Among the 57 patients, 44 had no measurable antiphage antibodies at any time during treatment (77%), and in 8 cases titers never exceeded 1:40 (14%). In only two cases (4%) high titers of antiphage antibodies were generated (1:320 – 1280). The authors conclude that immunogenicity of this type of phage usually does not impede therapy.
Weber-Dabrowska *et al.* (Weber-Dabrowska *et al*., 2003) conclude that the production of phage-specific antibodies is part of the normal body response to bacterial infection and the accompanying multiplication of phages. However the immunogenic effect of phages was weak in the majority of the patients (Kucharewicz-Krukowska & Slopek, 1987) and phage therapy is usually complete before specific immunity develops.

5.3 Bacterial cell lysis

Bacterial infections generally lead to inflammatory reactions which in turn may lead to serious complications in septic patients. Endotoxins are an important factor in the elicitation of an inflammatory response to infection. Endotoxins can be released as a result of the normal release of cell wall fragments from gram-negative bacteria or as a result of bacterial lysis either through antibiotics or bacteriophages.
There is little to no evidence that bacteriophage induced lysis leads to increased clinical problems in patients. Some studies even reported that lytic phages improved survival from sepsis (Merril *et al*., 1996, Sulakvelidze & Morris J.G, 2001). One study (Matsuda *et al*., 2005) describes the use of an amber mutant in the holin encoding *t* gene of a T4 phage. This LyD phage is deficient in the production of holin in a bacterial cell lacking an amber surpressor tRNA and could kill infected cells with significantly diminished lysis. Balb/C mice injected with B40sul *E. coli* were treated intraperitoneally with LyD phage, wild-type phage or β-lactam antibiotic and followed for survival. It

was concluded that “LyD phage therapy significantly improves survival and attenuates the systemic effects of bacterial sepsis……”. However *E.* coli levels in LyD phage treated mice are significantly higher in comparison to mice treated with wild-type phage or antibiotics.

6. Improvement of bacteriophages

The renewed interest in bacteriophages and phage therapy has lead to questions on the possible improvement of phages and their effectiveness. With current molecular biological techniques and the increasing knowledge of phage genomes it is suggested that phages can be engineered to improve effectivity, host range etc.

Only a limited number of studies on attempts to improve the therapeutical potential of bacteriophages have been published.
One study (Merril *et al*., 1996) describes the selection of so-called long-circulating bacteriophage mutants that were capable of a much longer survival in the blood of mice. Earlier it had been postulated that the rapid elimination of phages from the circulatory system had limited the efficacy of phage therapy, either by phage-directed antibodies (Stent, 1963) or by the reticuloendothelium system (RES; (Geier *et al*., 1973). Repeated serial passages of *E. coli* phage λ and *S. thypimurium* phage p22 through mice selected for phages that remained significantly longer in circulation. Two phage λ variants showed a 16,000 and 13,000 fold increase in evading the RES entrapment. Both variants contained an identical mutation in the bacteriophage capsid E protein (glutamic acid ➔ lysine). Also long-circulating mutants of p22 were obtained which prompted the authors to suggest that their method could be used as a general method to obtain phages capable of reduced capture by RES.

Another study describes the use of a nonlytic phage to specifically target and deliver DNA encoding bactericidal proteins to bacteria (Westwater *et al*., 2003). An M13 phagemid was used for the cloning of two genes encoding toxins Gef and ChpBK. Phage delivery of this recombinant phagemid to *E.* coli resulted in significantly reduced bacterial growth by several orders of magnitude *in vitro* and in a bacteremic mouse model of infection.

Bacteriophage lytic activity
Lysis of bacterial cell walls is based on the action of two phage encoded enzymes: holins and lysins.
Lysins are phage-encoded murein hydrolases that act on the bacterial host cell wall at the terminal stage of the phage reproduction cycle to release progeny phage. These enzymes are also known as phage lysozymes, endolysins, or muralytic/mureolytic enzymes. Their action is tightly regulated by holins, by membrane arrest, and by conversion from their inactive state. Their N-terminal catalytic domains are able to target almost every possible bond in the peptidoglycan network, and their corresponding C-terminal cell wall binding domains target the enzymes to their substrate. Owing to their specificity and high activity, endolysins have been employed for various *in vitro* and *in vivo* aims, in food science, in microbial diagnostics, and for treatment of experimental infections. Clearly, phage lysins represent great tools for use in molecular biology, biotechnology and in medicine, and we are just beginning to tap this potential.
There are at least two distinct mechanisms by which phages destroy the cell wall. Bacteriophages with large genomes use a holin-endolysin system, while bacteriophages with small genomes encode a single lysis protein. Some single protein lysis systems inhibit cell wall synthesis and are thus the phage analogs of antibiotics like penicillin (Bernhardt *et al*., 2002). Sometimes also phage capsid proteins are responsible for lysis (Bernhardt *et al*., 2001).

In gram-positive bacteria, (endo)lysins can also act as exolysins because the pepticoglycan layer of the bacterial cell wall is, in most cases, accessible from the outside. This is not the case for gram-negative bacteria, in which the presence of the outer membrane effectively prevents access by hydrophilic lytic enzymes. However, when the lipopolysaccharide layer is disrupted (by EDTA, detergents, etc.) cells become sensitive to external murein hydrolases (Loessner, 2005). Also high pressure seems to be effective, so was the inactivation of gram-negative bacteria described in milk and banana juice by lambda lysozyme under high hydrostatic pressure (Nakimbugwe *et al*., 2006).

Lysin domains can be cloned into non-host bacteria and purified after expression (Loeffler *et al*., 2001, Nelson *et al*., 2006) and used for different applications.
The use of the lytic enzyme of a pneumococcal bacteriophage, Cpl-1, was effective as an intravenous therapy for pneumococcal bacteria in a mouse model. Cpl-1 is also effective as a topical nasal treatment against colonization by S*. pneumoniae*. In vitro, the enzyme is active

against many serotypes of *S. pneumoniae*, independent of their penicillin resistance, and it is very specific for this species (Loeffler *et al*., 2003). The bacteriophage lysis PlyV12 was effective in killing *Enterococcus faecalis* and *E. faecium*, also antibiotic-resistant strains, and its development and use as an alternative therapeutic tool was proposed (Yoong *et al*., 2004). Phage enzymes proved to be highly efficient at killing pathogenic bacteria, therefore, they could be a valuable tool in controlling biowarfare bacteria. For instance, experiments with the lytic enzyme PlyG showed that at least ten strains of *Bacillus anthracis* were killed by this lysin.

Lysins have now been used successfully in animal models to control pathogenic antibiotic resistant bacteria found on mucosal surfaces and in blood. The advantage over antibiotics are their specificity for the pathogen without disturbing the normal flora, the low chance of bacterial resistance to lysins and their ability to kill colonizing pathogens on mucosal surfaces, capabilities that were previously unavailable. Thus, lysins could be an effective anti-infective in an age of mounting antibiotic resistance. A potential concern in the use of lysins is the development of neutralizing antibodies. Unlike antibiotics, which are small molecules that are generally not immunogenic, enzymes are proteins that stimulate an immune response when delivered both mucosally or systemically. It was found that highly immune serum slows, but does not block, the killing of bacteria by lysins (Fischetti, 2005).
Similar to other proteins that are delivered intravenously to animals and humans, phage enzymes have a short half life (ca. 15 min.). However, the action of lysins for bacteria is so rapid, that this might be sufficient time to observe a therapeutic effect (Fischetti, 2005, Loeffler *et al*., 2003).

Because of the specific action of lysins, they offer a unique possibility for the biological control of unwanted bacteria without having any effect on other organisms. The most obvious approach to the use of lysins for the biocontrol of pathogens in food and feed is to directly add purified enzyme to the food or to the raw product. A more elegant and also less expensive alternative is the production and secretion of specific endolysins by fermenting bacteria (see also (Gaeng *et al*., 2000). In this case the cell wall of the host bacterium must be insensitive to the produced lysin. By contrast, some applications aim to cause self-destruction, which is mediated by cells carrying endolysin genes that are able to degrade their own murein. In the production of cheese, it is hoped that controlled lysis of Lactococcus starter strains would result in leaky cells and might aid in accelerated cheese ripening (De Ruyter *et al*., 1997).

As bacteria get resistant to phages quite rapidly, they could also become resistant against lysins. However, repeated exposure of several bacteria grown on agar plates to low concentrations of lysins did not lead to the recovery of resistant strains. The cell wall receptor of lysins of *S. pneumoniae* is choline (Garcia *et al*., 1983), a molecule that is essential for pneumococcal viability. Although not yet proven, it is possible that during interaction of phage and bacteria over the millennia, to avoid becoming trapped inside the host, the binding domain of the lytic enzymes has evolved to target a unique and essential molecule in the cell wall, making resistance to these enzymes a rare event (Fischetti, 2005).

7. Alternative applications of bacteriophages

7.1. Phage-display systems

One of the most well-known applications of bacteriophages is their use in phage-display systems. Different types of phages have been used in phage display strategies including lambda and T7 and Ff filamentous phages (Danner & Belasco, 2001, Rodi & Makowski, 1999, Willats, 2002). The most commonly used phage however, is the filamentous non-lytic phage M13. This phage is a cylindrical particle of approx. 880 nm long and 6.6 nm wide, containing a circular single-stranded DNA genome. The genome encodes nine different proteins; five structural proteins and four non-structural. With prox. 2700 copies per particle, pVIII is the major coat protein (CP) while pIII, pIV, pVII and piX are the minor CPs with ~ 5 copies per phage particle. Insertion of extra DNA into the genome of M13 simply results in assembly of longer phage particles (Willats, 2002).
Recombinant phages are created by fusion between a foreign DNA fragment and any of the five CP encoding genes. Upon infection of a suitable host bacterium resulting recombinant phages express the fusion proteins on the surface of the phage particle. One drawback of this system is that the coat protein function of the recombinant CP is altered often resulting in reduced infection of the host cell (i.e. *E.coli*). This has been solved by the development of phagemid/helper-phage systems. Phagemids (plasmids with a phage origin-of-replication) carry the recombinant fusion sequences while the helper phage carries the majority of the genes required for the formation of phage particles. Co-infection of the *E. coli* host cells of recombinant phagemids and helper phages leads to phage particles expressing the fusion proteins on their surface. The most widely used systems employ fusion proteins with the pIII CP which is present on one end of the phage particle.

Phage-display systems have been used extensively for the selection of genes encoding the reactive sites of antibodies; the so-called single chain variable fragments (scFv). Cloning of scFv encoding DNA fragments in fusion with the pIII CP, results in expression of the scFv at the end of the phage particle. In a so-called panning procedure phage particles expressing the desired scFv are captured and used to infect *E. coli* cells. Repeated panning procedures will result in selection of the phage carrying the gene encoding the desired scFv. This scFv gene can then subsequently be expressed in a suitable prokaryotic expression system (Brichta *et al*., 2005).

The phage-display technology is generally regarded as a powerful technique to select molecules and ligands against purified target proteins. Infection of specific strains of *E.* coli cells depends on the recognition and binding of the phage to the so-called F-pili.These are encoded by an F-plasmid which needs to be present in the recipient *E. coli*.
Phage-display mediated selection of ligands on complex surfaces like living cells is much less effective and hampered by the inability of the phage to infect eukaryotic cells.
In an elegant study it was shown however that a filamentous phage engineered to display the basic fibroblast growth factor ligand FGF2, was capable of binding to mammalian cells through the FGF receptor (Larocca *et al*., 1998) and even deliver a gene through binding to the FGF receptor resulting in transduced cells (Larocca *et al*., 1999). In another study (Kassner *et al*., 1999) these authors displayed epidermal growth factor (EGF) on M13 phages that contained a fluorescent green protein (FGP) in its genome. These phages were capable of delivering the GFP gene to EGF-bearing Cos1 cells in a ligand-, time-, and phage concentration-dependent manner.
A similar study (Poul & Marks, 1999) describes growth factor receptor ErbB2 mediated endocytosis of F5 phages expressing this receptor. The phages were additionally engineered to express the GFP reporter gene driven by a CMV promoter. Endocytosis of these phages resulted in cellular GFP expression.
These studies show that filamentous phages can be used to screen for (genes coding for) mammalian cells binding ligands (in a way similar to the identification of scFv encoding genes in a phage-display system). It also demonstrates that filamentous phages can be engineered to deliver genes or genetic constructs to mammalian cells and shows their potential as targeted gene delivery systems.

7.2. Bacteriophage-based diagnostics

The specificity of bacteriophages for their hosts can be utilized for the very specific detection of bacteria. In 1987 Ulitzur and Kuhn (Ulitzur & Kuhn, 1987) constructed a recombinant λ phage by inserting the *luxAB* gen from *Vibrio fischeri* into its genome. Upon infection of *E. coli,* the *luxAB* gene was transduced and after addition of the requisite aldehyde substrate these cells showed a bioluminescent phenotype. Since then this technique has been applied to other phages for the low-level detection (10-1000 cells) of *L. monocytogenes* (Loessner *et al*., 1996), *S. typhimurium* (Chen & Griffiths, 1996), *E. coli* O157:H7 (Waddell & Poppe, 2000), enteric bacteria (Kodikara *et al*., 1991) and *S. aureus* (Pagotto *et al*., 1996) within a variety of food matrices. Other reporter molecules like the firefly luciferase (Sarkis *et al*., 1995), ice nucleation (*inaW*) (Wolber & Green, 1990), β-galactosidase (*lacZ*) (Goodridge & Griffiths, 2002), and green fluorescent protein (*gfp*) (Funatsu *et al*., 2002, Oda *et al*., 2004) have also been used to construct recombinant phages for the detection of food-borne bacteria like *Mycobacterium, Salmonella* and *E. coli*. Other fluorescent dyes have also been used for labeling of reporter phages (Mosier-Boss *et al*., 2003). Reporter phages were combined with immunomagnetic separation for capture, concentration and identification of bacteria (Favrin *et al*., 2001, Goodridge *et al*., 1999). Generally detection can be quite sensitive but requires the addition of a specific substrate and is also dependent of the matrix in which the method is applied.
As an alternative Ripp *et al.* (Ripp *et al*., 2006) have developed a binary recombinant phage system based on a *luxAB* recombinant λ phage and a specifically constructed bioreporter *E.*coli cell. Infection of the desired target bacterium with the *luxAB* recombinant λ phage will lead to the production and excretion of a acyl-homoserine autoinducer (AHL). This AHL will interact with the bioreporter *E.* coli which will lead to bioluminescence of the reporter cell. The initial phage infection of the target bacterium will lead to a autoamplified chemical signal.

7.3. Bacteriophage-based bacterial typing

Another application based on the high specificity of bacteriophages for their hosts is bacteriophage typing. This method has been used for many decades for the characterization and types strains of medically important bacterial species. In 1961 even an official World Health Organization (WHO) report was published (Blair & Williams, 1961) in which standardized methods for the typing of *S. aureus* strains and the interpretation of the test results were described. Newer techniques like pulse-field gel electrophoresis (PFGE) have now largely replaced phage typing of *S. aureus* (Bannerman *et al*., 1995, Zadoks *et al*., 2002).
Phage typing can still be a valuable method but will obviously depend on (the variability of) the bacterium and its strains under investigation and the availability of a large enough library of suitable phages capable of distinguishing bacterial strains or (eco) types. In an extensive study (Toth *et al*., 1999) in which different phenotypic and molecular methods were compared for determining diversity in the phytopathogenic bacterium *Erwinia carotovora* subspp. atroseptica, phage typing proved to provide the highest level of diversity within Eca.

8. General conclusions

The history of bacteriophage research is long and extensive. Ever since their first discovery as an agent of unknown etiology they have been studied and used for various purposes. With increasing advances in technology more and more became known about their structure and ecology. With the publication of the complete genome of page ΦX174 in 1977, the age of 'genomics' started and it opened up a new field of using bacteriophages as "tool boxes" for the study and manipulation of bacterial genes and genomes.
The study of bacteriophages has contributed greatly to the understanding of bacterial virulence and evolution. The availability of bacteriophage genome information has also contributed significantly to recognizing the role of prophage-encoded virulence factors in infectious bacteria and their contribution in bacterial genome diversity and horizontal gene transfer.

In addition bacteriophages have been used extensively as therapeutic agents against a wide range of bacterial pathogens of humans, animals and plants. This use started already early after their first discovery at the beginning of the 20th century and before the Second World War the therapeutic use of bacteriophages was studied intensively both in Western countries as well as in countries of the former Soviet Union.
After the Second World War the therapeutic use of bacteriophages was abandoned in the West but continued in Eastern Europe. Remarkable results in the cure of many serious bacterial diseases were obtained but little of that information ever reached the West.

The therapeutic use of bacteriophages is based on the use of naturally occurring phages. These phages can be obtained from various sources (sewage water is an apparently rich source) and their identification as a therapeutic against a particular bacterium is mostly based on screening bacteriophage libraries for the right lytic bacteriophage. The Eliavan Institute in Tbilisi, Georgia, still has one of the most extensive bacteriophage collections worldwide. Unfortunately, no information is available on the precise taxonomic position and characteristics of the successful therapeutic bacteriophages. Genomic information is sadly lacking but it would probably reveal a wide diversity of the bacteriophages employed.

From all available literature on the use of bacteriophages as anti-bacterial agents it becomes clear that there are very little to no adverse effects on humans, animals or plants, even after prolonged use. Bacterial resistance to bacteriophages develops very slowly, if at all, and since the bacteriophage is able to co-evolve, does not become a serious issue as with modern antibiotics. Immunogenic responses to bacteriophage treatment are rare and generally insignificant and even bacterial toxins which might be released upon bacterial lysis are not reported to cause serious problems. Phages disappear rapidly after their hosts are destroyed and leave no traces of their presence.

There is a renewed interest in the West in bacteriophages and since the 1980's many studies have been published showing their potential. Many companies have been started to pursue commercialization of bacteriophages, however, up to this point only a few bacteriophages are commercially exploited. Most notably are:

- LISTEX^{TM} P100 (EBI Food safety Wageningen, The Netherlands) a phage against *Listeria monocytogenes* which was recently approved by the US Food and Drug Administration as GRAS (generally regarded as safe) for use in all types of food products.
- Agriphage (Omnilytics Inc. Salt lake City, USA) against bacterial spot (*Xanthomonas campestris* pv. *Vesicatori)* or bacterial speck (*Pseudomonas syringae* pv. tomato) of tomato crops.

Many comments point to the problems they expect in approval of bacteriophages as human therapeuticals. The whole process of clinical trials will be long and expensive while the issue of intellectual property rights for a phage (which can easily be pirated and grown and distributed illegally) is still unclear. Despite the track record of bacteriophages so far, regulatory bodies are expected to raise (serious?) concerns about their safety upon public release (bacteriophages are after all viruses which are generally regarded as foes, not as friends). Some of these questions may be legitimate since there is still very little understanding what factor determine that phages

becomes lysogenic and incorporates its genome in its host. Can this be completely excluded for therapeutic lytic phages? Many unanswered questions remain.

There have been many speculations at the possibilities to improve the therapeutic effectiveness of bacteriophages through genetic modifications. Studies on the factors that determine host specificity of particular bacteriophages have been published but to our knowledge very few studies on extended host range through genetic modifications have been published. One study (Westwater *et al.*, 2003) describes a recombinant non-lytic M13 phage that was genetically modified to kill bacteria. Two other interesting studies (Poul and Marks, 1999; Kassner *et al.*, 1999) describe a modified M13 phage that was engineered so as to adhere to mammalian cells whereby it genetic material was transduced and expressed in these cells. Theoretically this opens up a lot of interesting applications. These phages could be modified so as to serve as gene therapy vectors but also to deliver specific toxins to for instance cancer cells. The possible risks involved in such applications are obviously unknown yet but appear to warrant serious attention.
Some studies report the selection of particular phages under certain circumstances however there appears to be no evidence that this will lead to changes in host range and only limited changes in other characteristics as longevity or virulence.

In contrast to complete genetically modified bacteriophages, many phage genes and phage genetic elements have been used to control harmful bacteria. Especially in the dairy industry many beneficial lactic acid bacteria (LAB) have been genetically modified with phage genes to express different types of phage resistance (see paragraph 4.3.2.). The genetic material of many phages in recombinant form is thus already present in a large number of food products. The bacteriophage encoded enzymatic lysins and holins may offer interesting alternatives to complete bacteriophages in controlling harmful bacteria. These enzymes can basically be expressed in industrial systems and offer an alternative to the use of ‘live’ bacteriophages.

Certain bacteriophages have been genetically modified for diagnostic purposes or for the expression of particular prokaryotic or eukaryotic genes (e.g. the phage-display systems). Generally these systems are used under very controlled conditions and there are no indications that these modifications lead to changes in host range or virulence.

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Appendix

This appendix contains the complete report as written by Prof Polyschuk and Dr. Shevchenko

Essentials information and transcripts of this report have been integrated into the study above

Bacteriophages in therapy and prophylactics of bacterial diseases: history, state-of-the-art, and perspectives

Valery Polischuk, Oleksiy Shevchenko, Lidiya Semchuk

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1. General conception of phage therapy. Existent preparations of bacteriophages, principles and issues of their production (*V. Polischuk, O. Shevchenko*)
2. Phage therapy and prophylactics of bacterial infections in human and veterinary medicine (*O. Shevchenko, V. Polischuk*)
3. Phages of phytopathogenic bacteria (*L. Semchuk*)
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Appendix – Interview with Head of Laboratory for Bacteriophages, State Research Center for Applied Microbiology (Obolensk, Russia) (*V. Polischuk*)
References

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Final revision and translation: O. Shevchenko

1. General conception of phage therapy. Existent preparations of bacteriophages, principles and issues of their production

The discovery of bacteriophages opened the whole new era of their practical use in human and veterinary medicine as tools for treatment, prophylactics and diagnostics of infectious diseases. Bacteriophages were successfully utilized for wound treatment starting from World War II, however there was nearly put a stop to their use in surgery since the discovery of antibiotics [Revenko, Megrabyan, 1975]. In clinics, the bacteriophages were first employed as therapeutics for infectious diseases in 1930ies [Samsygina, Boni, 1984].

Among the registered factors which drastically diminish therapeutic value of antibiotics are their toxic side effects on the patient's organism, development of allergic reactions, negative influence on normal microflora, reduction of bacterial susceptibility to them and hence the appearance of resistant forms of bacteria. Therefore the bacteriophages represent practically the only strongly specific and natural factor for biological control of bacterial diseases [Gogoladze *et al.*, 1971; Karavanov, Rachkevich, 1971; Proskurov, 1971]. Besides, as was supported with experimental data, pathogenic microorganisms isolated from patients with suppurative inflammatory and other diseases are normally more susceptible to phage therapy than to antibiotics [Menshikov *et al.*, 1980; Samsygina, Boni, 1984].

Phage therapy is also thought to be more preferable mean of treatment and prophylactics comparing to vaccination. The reason for this lies with the safety of phage use independently from the condition of the patient and his immune status [Kiknadze, 1972].

Bacteriophages active towards pathogenic microflora are usually acquired from the material obtained from a given patient (urine, faeces, pus, etc.). The other alternative which further gained wide spread was the isolation of virulent phage isolates from sewage waters (normally from the clinic). In particular, *Adelson* (1962) was the first to demonstrate successful purposeful isolation of phages lytically active against intestinal bacillus from the clinic sewage; phages often had wide spectrum of activity [Adelson, 1962a; Adelson, 1962b; Zaharova, 1962]. It has also been shown that when there is an occurrence of serotypes of typhoid bacteria resistant to present phages, rather fast isolation of active viruses from the sewage is achievable. Further on, active virus isolates were introduced into polyvalent preparation of typhoid bacteriophage for widening the diapason of its activity toward different strains and serotypes of *Salmonella typhi* [Mgaloblishvili, 1971]. It's worth saying that the numbers of isolated phages may be quite considerable. For instance, about 70 phage isolates of *Pseudomonas aeruginosa* were identified in sewage of Gorky city (Russia) in 1985 [Kulakov *et al.*, 1985]. Isolation and investigation of high numbers of bacteriophages pursues not only practical rationale, but issues of their ecology and circulation in nature as well.

There are several distinguished types of bacteriophage preparations basing on the localization of their application: ***wound***, for superficial treatment (against conditionally pathogenic microorganisms inducing suppurations and similar pathologies – staphylococcus phage, pseudomonas phage, streptococcus phage); ***intestinal*** (dysenteriae phage, typhoid phage, salmonellosis phage, coli-phage, proteus phage, coli-proteus phage, intestiphage). Separately stands the group of phage preparations intended for subcutaneous, intramuscular, intracavernous or intravenous injections for preventing the generalization of infections invoked typically by staphylococcus, streptococcus, and pseudomonas. In view of that, wide spread gained **pyophage** – a complex preparation composed of 5 components: staphylococcus phage, coli-phage, proteus phage, streptococcus phage, and pseudomonas phage [Samsygina, Boni, 1984].

The nomenclature of produced preparations was diverse and included more than 20 designations. Bacteriophages were produced in different medicinal forms: in liquid and dried form, in tablets with acid-resistant coating from acetyl phthalate cellulose or pectin, and also as rectal suppository [Peremitina *et al.*, 1979]. In due time, industry of USSR produced the following most important bacteriophage preparations for treating bacterial infections of humans [Kondratyev, 1977]:

1. **Staphylococcus bacteriophage** (pathogens – almost exclusively *Staphylococcus aureus*, rather rarely – *Staphylococcus epidermidis*). The preparation is a filtrate of lysed staphylococcus culture. The preps of staphylococcus phage existed: i) in liquid form, obtained on protein-enriched and protein-free medium – respectively, for local/enteric applications and parenteral injections; ii) as rectal suppositories with dried phage preparation and filling agent (polyethylene oxide) – for treating professional disbacteriosis; as paste – for personnel prophylactics in maternity hospitals, etc. [Darbeeva *et al.*, 1980; Anikina *et al.*, 1983; Severov *et al.*, 1983]. The way of prep application certainly depends on the localization of disease focus. Local superficial application is achievable via spraying of liquid bacteriophage preparation over the wounds and use of wetted bandages.

Subcutaneously the phage is applied in small doses in one or several sites. There were also practiced intracavernous applications (in pleural cavity, cavity of urinary bladder), intramuscular and intravenous injections.

2. **Streptococcus bacteriophage** (pathogen – *Streptococcus pyogenes*). Usually was produced in liquid form for superficial applications.
3. **Pseudomonas bacteriophage** (pathogen – *Pseudomonas aeruginosa*). Liquid preparation for treating suppurative infections, osteomyelities, abscesses and similar pathologies of pseudomonas etiology [Bekina, Sergeeva, 1983]. Means of application are similar to those of staphylococcus phage.
4. **Coli-bacteriophage** (pathogen – *Escherichia coli*). Preparation was produced in liquid form and used for controlling infections invoked by, or complicated by, *Escherichia*. Peroral and rectal applications were practiced.
5. **Proteus bacteriophage** (pathogens – *Proteus vulgaris, Proteus mirabilis*). Preparation was produced in liquid form and used for controlling infections invoked by, or complicated by, *Proteus*. Means of application are identical to those for coli-phage.
6. **Coli-proteus bacteriophage**. Combined preparation – mixture of filtrates of lysed cultures of *Escherichia coli* and *Proteus vulgaris, Proteus mirabilis*.
7. **Salmonella bacteriophage** (pathogens – *Salmonella typhi, Salmonella typhimurium*). Produced in liquid form. Peroral and rectal applications.
8. **Dysenteriae bacteriophage** (pathogens – *Shigella flexneri*, *Sh. sonnei*, *Sh. dysenteriae*, *Sh. newcastle*). Preparations produced in liquid form and in tablets (dried prep) coated with acetyl phthalate cellulose (APC) or pectin [Orlova, Garnova, 1975], and in suppositories. Peroral and rectal applications.

There were also known, albeit less spread, other phage preparations including those for controlling typhoid, cholera, *Mycobacterium* (tuberculosis) and *Yersinia* infections (plaque), etc. [Bystriy, 1974; Chernomordik, 1989].

Among the preparations deemed in due time to be most perspective and claimed were the ones designated for treating suppurative surgical infections of wound and burned surfaces – staphylococcus, streptococcus, proteus and pseudomonas phages, - in the first place because of observed difficulties in efficient control of such pathologies by other means [Peremitina *et al.*, 1979].

Examinations of etiology of suppurative inflammatory processes of orthopedic-traumatological [Krasnoschekova *et al.*, 1971; Krasnoschekova *et al.*, 1983] and burned [Panchenkov *et al.*, 1983] patients revealed that microbial associations (not the culture of single bacteria species) were isolated in 55-66% of all cases. In addition, from these isolated microorganisms about 61-92% was shown to be resistant to widely practiced antibiotics. With this, staphylococcus was most often detected, however normally followed by other concomitant bacteria. Analogical data was obtained for many cases of acute intestinal infections – affiliated microflora frequently played very substantial role together with the primary infectious agent [Gogoladze *et al.*, 1971].

Ubiquitous complex bacterial etiology of various pathologies (presence of different serotypes, strains of bacteria, and often different bacterial species) conditions necessity for developing ***polyvalent*** phage preparations (composed of a mixture of varied types/races of bacteriophages active towards **one** bacterial species), and even sometimes ***combined***, or ***complex***, phage preps (containing phages active toward **diverge** bacteria invoking pathology of a complex etiology – for instance, against acute intestinal infections or suppurative inflammatory processes) [Meypariani, 1971].

Good examples of produced polyvalent preparations were above-mentioned staphylococcus, streptococcus phages, etc. In fact, absolute majority of phage preps produced in the due time in USSR were polyvalent as a consequence of the way they were obtained. Conversely, coli-proteus bacteriophage preparation is complex. The same goes for prominent complex phage preparation 'Pyophage-80' composed of a mixture of staphylococcus, streptococcus, coli-, pseudomonas and proteus phages. 'Pyophage-80' was demonstrated to be lytically active against 99.3% of microbial associations isolated from wounds (*Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Proteus vulgaris, Proteus mirabilis, Streptococcus pyogenes*) [Krasnoschekova *et al.*, 1983; Soboleva *et al.*, 1983]. 'Intestiphage' is another example of complex prep designated for therapy of acute intestinal infections. By 1971, this preparation has contained bacteriophages to several species of *Shigella*, *Salmonella*, *Proteus*, and many strains and serotypes of *Escherichia coli*. Noteworthy, the composition of this particular preparation was regularly brought into correlation with the data on bacteria occurrence on a given territory before its use as therapeutics [Gogoladze *et al.*, 1971].

Technological process of production of bacteriophage preparation included the following steps:

- growing;
- filtration;
- conservation (sterilization);
- precipitation (usually with ammonium sulphate);
- stabilization;
- drying (for dried preps, tablet forms, pastes and suppositories – lyophilization or drying over calcium chloride);
- tabletting and applying of acid-resistant coating (for tablet forms of preparations) [Meypariani, 1971].

Acid-resistant coatings pursued the aim to escape the destruction of phage preparation in acidic gastric juices, and could have been from acetyl phthalate cellulose (APC) or pectin. APC makes an unbroken superficial coating over the whole tablet, and this does not allow divide the tablet for dosage (in children therapy, for instance). Pectin forms sort of 'individual' coating over each virus particle in the tablet, enabling use of pectin-coated tablets for therapy of intestinal infections of youngest children [Orlova, Garnova, 1975].

Production of medicinal series of bacteriophages was normally conducted using local freshly isolated bacterial cultures preferably considering species- and type-specificity of a pathogen. Hence, analysis of produced dysenteriae bacteriophage showed its incomplete virulence toward separate types of *Shigella flexneri*, *Sh. sonnei* and *Sh. newcastle*. However, the adaptation step allowed significant widening of lytic spectrum of the preparation – 445 from 575 freshly isolated bacterial cultures have been lysed (80-96% of *Shigella flexneri*, 91% of *Sh. newcastle*, and 38% of *Sh. sonnei*).

The production requires constant improvements and selection of active phages. Optimization of the composition of polyvalent preparations (and combined just as well) allows broadening the diapason of their lytic activity. This was exemplified by successful adaptation of phages lysing *Sh. newcastle*; after the optimization they had titer of 10^{-8} and were lytically active against ~60% of bacterial types/strains. In comparison, mother races of these viruses obtained at titer 10^{-8} from several profile research institutes were able to destroy only 8-21.6% of *Sh. newcastle* types/strains (8% for Khabarovsk Institute of Experimental Medicine (IEM), 2.8% for Ufa Research Institute for Vaccines and Antisera (RIVA), 12% for Tbilisi RIVA, 21.6% for Gorky IEM) [Viskova *et al.*, 1974; Voroshilova *et al.*, 1974].

Phage selection for their practical use must be based on wide employment of biological properties of viruses, and their interactions with homo- and heterological bacteria [Bykova, 1964]. Thus, active races of typhoid bacteriophages were obtained experimentally by passing *in vivo* in the organism of the 'diseased' patient. The phages were shown to be more virulent to the microbes comparing to viruses which were not passed in microbe-invaded organism. Active passed phages were also characterized by higher stability at elevated temperatures. Preparation made using these passed phages was shown to be 5-times more efficient in combating microbial infection. Moreover, there was noted a decrease in numbers of relapses, complications and cases of patients being bacterial carriers in acute phase [Bliznichenko, 1973].

Industrial production of bacteriophage preparations based on active phages is exemplified in the work of *Ivanov* and *Romin* (1963). For making paratyphoid phage preparation the authors utilized 5-8 different cultures of *S. enteritidis Gartneri* and *S. suipestifer*, and for coli-phage prep – 3-5 strains of *E. coli*. The technology itself consisted of the following steps. 30-50 ml of 12-h bacterial culture (up to 60 mln of cells per 1 ml) was introduced into 1 l of culture medium. Then respective bacteriophage was applied in 7-15 ml. Retorts were kept at 37˚C for 8-24 h, shaking periodically. After full lysis of bacteria, phages (without clouds and precipitate) were further used for retrieving preparation series. Cell lysates were filtrated via asbestos filter, mixing at that stage the lysates of all industrial microbes at equal volumes. Filtrates were then conserved (with Quinosol, phenol or mertiolate), packaged in flasks or ampoules, and further tested for sterility, toxicity and activity (by lytic titer equal to or higher than 10^{-7}) [Ivanov, Romin, 1963].

Different practical approaches were registered when producing phage preparations. In some cases, researchers practiced simultaneous growing of a row of bacterial strains and their bacteriophages on the same fermenter. *Voroshilova* (1974) in her work analyzed the efficiency of industrial production of five phages using five different bacterial strains at the same time on a single fermenter. She observed that processes of bacteria and virus interference and recombination events may drastically worsen the yield of phages and their lytic activity as well. The author also pointed at the importance of correct selection of bacterial strain serving as substrate for phage accumulation in industrial conditions, as bacterial hosts often define, or at least influence, lytic

properties of their viruses [Voroshilova, 1974]. In other words, it was unambiguously demonstrated that growing of different bacteriophages on respective bacterial cultures must occur separately, and mixing of various phages into single preparation may be done only at the final pre-sale stage.

In parallel to selection of highly active bacteriophages and elaboration of production technology, thorough selection of susceptible bacteria is of utmost meaning for industrial production of phage preparations. One of the possible issues which may complicate the production is the synthesis of bacteriocins by the culture. Research of *Abrosimova* (1977) was focused on colicinogenic activity of shigellas and its role in production of dysenteriae bacteriophage prep. Author revealed rather wide spread of colicins characteristic for the studied bacteria (~44% of explored strains) [Abrosimova, 1977]. This phenomenon should be necessarily considered whenever the co-cultivation of various bacterial strains occurs for production of polyvalent dysenteriae phage, as it may lead to prior growth of one of the strains cultivated. Consequently, antagonistic relationships conditioned by colicins release induce changes in lytic activity of bacteriophages; significant changes in quality indexes of the preparation may be seen.

The phage work during the production process should be provided with genetically homogenous lines, and constant control for the absence of culture contamination is essential. For example, analysis of industrial mother virus culture unexpectedly identified two morphologically different phages [Bliznichenko *et al.*,1972a]; even use of pure phage lines does not guarantee the preservation of the characteristics of initial viruses [Bliznichenko *et al.*,1972b].

Production of biological preparations was protected with patents in due time. For instance, production of medicinal bacteriophage preparations was patented with *Patent (19) RU (11) 2247151 (13) C2 (51) 7 C12N7/00)*. Main technicalities of this patent are quite similar to those stated in the method for obtaining polybacteriophage (also patented, *Patent RF №2036232, published 27.05.95*). Basic principles of prep production are described further, although it all comes to separate growth of bacteria (and phages) in liquid culture medium and then mixing them together. Main disadvantage of the approach was the use of meat-based culture mediums. These are expensive, difficult to standardize in their composition, and overloaded with ballast compounds. High-molecular weight proteins and peptides present in this type of media complicate the stage of prep purification. Release from ballast substances is one of the reasons forcing the need for purification stage in the phage production cycle, and this in turn requires specific equipment and decreases the yield of the preparation. The distinctive feature of the proposed solution was the use of casein acidic medium. Bacteria cultivation should be conducted in culture mediums at standard conditions in either reactors of flasks; mother phage and bacterial culture were introduced simultaneously. Protein content of casein acidic medium is 5-10-times smaller comparing to meat-based media. Thus obtained phage lysates (unpurified) are pretty much similar in their nitrogen content to purified preps achieved on meat-based mediums. Clear phage lysates are then filtered through a cascade of microporous caprone membranes with pore size of 3.0, 1.0, 0.8 and 0.2 μm. Then for achieving polyphage preparation, separately obtained virus cultures (previously tested for pH, toxicity, specific activity, and sterility for ensuring prescribed properties of the prep) are mixed in a single volume. Titer of any phage in the preparation cannot be lower than 10^{-5}.

It is essential to control activity of industrial phages on a large number of bacterial strains [Meypariani, 1971]. Such control allows selection of bacteriophages with high lytic activity toward freshly isolated bacterial strains obtained from different regions of the country. Additionally, this approach enables timely inclusion into the preparation of novel phages active against bacteria until that time undetected but shown to be spread at a particular moment on a certain territory.

Methods of phage cultivation are also significant for production. Comparison of two techniques – aeration and stationary growing of microbes/bacteriophage – clearly demonstrated an advantageous character of the aeration methodology: mean lytic activity of phage reached 10^{-9}-10^{-10}. However, this approach was not introduced into production of phage preparations as spontaneous lysis of bacterial culture was frequently observed (without the phage added) [Meypariani, 1971]. Furthermore, absence of corresponding technical conditions for growing phages on a large scale limited the use of this method at that time. Following the standard procedures, lytic titer normally settles between 10^{-7}-10^{-9}, which complied with demands to the bacteriophage preparations.

Obtained phage lysates required further processing allowing their purification. It was established that optimal virus precipitation could be reached with 69% of ammonium sulphate. In this way, 1.5-2.2 kg of dried preparation could be obtained from 200 liters of phage lysate. Yield of virus particles in this case was about 18-31%. These approaches greatly simplified following processing of the preparation.

Several substances were tested for their use as conserving agents for the preps, Quinosol and mertiolate among them. However this part of production still needed some research.
Drying was the most sensible step in the production of dried phage preparation. Stabilization of the phage is tightly linked to drying, thus both processes were considered in complex. Drying was carried out in two ways: lyophilization and drying over calcium chloride, the latter shown to be milder (phage activity remained practically untouched comparing to initial). However this technique is not suitable for production of large quantities of the prep. Hence special regime of lyophilization has been tailored: previously frozen mass (-35˚C) was gradually heated to 30˚C. In turn, glucose and magnesium sulphate were analyzed as stabilizing agents [Meypariani, 1971].
In case phage preparations are intended for parenteral (especially intravenous) use, a great deal more strict requirements are produced to the way they are made. Main condition is growing of bacteria and viruses on protein-free media. Also, purification techniques such as precipitation on DEAE cellulose were elaborated to ensure chemical purity of the preparation [Peremitina *et al.*, 1979].
During the production of phage preparation it is vital both scientifically and commercially to preserve mother phages. Such works were conducted in 1950-60. As exemplified by research on phages T3, T4 and φX174, low-temperature freezing and conservation does not significantly affect the quality indexes of the virus (i.e. number of infectious particles, their antigenic properties, adsorption time, spectrum of lytic activity, sensitivity to hyper- and hypotonic shock, etc.) and may thus be recommended for routine use [Vysekantsev, 1983].

To summarize existing data on bacteriophage preparations and principles of their production it should be said that generally there were several main requirements produced to phage preps intended for use in therapy and prophylactics of bacterial infections:
1) wide spectrum of lytic activity of the preparation toward existing biotypes of the pathogen (achieved via polyvalent or complex nature of the preparation);
2) high virulence of single components of the preparation (i.e., separate phages composing the prep) and their high concentration;
3) stability of lytic activity of the preparation with time;
4) tolerance of the preparation to acidic gastric juices and, conversely, capability for easy dissolving in intestines (for preparations concerning which peroral use is anticipated; achieved usually by covering the tablets with acid-resistant coating) [Kiknadze, 1972].
Historically, in production of phage preparations main attention was paid to virulence of virus isolates and spectrum of their lytic activity. As was said above, bacteriophages were selected empirically, practically without consideration of their biological features and peculiarities of interactions in system 'virus-host' [Kiknadze, 1974; Chanishvili, 1983]. Races of phages isolated for instance from sewage waters were then propagated on a limited number of laboratory strains of bacteria, and were not studied in their type composition [Meypariani, 1971]. Accumulated factual data points on obligatory control of such characteristics of phage preparation as its type composition, virulence, frequency of emergence of phage-resistant bacteria, changes in spectrum of lytic activity, and also interference among unrelated phages included into single preparation [Chanishvili, 1983].
Therefore, main weak spot of phage therapy and preparations used in due time is deemed to be the necessity to standardize bacteriophage preparations. The reasons for this are variability of bacterial agents and, more importantly, complex etiology of many diverse pathologies. Complexity of diseases, understandably, led to recognizing the need for use polyvalent or complex phage preparations for controlling them (that is, extensive widening the lytic activity spectrum of preparation by increasing ***number*** of types/strains/isolates of viruses included into the prep, **NOT** by broadening the spectrum of activity of each virus composing the preparation) [Peremitina *et al.*, 1979]. In other words, detailed virus composition for a given polyvalent preparation was practically unknown. In particular, this was further confirmed for polyvalent staphylococcus phage preparation [Soboleva, 1972], for preparation of mycobacterium phage [Aminov, 1973], cholera bacteriophage [Bystriy, 1974] and others. These preparations were shown to be heterogeneous in their composition, and viruses included into the prep were differing in their biological and morphological properties.

2. Phage therapy and prophylactics of bacterial infections in human and veterinary medicine

Use of bacteriophage preparations for treating bacterial diseases in human and veterinary medicine is called ***phage therapy***. There is vast experience of wide adoption of phage preps as therapeutics. We should note that phage preparations were most extensively utilized for curing acute intestinal infections, children's in particular, and also in treatment of suppurative inflammatory diseases of various localizations, especially in case of bacterial invasions of superficial burned wounds. Apart from aforesaid, phage preparations were successfully employed against antritis and suppurative otitis, suppurative conjunctivitis and blepharitis, in therapy of cholecystitis, endometritis, urethritis, pneumonia and other diseases [*Proskurov, 1971; Onanov et al., 1975; Samsygina and Boni, 1984; Zhilenkov et al., 2002*]. Interest to phage use in pediatrics and therapy is conditioned by increasing role of antibiotic-resistant bacterial microflora as etiological agents of perilous diseases.

In clinics, main struggle is focused on controlling **suppurative inflammatory infections** developed as traumatic or surgical complications, as also in burned wounds. These infections are mainly invoked by staphylococcus, and besides by streptococcus, pseudomonas and proteus (normally as associated microflora). Therefore, principal efforts in therapy are directed on staphylococcus as primary casual agent. It should be said that staphylococcus may induce both local pathological processes and generalized infection of the organism spreading with blood and inseminating different organs. Based on this, means of application of staphylococcus phage preparations (and those of phages lysing associated bacteria) vary greatly from local superficial to parenteral (including intravenous) in therapy of suppurative processes in pliant tissues; of osteomyelities; of suppurative infections in lungs, pleura and abdominal cavity; and in treatment of infected burned wounds [*Matusis et al., 1974a, 1974b; Nikolaeva, 1974; Vasilkova, 1975; Revenko, Megrabyan, 1975; Samsygina and Boni, 1984*].

Generalized forms of staphylococcus infection are major reason for death of heavily burned patients in clinic. Logic for utilizing phage therapy in prophylactics of staphylococcus infection lies with the following points. Injected intramuscularly and circulating in patient's blood, bacteriophages may immediately act against microbes disseminated in the blood system. In such a way viruses may confer specific antimicrobial defense to non-immune or insufficiently immune organism in the very first days of the disease, preventing thus the generalization of infection [*Matusis, Pylaeva, 1982*].

Local wound application of staphylococcus phages or a mixture of different phages (staphylococcus, streptococcus, pseudomonas, proteus and coli-phages) as a measure of sepsis prophylactics aims at two interconnected objectives. Firstly, this treatment a hundred times decreases numbers of microbes vegetating in the wound which lessens the probability of their invasion into deep layers of the lesion and in the blood. Secondly, phage therapy-induced decreased numbers of wound microflora lead to reduction of pus matters and improvement of granulations [*Matusis, Pylaeva, 1982*].

In addition to parenteral and local wound applications, staphylococcus infections were also successfully treated with peroral applications of liquid phage preparation [*Feklisova et al., 1983*].

There is significant experience of local use of staphylococcus bacteriophage for curing such diseases as hematogenic and chronic osteomyelities of children, odontogenic and chronic antritis, suppurative inflammatory diseases of maxillofacial area, lungs, pleura and pliant tissues, burns, conjunctivitis, pneumonia, and also for prophylactics of postoperative wound suppurations [*Proskurov, 1971; Garsevanishvili, 1974; Revenko, Megrabyan, 1975; Ermakov et al., 1983; Ermakov et al., 1984; Samsygina and Boni, 1984*].

Local application of staphylococcus phages normally presumes spraying of superficial suppurative cavities (in case of osteomyelities, for instance), wetting of bandages, etc. Antiseptic treatment of the wound preceding phage therapy is recommended. For chronic diseases, intramuscular injection of sterile staphylococcus phage is practiced, too. 80% of patients subjected to this way of phage therapy demonstrated visible improvements in their condition by 3 day (relaxation of inflammatory reactions in suppurations, diminution of necrobiotic changes).

Staphylococcus bacteriophage has been also successfully utilized in prophylactics and treatment of postoperative suppurations, particularly for high-risk persons. For this, suture was followed by wound infiltration with sterile phage solution. Sometimes injection of bacteriophage into parenteral cavity was practiced. After such phage therapy the risk of development of postoperative suppurations was shown to lessen thrice (from 6.2% to 2.13%); correspondingly, time of treatment for patients was decreased [*Samsygina and Boni, 1984*].

There is data on successful bacteriophage use when treating heavy patients with infected burned wounds. For example, use of phage therapy against staphylococcus (as compared to antibiotics combined with antiseptics) led to great decrease of quantitative indexes of total wound microflora, pH increase of tissue exudation, shortening of period of patient's preparation to surgical intervention from 12-16 to 6-8 days.

In therapy of patients with suppurative infections of lungs and pleura, staphylococcus phage was introduced dropwise via microtracheotomy for 5-10 days. Improvements have been recorded by 2-3 day (temperature normalization, lessening of pus production, improved roentgen picture). Staphylococcus bacteriophage preparation can be also applied directly into abscess cavity after its draining and washing of suppurations. In case of acute and chronic empyema of pleura, bacteriophage therapy was shown to be efficient when the prep was regularly introduced for 10-12 days into pleural cavity after draining and washing [*Samsygina and Boni, 1984*].

In therapeutic practice, staphylococcus phage preparation was also successfully utilized for treating cholecystitis. Application was made for 3-4 times through a probe at intervals of 2-3 days. Analogically urethritis was cured; the prep applied locally into the cavity of urinary bladder [*Proskurov, 1971*].

There are many examples of good treatment of odontogenic and chronic antritis with sterilized preparation of staphylococcus bacteriophage [*Biberman, Plotnikova, 1976; Samsygina and Boni, 1984*], as well as with a mixture of staphylococcus and streptococcus phages [*Biberman, Starodubtsev, 1983*]. When treating odontogenic antritis, maxillary sinus was washed with phage preparation. Notable improvements could be seen after 2-3 applications: lessening of nasal secretions, breathe relief, negative results of microflora tests. Best results for chronic antritis were achieved when using dense deposited preparation of staphylococcus phage mixed with equal volume of lanoline and vaseline. Therapeutic effect of such preparation was preserved for 7-8 days after introduction into sinus.

Similarly, staphylococcus phages, along with phages lysing streptococcus and pseudomonas, were recently utilized with good results for treating chronic otitis [*Zhilenkov et al., 2002*].

Phages of staphylococcus were in addition used for curing staphylococcus-induced diseases of eyes – for conjunctivitis and ulcerous blepharitis [*Proskurov, 1971*], and also for ulcer of cornea [*Protopopov, 1974*]. Local application was employed in the form of either drops or lubrication of eyelids' edges.

Phages of staphylococcus and streptococcus recently found use in dentist practice for controlling parodontosis [*Chubatova et al., 2000*].

Intracutaneous injection of staphylococcus phage was adopted for treating deep staphylodermy [*Vartapetov, 1974*].

Staphylococcus enterocolitis was shown to be efficiently treated with staphylococcus phage preparation applied orally or rectally for 3-4 days [*Proskurov, 1971; Pogorelskaya et al., 1983*].

Reports on intravenous application of staphylococcus phage are not numerous and mainly connected with curing suppurative infections of lungs and pleura [*Samsygina and Boni, 1984*]. Parenteral injection was usually combined with local phage therapy (inhalations, sprayings). In a group of patients treated with intravenous infusions of staphylococcus phage in addition to antibacterial therapy and local sanifying phage therapy, firm improvements were observed in 95.3% of cases; lethality 3-times decreased. In comparison, in control group treated with antibiotics only, recovery was seen only in 64.1% of patients. Patients taking intravenous injections of phage prep demonstrated also normalization of immunity indexes by 20-21 day. No side effects were observed for this type of staphylococcus phage application.

At the same time, some authors argue about inexpediency of intravenous use of bacteriophage preparations when treating staphylococcus sepsis as it may be often seen that staphylococcus cells are masked in human organism by immunoglobulins and complement during generalized infection which, in turn, complicates virus adsorption to bacterial cells [*Bykov et al., 1983*]. Other authors, conversely, recommend intravenous introduction of staphylococcus phages as rather efficient measure for prevention of generalized infection [*Georgadze et al., 1974*]. In experiments on laboratory mice, *Anikina* (1974) demonstrated that when introduced intravenously staphylococcus phage circulates in the organism for a longer period of time. Studies on the efficiency of treatment of staphylococcus infection in rabbits with intravenous application of phage preparation showed that regular injection of the phage at the first stages of bacterial infection leads to notable prolongation of life time-span of experimentally diseased animals (from 4-10 days to 18-20). Even more, intravenous phage therapy prevented the generalization of infection, postponed the development of sepsis and decreased bacterial invasion of internal organs. However, despite high efficiency of staphylococcus phage use, full rescue of the rabbits from the infection could be

achieved only by combination of phage therapy with specific antibiotic therapy [*Anikina et al., 1982; Anikina, Bubashvili, 1983*].

Clinic-laboratory analysis on the effectiveness of phage therapy against staphylococcus infection showed that use of bacteriophages allows reaching a decrease in the rate of formation of staphylococcus sensibilization of patients, and also a 1.5-time decrease in frequency of bacterial sepsis development. Thus, phage therapy efficiently prevents the generalization of staphylococcus infection. Combined action of staphylococcus bacteriophages, immune preparations, and antibiotics elevates the effectiveness of treatment [*Matusis et al., 1983*].

Proteus (*Proteus vulgaris, Proteus mirabilis*) invokes intestinal infections as well as suppurative inflammatory processes (mostly after surgical interventions), meningitis, encephalitis, pneumonia and sepsis. Rather often the association of proteus infection with staphylococcus, streptococcus, pseudomonas and coli is seen [*Peremitina et al., 1983*]. Large efforts were paid to isolation of virulent races of proteus bacteriophages with as wide lytic spectrum as possible from sewage and river waters, faeces, pus and urine of patients. From 209 isolated phage races, some series of preparations of polyvalent proteus bacteriophage were made; its efficiency toward proteus strains reached 95.2%. As expected, this polyvalent bacteriophage was shown not to be active against salmonella, escherichia and shigella. Prophylactic subcutaneous injection of this prep to mice preserved them from the proteus infection with almost 100% guarantee, and therapy of experimentally infected animals was demonstrated to possess the effectiveness of 98.4%. Treatment of patients with the preparation of polyvalent proteus bacteriophage was very successful in therapy of suppurations of burned wounds, cholecystitis, etc. of proteus nature [*Sakandelidze, 1971a, 1971b*].

Attention of scientists was also devoted to biological properties of pseudomonas phages. In their work, *Burbutashvili* and *Gachechiladze* (1971) employed 120 strains of *Pseudomonas aeruginosa* isolated from clinic material (pus, bile, exudations) and 20 isolates of bacteriophages, 15 of which were obtained from laboratory cultures and 5 – from sewage waters. Some phages, in particular those isolated from sewage, demonstrated wider spectrum of lytic activity toward various strains of pseudomonas, and lysed about 37.5% of all strains used in investigations.

Microorganisms *Klebsiella*, *Enterobacter*, *Hafnia* and *Serratia* are known as agents inducing infections of respiratory tract (pneumonia), intestinal infections, inflammations of urogenital tract (pyelonephritis, endometritis), bile-excretive pathways, cerebral membranes, eyes, joints, etc. along with staphylococcus, they may be a cause for suppurative postoperative complications, primary sepsis, and so on. Search for polyvalent bacteriophage preparations led to isolation of different virus strains from clinic sewage. According to *Sergeeva et al*. (1988), about 65% of all prepared complex phage preparations were highly virulent and lysed 94-100% of identified species of *Klebsiella*, *Enterobacter*, and *Hafnia*.

As we mentioned before, suppurative processes are rarely invoked by a separate microorganism and, on the contrary, often bear complex etiological origin. In such situations most efficient was the use of complex bacteriophage preparations containing viruses to different bacteria species.

Particularly, combined preparation composed of 55% of staphylococcus phage, 30% of streptococcus phage, and 15% of coli phage was successfully employed when treating pneumonia in younger children. The prep was applied locally in form of aerosol inhalations [*Garsevanishvili, 1974*].

Similarly, therapy of acute bacterial lung abscesses was conducted with complex phage prep containing viruses to staphylococcus, streptococcus, coli and pseudomonas. Introduction of the preparation was done under anesthetic bronchoscopy [*Ermakov et al., 1983; Ermakov et al., 1984*].

Polyvalent phage preparations against staphylococcus and pseudomonas were also successfully utilized in combination when curing postoperative complications of oncological patients [*Sokolova et al., 1983*].

Good results of local application of complex phage preparation (toward coli, proteus, and staphylococcus) were obtained for therapy of suppurations in patients with fractures [*Melnikova et al., 1975*].

Complex preparation ‘Pyophage 80’ contains a mixture of staphylococcus, streptococcus, coli, pseudomonas and proteus phages, and it has been shown to be tremendously efficient lytically against 99.3% of microbial associations isolated from wounds with suppurative inflammatory processes (*Staphylococcus aureus, Staphylococcus epidermidis, Pseudomonas aeruginosa, Proteus vulgaris, Proteus mirabilis, Streptococcus pyogenes*) [*Krasnoschekova et al., 1971; Krasnoschekova et al., 1983; Soboleva et al., 1983*].

In pediatric in specific and in clinic of infectious diseases in general, **acute intestinal infections** pose a great problem, especially dysenteriae and salmonellosis. Bacteriophages were extensively

utilized for treatment, prophylactics and diagnostics of infectious intestinal diseases. Particularly, evaluation of treatment effectiveness of child's dysenteriae of complex etiology (*Shigella sonnei* in 58% of cases and 42% of patients with unascertained invoking agent) with polyvalent phage prep in tablet form was conducted. Results showed that phage therapy was effective in 80.4% of cases which approximately corresponded with the effectiveness of antibiotic therapy [*Kozlova, 1972a*]. Similar investigations on the expediency of polyvalent phage use for treating dysenteriae of adults (mainly invoked by *Sh. flexneri* and *Sh. sonnei*) demonstrated that 93.3% of identified bacterial strains were lysed by viruses [*Barinova, 1970*]. Acute dysenteriae was also successfully treated with another polyvalent dysenteriae bacteriophage which was characterized with rather wide lytic spectrum – 67-89.5% of serotypes/strains of *Sh. sonnei* and *Sh. Flexneri* circulating in due time. Preps of dysenteriae bacteriophage, due to normally peroral use in kids, were mainly produced in tablets with pectin coating, and more rarely with APC coating [*Birkovskiy, 1974*]. Spread and effectiveness of dysenteriae bacteriophage was also confirmed by many other authors [*Belyaev et al., 1971; Nasonova et al., 1971; Orlova, Garnova, 1971; Soldatova et al., 1971*].

For coli infections (*Escherichia coli* O-55), single use of coli-proteus bacteriophage stopped the isolation of escherichia in 93% of patients. Remaining patients required the second course of phage therapy for full recovery and stopping of being a carrier. Phage therapy efficiency was 1.7-1.8 times more successful comparing to antibiotics [*Kozlova et al., 1972b; Samsygina and Boni, 1984*]. Similar outcomes on use of coli-proteus bacteriophage preparations were obtained by *Bogolapova et al*. (1971) and *Naftulyeva et al*. (1971).

In therapy of salmonellosis, polyvalent preps of salmonella phages found wide use. *Zhuravleva et al*. (1973) noted that utilization of liquid polyvalent phage for treating salmonellosis in children was comparable to antibiotic therapy in its effectiveness. When using salmonella bacteriophage, full decontamination of the organism from salmonella was achieved in 78% of patients; antibiotics' use led to recovery of 70.2% of patients. Similar data was presented by other authors [*Hahareva et al., 1971; Cereteli et al., 1983*].

Polyvalent bacteriophage preparations were also employed with good results in therapy of typhoid [*Mgaloblishvili, 1971*].

Main complication physicians face when treating intestinal diseases of younger children is the duration of disease etiology' establishment. This conditioned the need in complex bacteriophage (mixture of various phages) – 'Intestiphage'. Employment of such complex preparation allowed highly effective therapy without firm results of bacteriological study. In 1971, this preparation consisted of bacteriophages to several species of *Shigella*, *Salmonella*, *Proteus*, and many serotypes/strains of *Escherichia coli*. It's worth noting that content of 'Intestiphage' was regularly brought into correlation with data on spread of agents of intestinal infections on a given territory [*Gogoladze et al., 1971*].

Phages of bacteria belonging to particularly dangerous infections attained significant attention. They are of importance now as well in the view of international terrorism threat. Specifically, great efforts were focused on cholera phages. It is known that in 1957, these bacteria invoked 7th pandemic in Europe, Africa and South-East Asia. Based on this, cholera bacteriophages were of significance as components of polyvalent preparation against this disease. Apart from therapy, cholera phages were extensively utilized for fast diagnostics of the agent [*Agapova, 1973*].

Research on *Vibrio cholerae* bacteriophages revealed that there were viruses lysing up to 65-100% of laboratory *V. cholerae* strains of both homological and heterological serovars. However, testing of these phages on biovars isolated from nature showed their activity toward only about 30% of strains of homological biovar. Despite the difficulties, given the danger this disease presents, selection of virulent bacteriophages for controlling cholera was deemed to be expedient [*Ostroumova et al., 1983*].

In medicine, phage therapy was shown to be advisable toward agents of tuberculosis. When introduced subcutaneously, intravenously or intranasally to infected laboratory animals, mycobacteriophages (active *in vitro* against some strains of *Mycobacterium tuberculosis*) were capable of delaying the development and progressing of tuberculosis [*Sharov, 1974*].

Majority of studied mycobacteriophages were demonstrated to possess strict specificity to *Mycobacterium tuberculosis* and *Mycobacterium bovis*, at the same time being a powerful factor of variability of these microorganisms [*Kosobutskiy, 1974*]. Nevertheless, some authors noted converse data on unusually wide lytic spectrum of some mycobacteriophages toward different groups of mycobacteria (*M. tuberculosis, M. bovis, M. fortuitum, M. friedmannii*, etc.) [*Aminov, 1973*]. It was demonstrated that contact of mycobacteria with bacteriophage may lead to the development of phage-resistant bacteria forms, and hence combination of phage therapy with antibiotics is recommended. Moreover, co-cultivation of phages with susceptible bacteria can also

lead to the appearance of novel phages with shifted spectrum of lytic activity [*Kosobutskiy, 1974*]. It should be stressed that it was not possible to achieve full recovery of patients from pathogenic mycobacteria by mean of bacteriophage therapy [*Rodionova, 1969; Sharov, 1974*].
There were also phage preparations for controlling pseudotuberculosis induced by *Yersinia pseudotuberculosis*. However, studied viruses had quite narrow specificity and therefore the preparations did not gained much spread [*Leshkovich et al., 1983*].

Use of bacteriophages for preventing the rise of bacterial infections is called ***phage prophylactics***. It is carried out in human groups of professional of social-age risk with the same phage preparations, and is the greatest advantage of phage preparations comparing to other means of controlling bacterial diseases.
For instance, liquid form of staphylococcus phage and its dried form (paste) were successfully employed for staff prophylactics in maternity hospitals [*Darbeeva et al., 1980; Anikina et al., 1983; Severov et al., 1983*].
Dysenteriae, salmonella and typhoid bacteriophages were used with good results for sanitation of pre-school and school institutions (including spraying of premises) aiming at decrease of incidence of intestinal infections. Similar preparations were utilized at factories of food industry, however mainly for controlling carriers [*Mgaloblishvili, 1971; Hahareva et al., 1971; Drozdova, 1974; Katashina et al., 1974; Kiknadze, 1974; Kondratyev, 1977; Cereteli et al., 1983*]. Complex bacteriophage preparations – dysenteriae and coli-proteus phages – also found wide use in prophylactics of intestinal bacterial infections [*Belova et al., 1971; Soldatova et al., 1971*].

In **veterinary medicine**, treatment of bacterial infections of animals with bacteriophage preparations was not significantly spread as comparing to humans. However, there were some positive outcomes and scientific directions worth of saying.
First attempts of using phages against infectious animal diseases were dated back to 1930ies. D'Herrelle tried bacteriophages for controlling pullorosis-typhoid of chickens and for pasterellosis of buffalos. Promising results were obtained.
In veterinary, vast experience of phage therapy of intestinal infections was accumulated by *I.F. Kvesitadze* (1947) and *K.N. Sherstoboev* (1947). From 1940, bacteriophage produced by Kvesitadze was utilized for treating calf infected with paratyphoid. Till end of 1940ies, more than 6,000 calf in total were successfully cured with phage therapeutics. Moreover, prophylactics and early treatment were shown to be especially efficient. When phage therapy was in place from the very first days of the disease, 90-100% of diseased animals fully recovered. *Sherstoboev* was involved in production of phage preparations for controlling dysenteriae and coli infection. According to his data, 95.6% of young pigs with dysenteriae infection and 98.1% of those with coli infection were cured with good results; even more, about 85% of calf showing paratyphoid infection were treated successfully. This information received further support from other researchers as well [*Muromtsev, 1953*].
Bacteriophages were also employed against brucellosis of cattle and pullorosis of chickens. Experimental inoculation of laboratory animals with *Brucella abortus* and its following treatment with specific phages showed that brucellosis bacteriophages possess potent curing and sanifying effects. However the preparation was very strictly specific to a definite *Brucella* species (i.e., despite being efficient, had a very narrow spectrum of lytic activity). Use of phage therapy decreased the incidence of brucellosis generalization almost twice (from 85% to 43.4%). Therapeutic effect of virus preparation could be amended by additional treatment with brucellosis vaccine and/or specific γ-globulins. Combined action of both bacteriophage and γ-globulins decreased the frequency of infection generalization down to 25%. Nevertheless, even combined therapy did not allow reaching full release of experimental animals from brucellas, i.e. they remained carriers of the infection [*Pophadze, 1974; Pophadze et al., 1974*].
Apart from *Brucella abortus*, *Kiseleva* (1970) succeeded in selection of virulent bacteriophages to *Brucella melitensis* by isolation of *c*-mutants of moderate phages.
Bacteriophage preparations were also irregularly used for treating other infectious diseases of animals. For instance, *Filimonenko* (1960) conducted research in 11 salmonellosis-adverse villages in Georgia. In farms of these villages, cattle were preventively treated with salmonella phages to reduce the incidence of transmission the infection during calving. First introduction of the preparation was done a month before, and the second one – 5-7 days prior to calving. Outcomes demonstrated that only 3% of calf born by phage-treated cattle did have the salmonellosis, in contrast to 29% of those from control group.

In some cases, phage therapy was known to be more efficient when combined with antibiotics. Hence, *Samadashvili* (1974) observed that despite principal effectiveness of bacteriophage preparation for controlling chicken pullorosis, better results were achieved by its combination with antibiotic therapy.
Nikolaenko and *Egorova* (1957, 1958) proposed specific bacteriophage against paratyphoid of sheep. It was designed to be introduced to sick lambs either intramuscularly (10-15 ml a day) or perorally (thrice a day, after taking soda). For preventive use, the phage preparation could be given to healthy lambs perorally (20 ml, 2-4 h before feeding after taking soda, 3 days in total).
Saying about prophylactics, it was also demonstrated that preventive treatment of chickens with active pullorosis phage preserved them from future infection with efficiency close to 90% in laboratory conditions and up to 80% in industry. Periodical introduction of this bacteriophage preparation to chickens and hens significantly contributed to a decrease in numbers of disease carriers (3-6 times) in industry.
Regardless of stated examples of successful control of animal bacterial infections with phage preps, industrial production of bacteriophages for needs of veterinary medicine was never put on a large scale. Two main commercial bacteriophage preparations for veterinary use were known – brucella phages against brucellosis of cattle (pathogen – *Brucella abortus*) [*Pophadze, 1974*], and pullorosis phages for controlling chicken pullorosis (pathogen – *S. pullorum*) [*Samadashvili, 1974*].

Separately stand studies focused not on veterinary use but on effect of phage preparations (designed for controlling human disorders) on laboratory animals. Hence, *Barinova* (1970) analyzed therapeutic effects of typhoid and dysenteriae phages on guinea pigs being at the same time infected with these casual agents. In 24 hours, she observed pronounced effect of phage use depicted in drastic decrease of pathogens' numbers or even full release of animals from the infection.
In turn, *Kiknadze* and *Chanishvili* (1972) tried bacteriophage preparation against O-salmonellosis in experiments with mice infected with *S. typhimurium*. They noted that phage secured a 9.8-fold decrease in lethality of mice infected with lethal dose of microbes.
All presented here data on the use of phages in veterinary medicine are applied to warm-blooded animals. However, among literature focused on this subject there are also references on bacteriophage therapy of infections common to fish and insects, especially bees.
Phages active toward animal-pathogenic bacteria can be often seen in sewage, manure, excrements etc. They are widespread in intestines of wild animals and birds. Thus, bacteriophages lysing dysenteriae and typhoid bacteria were regularly found in excrements of 29.3% of inspected animals and 62% of birds (497 species in total). In silt and water of ponds and lakes, there were also identified viruses able to lyse agents invoking bacterial diseases of fish. Same bacteriophages were then isolated from the intestine content of carps. It was further established that these phages were specifically involved into rises and falls of infectious dropsy of carps. According to *Sheperclaus*' data, application of polyvalent bacteriophage active towards *Ps. punctata* (agent of infectious dropsy of carps) greatly reduced the mortality rate of fish – by 58% – when comparing to control non-treated group.
Bacteriophages active against the agent of European rotten of bees, *Bac. alvei*, are frequently found in flowers of melliferous plants where they're brought by bees. Good results were achieved when treating of bee families with specific phages. *Smirnova* (1968) studied the effectiveness of phage therapy of entomopathogenic bacteria including American and European rotten. In particular, phage preparations were utilized in large-scale field studies at the apiaries in different regions of USSR. Bacteriophage was applied as an additive to sugar syrup (100 ml per 1 l of syrup). Phage therapy reduced the incidence of the disease (European rotten of bees) by 4-4.4 times, in contrast to control bee families. Scientists also noted higher efficiency of phage therapy comparing to antibiotics.
At the same time, many works of soviet and western investigators stated incompetence of bacteriophage preparations to control bacterial diseases of insects. One of the main reasons for such severe discrepancy in the evaluation of expediency of phage therapy was, as sounded by many researchers, great inaccuracies in selection of phages [*Rautenstein, 1967*].

In summary of presented experience on phage use in human and veterinary medicine, we should say that maximum effect of bacteriophage therapy may be achieved observing the following preconditions:
1) use of highly virulent bacteriophages in high concentration (usually 10^7);

2) test on susceptibility of bacterial culture to bacteriophage preparation is strictly recommended. In case patient's microflora is insensitive to phages from complex preparation, isolation of new virulent bacteriophages is needed (as well as following elevation of its activity towards this bacterial pathogen by adaptation);
3) phage therapy is considered as ONE of the components of complex treatment. In practice, phage therapy is very often combined with antibiotics and immunotherapy [*Karavanov, Rachkevich, 1971; Kondratyev, 1977; Komahidze et al., 1974; Pokrovskiy, 1979*].
Therefore, success of bacteriophage therapy is defined by timeliness of treatment, susceptibility of patient's microflora to the phage preparation utilized, and by optimal scheme of treatment (doses, mean of phage introduction, combination with other kinds of therapy).

3. Phages of phytopathogenic bacteria

Until recently, role of populations of bacteriophages in plant biocenoses was not studied adequately. Exploration of their characteristics is deemed to be an important task both for our understanding of processes taking place in nature and for controlling number of bacteria invoking substantial economical losses. Information of this kind should allow formulating criteria for selection of bacteriophages with "predetermined" qualities. This approach considers phages as feasible biological agents for controlling bacterial diseases, and even more – as ecologically safe means alternative to chemicals.

First endeavors focused on using phages of phytopathogenic bacteria in USSR are dated back to 1930ies. Investigators developed control methods for bacterial cancer [*Izrailskiy, Vinogradova, 1935; Kalyaev, 1935]*, gummosis of cotton plants [*Lebedeva, 1936*], wilt of kok-sagyz [*Novikova, 1950*]. In particular, *Izrailskiy* was involved in studying the perspectives of '***phage prophylactics***' and '***phage therapy***' related to tumors on *Pelargonium zonal* plants infected with *B. tumefaciens*. It has been shown that phage treatment leaded to more intensive shrinkage of tumors comparing to non-treated plants. However, the difference between treated and non-treated plants was evident for only a month and then decreased nearly to zero. The authors noted high degree of phage inactivation by direct sunlight. Investigations in the field of practical use of bacteriophages were rather empirical. They allowed obtaining definite positive outcomes, however had many limitations requiring additional detailed studies and summarizing of accumulated practical evidences. For example, prophylactic treatment of soil with bacteriophages against bacterial wildfire of *N. rustica* reduced the appearance of lesions for up to 67-74% at the first stage of vegetation, but the difference in disease development between treated and non-treated plants was negligible at the end of vegetation. Double treatment of plants with phage preparation allowed achieving steady improvement of plant health (up to 66-67%). And finally, combination of soil treatment with double treatment of plants demonstrated an increase of phage prophylactics' efficiency to 74% when comparing to normally grown non-treated plants [*Novikova, 1938*].

One of the important factors influencing the efficiency of phage therapy and prophylactics noted by many researchers was the preventive way of their use [*Lebedeva, 1936*]. This approach was employed by *Tokarchuk et al.* in their work. They adopted presowing treatment of sugar-beet seeds with phage lysate [*Tokarchuk et al., 1994*]. Afterwards, seeds were dried and sown. During the vegetation, authors treated the plants twice with phage preparation, and these measures significantly improved the qualitative indexes of sugar-beet yield and sugar extraction [*Tokarchuk et al., 1993*].

Existing data point on the crucial role of criteria for selection of bacteriophages for practical purposes. Spectrum of lytic activity takes important place here. *Novikova* was one of the first who recognized the necessity to use highly virulent phages [*Novikova, 1938*].

Practical deployment of phages assumes their isolation from nature as well as thorough analysis of preconditions allowing to discover the viruses in focus of infectious process. *Gvozdyak* pointed out that occurrence of a given bacteriophage on a plant is very much related to the development of the disease invoked by bacteria. At the beginning of infectious process the phage is barely detected on diseased plant; further on the virus can be found only in low titers; and the maximum of phage concentration can be seen normally only in full swing of the disease and immediately afterwards. At the end of the disease, phage titers decrease dramatically as there no susceptible microorganisms left to lyse [*Gvozdyak, 1981*].

In order to select phages permitting influence on bacterial infectious process it is important to take into account species diversity of bacteria inducing plant disease. Thus the correct identification of bacterial agent(s) is an essential part of successful phage therapy. For this purpose, both classical approaches and express methods may be utilized [*Kapyrina, 1974*]. It is known that bacteriophages are characterized by very high specificity to their respective bacterial hosts and thus may appear effective indicators of bacteria [*Samoylenko, 1969*]. The author showed the possibility to develop collections of phages for diagnostics ('***phage diagnostics***') of bacteria belonging to different species (*Pseudomonas*, *Xanthomonas*, and *Corynebacterium*). In his other work, *Samoylenko* investigated phages used for identification of definite bacterial agents inducing bacteriosis of beans. It is worth mentioning that collections of phages used routinely for phage diagnostics should include viruses with well-studied and stable properties [*Samoylenko, 1972*]. Correspondingly, for wide phage diagnostics of bacterial diseases of plants it is essential to supplement the collection of viruses with bacteriophages newly isolated from nature. Data on the efficiency of bacteriophage isolation are rather controversial. In several foreign publications authors noted difficulties they experienced when isolating new active phages from natural environment. At

the same time *Samoylenko* [*Samoylenko, 1972*] reports on successful isolation of more than 90 isolates of bacteriophages from soil, sewage waters, infected plants. About 30 strains of *Pseudomonas* were used to determine the spectrum of lytic activity of these phage isolates. Conducted work allowed detection of bacteriophages which were suitable for fast and reliable identification of taxonomic status of some bacterial strains. Phage diagnostics was also suggested as quite reliable technique in the means of constancy of phage activity over time. Duration of conservation of bacterial cultures did not affect their susceptibility to viruses. Isolated bacteriophages demonstrated similar activity toward both newly isolated bacterial strains and those which were preserved in museums for up to 20 years [*Samoylenko, 1972*].

Another issue is the selection of proper susceptible bacterial hosts for isolation of active bacteriophages. Selection of bacteria sensitive to some phages proved to be very difficult. In particular, *Burova* and *Tovkach* [*Burova, Tovkach, 2006*] reported on low frequency of isolation of phages active toward strains of *Erwinia carotovora*. *Gorb* and *Tovkach* deemed that "one of the phenomenal properties of *Erwinia carotovora* is the existence of bacteriocins", which in their opinion might represent genomes of defective phages built into the cell genome, hence conferring 'immunity' to bacteria. The authors also suggested a putative role of bacteriocins in ecology of natural *Erwinia* populations as factors indirectly influencing the pathogenicity of bacteria [*Gorb, Tovkach, 1997*]. It's worth saying that small number of *Erwinia* phages isolated might be due to the absence of susceptible bacterial strains used in the study.

Traditionally, studies of bacterial viruses were carried out for a given taxonomic group of bacterial hosts. However, it is thought that research including phages of phytopathogenic bacteria having ***different*** taxonomic status is more objective. In one of such works, distribution analysis of phages infecting *Pseudomonas*, *Xanthomonas*, and *Erwinia* was conducted for various geographical areas in Ukraine [*Boyko et al., 2002; Boyko et al., 2004*]. These investigations revealed dominance of bacteriophages to one of the three bacterial strains used in the work. Presented data also allowed determining phages, and thus their respective hosts, occurring in nature in higher titers. Among three bacterial strains tested (*Xanthomonas beticola st. 7325* (IMV), *Pseudomonas syringae pv. lachrymans st. 7591* (IMV), *Pseudomonas syringae pv. tabaci st. 223* (IMV)), majority of isolated bacteriophages were lytically active (and hence found in higher titers) to *X. beticola st. 7325* – bacterial pathogen invoking tuberculosis of sugar-beet. According to the specifications of this species [*Bilay, 1988*], initially the agent has been isolated in USA, and generally is typical for Northern America, not to Europe. One of the possible reasons for frequent detection of bacteriophages to *X. beticola*, in authors' opinion, lies within the delivery of bacterial strains via export/import of food production (sugar-beet roots) and, more importantly, seed material [*Boyko et al., 2001*]. Another explanation could probably be wide spectrum of lytic activity of these phages; however it was proved to be wrong. Available data allow considering bacteriophages of phytopathogenic bacteria as perspective tools for controlling imported agricultural production and infected plant material.

To see the whole picture of interactive processes taking place in nature between phages and their corresponding hosts, it is deemed essential to know the numbers of the organisms in the ecosystems; moreover, this kind of data should not be one-time measurements, it should have better be of dynamic nature allowing to determine fluctuations. *Andriychuk et al*. [*Andriychuk et al., 2006*] conducted such type of research composed of continuous cycle of observations (14 months in total) of sugar-beet plants during the vegetation and of sugar-beet roots in winter [*Andriychuk et al., 2004*]. This led to identification of 'free phages' (not necessarily virulent) isolated directly from samples, and to that of moderate phages achieved by UV induction of represented lysogenic microflora. Interestingly, frequencies of phages' detection were shown to fluctuate and to have season-dependent nature. For instance, bacteriophages to *P. syringae pv. atrofaciens* (wheat bacteriosis) and *P. viridiflava* (bean disease) were identified only for a very short period of the year. On the contrary, other bacteriophages, in particular to *P. syringae pv. aptata* (sugar-beet leaf mottling), were detected on a regular basis. Same tendency for fluctuation was demonstrated for titers of bacteriophages detected. Often bacteriophages could be isolated in titers of about 10^6 PFU/ml at one time point, and were not detected at all at the next one, and vice versa [*Andriychuk et al., 2004*]. This process could possibly be related to the availability of bacterial populations capable of supporting bacteriophages' replication.

Same authors pointed out that it was necessary to consider technogenic pressure on the ecosystem. For example, about 90% of bacteriophages retained their lytic activity at pH 5-11 in laboratory conditions; and, conversely, phages were easily inactivated by more acidic/basic pH. Based on this, the authors suggested probable that 'acidic' rains may significantly influence the

numbers of phage populations and lead thus to more extensive development/spread of bacterial diseases [*Semchuk, 2003*].

Quantitative evaluation, however, was seriously complicated when isolating moderate phages from natural ecosystems. Hence only the specificity of detected inducible bacteriophages has been analyzed. The majority of moderate bacteriophages were isolated in rather low titers, probably because of the irregular UV induction happening in individual cells [*Andriychuk et al., 2004*].

It was established that normally phage populations isolated from natural ecosystems were characterized by wide spectrum of lytic activity – their overall lytic spectrum covered all tested bacterial strains [*Andriychuk et al., 2004*]. High titers of 'free' phages detected in biocenoses were rather transitory and untypical. In authors' opinion, hypothetically it is possible that constant spontaneous production and further 'incoming' of phages generated from lysogenic bacterial hosts occur in the environment [*Andriychuk et al., 2004*].

Lysogeny of phytopathogenic bacteria is underinvestigated. Only individual representatives of *Pseudomonas* and *Erwinia* were studied in some details [*Samoylenko, 1976; Tovkach, 2002; Spivak, 1972; Didyk, 1988*], and polylysogeny was recognized for several strains of these bacteria [*Kyshko et al., 1974*]. However, according to results presented by *Samoylenko* [*Samoylenko, 1975*] and *Spivak* [*Spivak, 1972*], nearly 80-90% of bacterial strains may be lysogenic. To date, many physical and chemical factors leading to induction of moderate phages from lysogenic bacterial cultures are deciphered. *Gvozdyak* and *Volkova* suggested an appealing hypothesis, pursuant to which the plant itself has the capability to induce bacteriophages of lysogenic bacteria [*Gvozdyak, Volkova, 1977*]. Such suggestions still require valid confirmations. In case these effects do exist, they may be not only of biological, but mainly of practical value – for instance with the purpose of generating new plant cultivars with prescribed activities of such 'inductors'.

Phage research assumes our understanding of mechanisms securing their preservation in nature. In natural ecosystems there are many processes mediating interactions between bacteriophages (recombination), and between bacteriophages and their hosts – bacteria (virus reproduction, lysogenization of bacteria, generation of bacterial forms resistant to definite phage(s), etc.). Normally, reproduction of a virulent phage leads to cell lysis; conversely, integration of phage genome into bacterial genome makes it lysogenic and changes its properties, for example conferring immunity against infection with closely related bacteriophages. The surviving non-lysogenic cells obviously have the potential to form populations resistant to the phage(s). Evidently there is plenty of mechanisms in nature allowing the bacteriophages to adapt to ever-changing hosts.

Using phytopathogenic bacteria species *Pseudomonas*, *Andriychuk et al*. analyzed the efficiency of phage recombination as a model for processes ongoing in nature and allowing the virus to're-adjust' to another host and hence possibly broaden its host range [*Andriychuk et al., 2006*]. In this study authors employed bacteriophages newly isolated from natural ecosystems. Indicator strains *P. syringae pv. tabaci 8646* (IMW), *P. syringae pv. aptata 8545* (IMW*), P. savastanoi pv. phaseolicola 4013* (IMW), *P. alliicola 8494* (IMW) were utilized. For recombination study there were bacteriophages used which did not demonstrate any lytic activity toward above mentioned bacteria strains. After mixed infection of bacteria with pairs of bacteriophages, the appearance of novel lytically active phage recombinants has been often seen. For about 100 pairs of bacteriophages tested in mixed inoculation, about 50% induced lysis of bacterial culture, i.e. gave rise to lytic viruses. The recombinants were stable and were easily passed further. Evidently such 'plasticity' of bacteriophages represents very capable evolutionary adaptation which allows bacterial viruses 'swap' hosts successfully. Retrieved data suggest a possibility of a never-ending ongoing natural sequence of recombination events among bacteriophages.

Serological relationships of bacteriophages may be an indirect indicator of such processes. In particular, *Ruban et al.* studied 6 phages of phytopathogenic pseudomonades. They established that 5 of them did possess large number of common antigens. Only one phage, despite having some serological commonalities with the others, was shown to be considerably different in terms of constant of neutralization [*Ruban et al, 1974*].

It was demonstrated as well that phages having differing particle morphology (with short tails and long non-contractible tails) and reproducing, respectively, in *Pseudomonas* and *Xanthomonas* bacteria, were closely related serologically [*Andriychuk et al., 2005*]. At the same time, in the limits of *Pseudomonas* phage group with similar morphology there were several bacteriophages having insignificant relatedness in their antigenic properties. These particular phages were also differing in their spectrum of lytic activity.

There were also other ecological mechanisms recorded by researchers serving hypothetically for 'preserving' quantity of phage populations in nature. Efficient isolation of bacteriophages specific to phytopathogenic bacteria *Pseudomonas*, *Xanthomonas*, and *Erwinia* was carried out from fish gills from Black Sea, and also from water samples taken during Ukrainian Antarctic Expedition. There is a possibility that bacteriophages were flushed into the sea with rain waters, and gills being a filtering apparatus did accumulate and concentrate total load of bacteriophages the fish came across [*Semchuk et al*., 2003]. The situation with phages detected in Antarctica **(**region of Argentina islands) seems to be more complicated as there were no susceptible bacterial hosts identified there [*Boyko et al., 2003; Boyko et al, 2004*]. It was rather typical for the phages isolated in Antarctica that majority of them were lytically active toward respective bacteria just for a couple of first passages. Afterwards their lytic activity decreased dramatically. It's worth saying that similar results were obtained by other investigators, for example by *Tovkach* for *Erwinia carotovora* [*Tovkach et al., 2003*]. *Mkrtumyan et al.* also showed that viruses of actinomycetes demonstrated only one efficient cycle of reproduction on susceptible host, which was then followed by 'attenuation'. The authors argued that main reason for 'attenuation' of phage reproduction was restriction-modification of virus genome inside the infected cells; hence in their opinion the only successful round of phage replication did look as the last attempt of the virus to preserve the number of population [*Mkrtumyan et al*., *1985*].

Another mechanism supporting the quantity of phage populations in nature is their stability in the environment, i.e. period of time, during which they are still capable of infecting a susceptible bacterial cell. These time spans may vary significantly for different viruses. *Gvozdyak* in his work [*Gvozdyak, 1981*] observed rather high tolerance of phages to drying and good stability in soil. Investigations of *Andriychuk et al.* [*Andriychuk et al., 2006; Romashov et al., 2005*] with several phages infecting *Pseudomonas*, *Xanthomonas*, and *Erwinia* demonstrated opposite results as the infectivity of viruses diminished soon. Obviously stability of the phages in the environment is an individual characteristic, which was already noted by *Beltyukova* in the mid-1930ies [*Beltyukova, 1935*]. In nature seemingly the quantity of phages depends on presence of susceptible bacteria. In turn, it is known that phytopathogenic bacteria normally are inactivated at the surface of soil in several days, however being able to persist for quite a long period of time on plant debris [*Bilay, 1988*]**.**

Phage research necessarily includes the peculiarities of their reproduction. For instance, *Faltus* and *Kyshko* analyzed the transfecting properties of DNAs of moderate and virulent phages of *Erwinia carotovora*. The outcomes stated that DNA of the moderate phage was more sensitive to UV irradiation. In case of mixed infection with these viruses, virulent bacteriophages gained the dominance [*Faltus, Kyshko, 1983*]. Data of this type shed light on possible recombination and competitive relationships among different bacteriophages in nature.

Didyk et al. analyzed in their work the induction of *clear* mutations of *Erwinia carotovora* phage under effect of chemical mutagens nitrosoguanine and hydroxylamine; they obtained large collection of *c*-mutants. The results are of interest from the point of view of 'construction' of bacteriophages with 'prescribed' properties. Authors deemed that both virulent and moderate phages might serve as 'starting material' for phage design [*Didyk et al, 1988*].

Research conducted on properties of phages infecting phytopathogenic bacteria isolated from various natural ecosystems allows discussing issues of virus circulation in nature. *Boyko* presented results on comparative investigations of serological and physical/chemical characteristics of *Pseudomonas syringae* phages 9B, 123 and 788/8. Despite these viruses were isolated at different locations and at varied time points, they were shown to be very similar [*Boyko et al., 1997*]. Two of them were isolated via induction of lysogenic microbial cultures. Based on the evaluation of protein composition and genome structure (restriction analysis) of these phages, the authors concluded that these bacteriophages belonged to closely related group; as the same tendency was observed regularly, authors argued that this group of phages may have gained significant spread in natural ecosystems.

Many scientists noted the occurrence of phage-resistant forms of microorganisms. *Romashov* analyzed this issue in the laboratory conditions. He demonstrated that normally virus-resistant forms of *Pseudomonas*, *Xanthomonas*, and *Erwinia* were generated in case of multiple phage infection of single cells. In laboratory conditions, phage-resistant forms of bacteria could be easily developed only when the titer of the phage came practically to 10^9 PFU/ml or more [*Romashov et al., 2004a*]. As such high virus concentrations are rather untypical for natural ecosystems it is deemed that probability of appearance of phage-resistant forms of microorganisms in nature is unlikely. These observations are in agreement with the assessment of efficiency of generating phages with shifted spectrum of lytic activity (*h*-mutants) toward phage-resistant

bacteria. For model T-even phages, *h*-mutants were simply selected after the multiple infections of phage-resistant bacteria. However, in case of bacteriophages of phytopathogenic bacteria the authors had to refer back to mutagens. Subsequent analysis of *h*-mutants (protein composition and restriction analysis) revealed differences probably responsible for phage adsorption and reproduction in 'resistant' bacterial cells [*Romashov et al., 2004b*].

Regardless of minor possibilities of occurring of virus-resistant bacteria in natural ecosystems, they may be a serious issue in industrial production of phage preparations. Methods for phage accumulation presume the use of liquid culture mediums. In fermenters, where bacteriophage reproduction in susceptible cells and accumulation of mature virus particles take place, high titers of bacteriophages reached with time can lead to full lysis of sensitive bacterial culture and further selective multiplication of microorganisms resistant to the viruses in the medium. In result, full growth of phage-resistant microflora may often happen in reactor, putting the production of desired bacteriophages to an end [*Sergienko et al., 1939*].

Apart from phages discussed above, viruses of cyanobacteria are of interest as well. These microorganisms frequently may compose a great deal of total mass of microbes inducing 'spoiled' water in reservoirs with slow currents. This, in turn, makes almost impossible to utilize such water reservoirs in economical activity. Due to this and other similar vital problems, identification of phages capable of infecting these bacteria, and elaboration of optimal conditions for their accumulation are important issues. *Goryushin et al*. in his work focused on the assessment of physical and chemical parameters of cyanobacteria cultivation necessary for successful phage production. In the system '*Synechococcus cedrorum* – phages AS-IK, S-SK' he established the significance of growth stage of bacterial culture for efficient phage infection, as well as requirements for mono- and bivalent cations, pH value, illumination intensity, diapason of optimum temperature, etc. [*Goryushin et al, 1986*].

Viruses infecting actinomycetes are also widely spread in nature, and besides often isolated during the production of antibiotics. *Mkrtumyan et al.* presented investigations directed on moderate and virulent phages of actinomycetes. 68 strains of actinomycetes tested for their susceptibility to 4 different moderate bacteriophages demonstrated various reactions on infection. Authors pointed on strongly specific type of relationships between the microorganism and the phage. Bacterial strains could rather easily turn from susceptible to resistant form and back. In authors' opinion, this mechanism may serve for maintaining the fragile balance in 'virus – cell' system, consequently permitting co-existence of both the phage and the host [*Mkrtumyan et al, 1985*].

4. Advantages and drawbacks of bacteriophage preparations in therapy of bacterial diseases

Advantages of phage preparations are as follows:
- high specificity (achieved by natural biological properties of separate viruses in preps);
- usually wide spectrum of lytic activity (resulting from polyvalence or complexity of preparations);
- reproductive capability;
- harmlessness;
- absence of side effects;
- possibility to use in combination with other forms of therapy (antibiotics, immunotherapy) [*Proskurov, 1971; Komahidze et al., 1974; Chanishvili, 1982*];
- ability to utilize for prophylactics.

Among main ***drawbacks*** of bacteriophage preparations and phage therapy itself are:
- polyvalence and/or complexity of almost all existing preparations and resultant biological heterogeneity, insufficient data of their typical composition;
- need for regular verification of preps for their effectiveness towards microflora of patients and following adaptation according to appeared strains of bacteria;
- own antigenic properties of bacteriophages and possibility of generation of specific anti-phage antibodies in organism of human or animal when the same preparation is used several times;
- probability of individual adversion of phage preparations (allergic reactions);
- short-time temperature rise in patients to 38-39˚C conditioned by absorbance of decay products and toxins released from lysed bacteria into blood (was observed during phage therapy of heavy cases of generalized staphylococcus infections) [*Gorshkova, Pogorelskaya, 1971; Meypariani, 1971; Belaya, Averina, 1974; Bregvadze, 1974; Leontyeva et al., 1974; Filichkin, 1974; Peremitina et al., 1979; Chanishvili, 1982; Samsygina, Boni, 1984*].

We should say that despite stated disadvantages of phage preparations and the therapy, their benefits are much more noteworthy as, after all, use of bacteriophages for curing bacterial infections has been proved to be very efficient ***in clinical practice*** on the whole. Certainly, individual reactions of patients cannot be ruled out, however they're rather rare and are not common. Adverse effects of phages on human or animal organisms are drawing to zero in comparison to antibiotics. In our opinion, main drawback of virtually every phage prep was the obscurity of its content, so in theory it was almost impossible to reproduce same preparation for the next time.

5. Some properties of bacteriophages influencing lytic process

Use of phages for practical purposes, in the end, assumes the interaction between the virus and the bacteria with following lysis of the cell. Investigations on phage biology give data allowing better understanding of processes underlying the system 'virus-cell'. Particularly, in discussion of expediency of phage therapy the accent was made on importance for control of high content of virus particles in the preparation during its industrial production. Research shows that cell lysis may happen even when there is no infectivity observed in phage prep but at the same time when virus particles are presented in high titer.

Two different bacteriophages – λ (*c*-mutant) and T2 – were employed for such study [*Zuyev, 1966*]. Outcomes demonstrated that both viruses (in high concentration) were able to kill bacterial cells with almost equal efficiency (~98%) independently whether the preps contained intact virulent particles or UV-inactivated virions. This data shows importance to achieve high titers of phages in industrial production of the preparations, as the cells would be affected even without any reproductive cycle of the virus.

Physical and chemical factors also influence the activity of virus particles, and our knowledge of these will enable to elaborate proper technical specs in order to avoid virus inactivation during production of the preps. Bacteriophages were shown to be quite stable. Their inactivation occurs typically by heating over 60˚C, UV irradiation, treatment with solvents of acidic pH [*Bliznichenko et al., 1972a; Bliznichenko et al., 1972b; Samsygina, Boni, 1984*]; phages are less sensitive of radioactivity.

Many phages show sensitivity to urea and alkylating agents [*Kosobutskiy, 1974*]. Some authors also observed adverse effect of magnetostaticfield on bacteriophages including those lysing *Sh. flexneri, Sh. sonnei* and *Sh. newcastle* [*Barinova, 1970*]; constant magnetic fields decreased virulence of staphylococcus phages as well [*Granstrem, Vorobeychikov, 1985*].

At the same time, UV irradiation may serve a powerful tool for obtaining phages with novel characteristics. Hence, *Ktsoyan* (1970) noted that λ phage, been irradiated by high-rate UV, produce mutants. After UV induction, reparation of lethal 'damages' of the phage by *E. coli* was observed, as well as cross-reactivation of inactivated bacteriophages (when infecting λ-lysogenic bacteria strains). Whereas, after ionizing irradiation of λ phage, *E. coli* cells were not capable of repairing lethal and premutational damages. Such cells also demonstrated no evidences for cross-reactivation. We deem that investigations in this field utilizing models based on phytopathogenic microflora may be of use for analysis of these processes in nature (for example, in Chernobyl zone, where the gradient of radioactive irradiation is clearly observed).

Essential factor influencing the success of phage selection and following production is the process of restriction-modification in a susceptible cell. *Akurashvili* (1987) showed that phages of *Staphylococcus aureus* do not undergo an intracellular limitation in their reproduction in bacterial strains with DNA restriction sites. As was identified, base of this mechanism lies in the elimination of recognition sites for restriction endonucleases of staphylococcus.

Finally, study on serological traits of phages enables their easier selection for industrial production. However, at the same there has been no significant correlation observed between lytic activity of bacteriophages and presence/absence of common antigens. For instance, *Borisov* and *Petuhova* (1962) revealed no serological relationship among many phages infecting *E. coli*.

6. Changes in bacteria characteristics during lysogenic conversion

Practice of lysogenic phage use required extensive research of theoretical bases on interactions of the virus with susceptible cell. Investigations of this type also shed light on possible risks. One of the notoriously known events elevating the pathogenicity of bacterial microflora is lysogenic conversion, when moderate phage incorporates its genome into the genome of bacteria. With this, the cell acquires immunity to further infection with homologous bacteriophage, and may also obtain new features which often amplify cell's pathogenicity. Phenomenon of lysogenic conversion was well studied for agents invoking diphtheria. Hence, search for specific moderate phages not conferring toxigenicity to diphtheria bacteria was of great practical interest. Such investigations were conducted by *Chistyakova* (1967) with many 'Ukrainian' strains of bacteria. Diphtheria phages differing in their lytic spectrum from known before were utilized in this work. It was assumed that moderate phages would not change bacterial properties (i.e., pathogenicity) during lysogeny, reserving them non-toxigenic but rendering immune to any future superinfection with homologous virus. This work led to identification of many phage variants, some of which conferred immunity and no additional toxigenicity.

Practical use of phages envisages probable presence of mixed bacterial infection in the organism of the patient. Target use of bacteriophages specific to a definite bacteria species may sometimes lead to a 'side effect' – lysogeny of other bacteria species, rarely with attaining ability to produce toxins. Therefore, in 1960ies it was established that lysogenic conversion of atoxigenic diphtheria strains toward toxigenicity may be mediated not only by their 'own' diphtheria phages, but by staphylococcus viruses as well. Even more, the last ones did not require previous adaptation [*Stratienko, 1966*]. In turn, *Bobkov* and *Borisov* (1977) revealed that antigenic variability of *Shigella flexneri* resulting from lysogenic conversion may be controlled by *E. coli* phages.

In some cases, lysogeny leads to changes in group-specific antigens of bacteria and, respectively, shifts the sensitivity of microbes to phages. Changes in cells' adsorption characteristics, in this case, reduce to zero the efficiency of previously active bacteriophages. Such changes were observed for *Shigella flexneri*. Changes the cells undergo in result of lysogeny were studied in details. It was shown that lysogenic conversion might 'transform' y-variant of bacteria (antigenic characteristic -3,4) into x-variant (antigenic characteristic -7,8). Antigenic shifts of bacteria invoked by moderate phages, obviously, condition the necessity for proper correction of virulent phage preparations used in therapy taking into account novel adsorption properties of microbial cells.

7. Phage use in other scientific elaborations

Long range of issues may be solved with the help of bacteriophages. One of them is investigation of infection transmission mechanisms. This work was carried out for agents of intestinal diseases [*Alavidze et al., 1977*]. Research in this focus may help tracing sources of food poisoning and safety control of food in general. For instance, it may be quality control of fruits and vegetables grown utilizing ecologically friendly technologies. This growing assumes use of manure as fertilizer (which is a source for vast number of bacteria); at the same time it is rare case when collected fruits or vegetables undergo thermal treatment.

Another non-standard way of phage use was their involvement into testing for toxic effects of chemical compounds. Specifically, activity of chlorine-derived substances was analyzed in parallel on both coli phages and cell culture. Authors revealed correlation between anti-phage and anti-mitotic activity of studied compounds, and hence proposed to utilize bacteriophages as test objects for analysis of similar substances [*Borisov, 1964*].

Ideas on practical use of phages apart from typical therapy agents were sometimes courageous and amusing. One of them suggested use of phage 62 of *Erwinia carotovora* for growth regulator of malignant tumor. Ehrlich carcinoma transplanted to males of white laboratory mice was the object of treatment. Two schemes of phage introduction to mice were employed. One assumed phage use before, and the other – after tumor transplantation. When the first scheme gave no notable results, the application of the second one was shown to reduce the weight of tumor by 2.7. Authors argued that probable mechanism might lie in capability of phages to induce interferon synthesis, however this research was never continued [*Lisovenko et al., 1977*].

8. Specific adsorption of bacteriophages on bacterial cells

Phages show striking specificity toward their bacterial hosts. Existence of several hosts for one phage is rare in nature. This characteristic of phage is appealing for practical use as it allows rather easy selective influence on bacterial microflora. Specificity of interactions between the phage and bacteria resembles fine-tuned mechanism in 'enzyme-substrate' system. Majority of bacterial viruses demonstrate high specificity to the hosts. Hence, it was observed for *Klebsiella* phages [*Gabrilovitch et al., 1983*], many phages of *Yersinia enterocolitica* and *Yersinia pseudotuberculosis* [*Zhugova, Gabrilovitch, 1983*], etc. Stage of phage adsorption to bacterial cell is deemed to play major role in conferring virus specificity toward its hosts – irreversible binding of bacteriophage with outer membrane of the cell is possible only when respective receptor areas are fully complimentary.

By 1958, it has been established that ***predominant*** superficial bacterial receptors for phages are carbohydrate components of lipopolysaccharide (LPS) layer and surface proteins of the outer membrane of bacterial cell. For instance, it was shown that LPS isolated from *Shigella sonnei* can irreversibly bind T3 and T4 phages.

Relationship was revealed between disruptions of LPS structure and capability of bacteriophages to adsorb on the surface of bacteria. Particularly, defects in LPS of *E. coli* K12 in the form of loss of phosphate bond conditioned its resistance to T4 phage. Additional loss of heptose led to acquisition of further insusceptibility of these bacteria to T3 and T7 phages. We should also say that LPS defects leading to loss of bacterial susceptibility to viruses also invoked hypersensitivity of these microorganisms to antibiotics novobiocin, spiromicin and actinomycin D.

Similar experiments with *E. coli* K12 mutants characterized by significant loss of rhamnose, galactose, and glucose in their LPS confirmed higher sensitivity of bacteria to ampicilline and simultaneous loss of susceptibility to bacteriophages MS-2 and φW at adsorption stage.

There is also literature data proving similar role of *Salmonella typhimurium* LPS in adsorption of phages 6SR and Br2. *R*-forms of *Salmonella typhimurium* (deficient in glucose and galactose in LPS) demonstrated decreased ability to adsorb P1 phage, too.

Comparable outcomes were shown for *R*-forms of *Klebsiella aerogenes* deficient in capsule polysaccharide: mutants were insensitive to specific phage due to loss of part of galactose molecules in their LPS [*Shoshiev, Novoseltsev, 1980*].

Some peculiarities of LPS chemical structure responsible for phage sorption were noted. It was shown that core regions of LPS of *Escherichia coli* and *Salmonella typhimurium* possessed receptor functions for φX174 and S13 bacteriophages. These regions contained *N*-acetylglucosamine, glucose, galactose, ethanolamine and other carbohydrates. Correspondingly, LPS-deficient mutants (loss of terminal galactose) became resistant to these viruses. However, researchers also observed that virus receptors 'recognize' not only terminal galactose on bacterial cell wall, but also spatial configuration of molecules surrounding this terminal glucosidal bond (3D environment).

Dependency of adsorption of T3 and T4 phages on presence of terminal glucose was also revealed. As turned out, glucose, glucosamine and some other carbohydrates of *O*-side LPS chain may serve as receptors for T4 on *Escherichia coli* surface. Experiments with *Escherichia coli* B mutants characterized with defects in LPS structure demonstrated, that absence of single or several side residues (phosphate, heptose or nitrosophosphorylethanolamine groups) connected with main carbohydrate chain, as well as presence of a single terminal residue of main carbohydrate chain, did not lead to loss of T3 and T4 phages' capability to recognize and specifically adsorb on bacterial cells. However, absence of two glucose residues in LPS did induce resistance of *Escherichia coli* B cells to phages.

There is also data on relationships between changes in LPS content and its structure of outer membrane of *Salmonella typhimurium* and *Escherichia coli* and susceptibility of these microbes to U3, S21, R1, T4, T7, φ5, φW and φ3 phages. Shift in bacterial sensitivity was shown to be tightly related with changes on LPS monosaccharide composition.

Investigations of phage receptors of *Shigella* mutants showed that different regions of bacterial membrane often represent receptors for different viruses. For instance, 2-keto-3-desoxyoctanate was confirmed as cell receptor of outer membrane for FR3 phage; heptose or glucose – for V phage; glucose – for H^+, P1 klevir, T4 and T3 phages; and core regions of LPS – for 6SR phage [*Shoshiev, Novoseltsev, 1980*].

In some enterobacteria (*E. coli* mainly), proteins of outer membrane may serve as receptors for bacteriophages. As was demonstrated, receptor protein for colicins E2 and E3 with molecular weight of 60 kD plays important role in phage adsorption; moreover, activity of this protein

developed only in presence of LPS polysaccharides. Further on, conjugation-deficient *Escherichia coli* K12 mutants were also shown to have defects in protein of outer membrane, and this defect affected capability of viruses to attach to these mutant cells.
Similarly, surface protein (44 kD) of *Salmonella typhimurium* is known to be involved in C-21 phage adsorption process. *Salmonella typhimurium* mutants (deficient in surface proteins, 33 kD and 36 kD), in turn, were significantly less susceptible to P221, PH105 and PH51 bacteriophages [*Shoshiev, Novoseltsev, 1980*].
Apart from its participation in phage sorption, LPS is also involved in penetration of virus nucleic acid into bacterial cell. Enzymes allowing viruses partially disrupt the integrity of cell wall were identified for many bacteriophages. Enzymes of T2, T4, P22, M-1 and some other phages are particularly well known. Among the phage enzymes detected, endorhamnosidase, gluconase, endoglicanohydrolase and several others are most common. Their activity induces release of corresponding reducing sugars from LPS and depolymerization of polysaccharides of outer bacterial membrane. As shown, Ca^{2+} and Mg^{2+} ions facilitated these reactions [*Shoshiev, Novoseltsev, 1980*].
Presented information permits a suggestion that the nature of *some* bacterial receptors for phages is now uncovered. In most cases, these are outer carbohydrate LPS components, or capsule polysaccharide, or, less commonly, proteins of outer membrane of bacteria. Up to date, about 150 monosaccharides (composing polysaccharides) of bacterial cell wall are revealed, and some of them are unique to bacteria. Various combinations of these carbohydrates condition chemical and serological differences among bacteria. Seemingly, these sugar combinations are unique for bacteriophage receptors as well, and this may be the mechanism conferring high selectivity of virus adsorption on the surface of bacterial host (and thus strict specificity of these phages). Logically follows that only very close similarity between chemical structures of LPS of two microbes may enable bacteriophage to infect 'new' microorganism and widen its host range. Examples of such 'coincidences' of oligosaccharide subunits of *O*-side LPS chain were noted for some yersinia and salmonella. Similarity of subunits of capsule polysaccharide between *Escherichia coli* K42 and *Klebsiella* K63 was also recorded.
Therefore, commonality of structure and chemical composition of LPS layer, and some of its superficial components in particular, governs susceptibility of bacteria to homo- and heterological bacteriophages, in other words – cross-reactivity of phages. Specifically it was established that some *E. coli* strains were characterized with high susceptibility to plague phages adequately comparable to sensitivity of natural bacterial host – *Yersinia pestis* – to this virus. It is also known that plague phage is able to lyse agents inducing pseudotuberculosis, typhoid, dysenteriae and so on. Similar activity to these pathogens was also shown for pseudotuberculosis phage. In turn, phages intestinal of microorganisms lyse plague and pseudotuberculosis bacteria [*Shoshiev, Novoseltsev, 1980*].
Data of this type were obtained for escherichias and shigellas [*Babkov, Borisov, 1977*], and for salmonellas [*Hahareva et al., 1971*]. Among many serological groups of enteropathogenic *Escherichia*, there are groups antigenically related to *Shigella*. Specifically, serogroup *E. coli* O129 contains O-antigen (and also type antigen V and group factors 3, 4 or 7, 8 which confer activity of O-antigen) analogous to that of *Sh. flexneri* 5. Moreover, *Sh. flexneri* antigens were revealed in recombinants generated by *E. coli* O129 with *E. coli* K12 [*Babkov, Borisov, 1977*].
What is more, in clinics were isolated bacteriophages lysing both *E. coli* and *Sh. flexneri*, which is rather untypical [*Dzidzishvili, 1971*]. As nowadays role of converting phages in determining synthesis of type-specific antigens and group factors by *Sh. flexneri* is fully confirmed, a suggestion was proposed that *E. coli* serogroups antigenically related to *Sh. flexneri* may arise from lysogenic conversion mediated by moderate bacteriophages.
Therefore, simultaneous occurrence of *E. coli* and *Sh. flexneri* in patients, sewage, etc., which is so typical for clinics, can lead to exchange of antigenic complexes among groups of these pathogens; this process may possibly be mediated by various mechanisms including moderate bacteriophages. The appearance of novel surface antigenic complexes stimulates potential newly acquired susceptibility of these bacteria to phages whose receptors are complementary to such antigenic structures. Further recombination of convertants with representatives of other related serogroups can facilitate wider spread of antigenic complexes in microbe population. Results of numerous experimental works state that not only viruses but also other mechanisms (conjugation/recombination) are partially responsible, and sometimes play even more significant role, for transfer of genetic information among various bacteria genera [*Dzidzishvili, 1971; Babkov, Borisov, 1977*].

Likewise, polyvalent salmonella bacteriophage was demonstrated to lyse 2-6% of circulating escherichias; most of them did possess salmonella superficial antigens of B and C group [*Hahareva et al., 1971*].
Issue on phage specificity is of interest in this context. T4 was established not to adsorb on representatives of *Vibrionaceae*, *Pseudomonas* and *Rhizobiaceae*. As about *Enterobacteriaceae* family, only *Escherichia coli* B is susceptible to this virus. However, urea treatment of bacteriophage caused changes in virus specificity and conferred him capability of infecting other enterobacteria and *Y. enterocolitica*, *Erwinia carotovora* and *Citrobacter intermedius*.
Korotyayev (1982) revealed that acquisition of plasmids by bacteria may change the adsorption properties of bacterial cell. *E. coli* together with T3 and T7 phages were utilized for this study. Author showed that some plasmids conferred bacteria ability to generate donor **pile**. This in turn 'made' cells sensitive to T3 and T7 bacteriophages. We should stress that phage adsorption occurred after its interaction with receptors on bacterial cell wall, not the **pile**.
Ivanitskaya studied biophysical regularities of reversal adsorption mechanism for T4 phage. Achieved outcomes revealed that adsorption of a definite number of virus fibrils facilitates the attachment of remaining fibrils [*Ivanitskaya et al., 1982*].
Adsorption and further lytic process of bacteriophages may be 're-adjusted' from one bacterial host to another. Experiments in system '*Yersinia pestis* – T1, T3 and T7 phages' demonstrated that induced mutagenesis may generate mutant phages being capable of replicating in an unusual host – plague microbe [*Kunitsyna, 1973*]. These conclusions are of significance for future 'directed' design and selection of bacteriophages for controlling agents causing perilous disease, and for modeling the spread of microorganisms.
Basing on aforesaid, it is assumed that bacteriophage specificity toward microbes is somewhat conditional and largely depends on chemical composition of receptors on bacterial surface and complementary receptor structures of viruses. Moreover, some experimental data point that phage genomic nucleic acid may not possess high specificity toward the interior of bacterial cell, and hence its artificial introduction into live cell of microorganism should lead to successful replication of the virus in its 'host' [*Shoshiev, Novoseltsev, 1980*].

Appendix

Interview
with ***Eugeniy L. Zhilenkov***
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Q.: What is your opinion on perspectives of phage therapy for control of bacterial infections?
A.:
Issue of practicability of bacteriophage use in treatment of diseases with bacterial etiology has been discussed extensively for several decades which is confirmed by tremendous number of publications. Thus, about 800 scientific works were published from 1917 till 1956 just in USSR. With the opening of antibiotic era, the interest to phage therapy gradually decreased, and only about 100 papers were issued in 1957-1965. However, recent publications and filed patents are all evidences for renewal of great attention to use of bacteriophages in, primarily, surgery, gastroenterology, etc.
Thorough evaluation of preceding experience in phage therapy permits explaining the reasons for such state of affairs, just as formulating specific demands to development of novel efficient phage preparations. When elaborating these preparations, researchers should take into account the following:
1. Being able to lyse bacteria *in vitro*, bacterial viruses sometimes are not active against them *in vivo*.
2. There recognized the need to develop mixed (complex) phage preparations obligatory composed of different species-specific virulent bacteriophages. It is inadmissible to use phages of one race only.
3. The preparation must contain only virulent viruses, lytic activity of which is preserved for the time of storage and application.
4. It should be considered that bacteriophages may sometimes got inactivated by the components of physiological fluids (enzymes, blood elements, etc.).
5. Stringent laboratory control of phage therapy efficiency is imperative (obligatory identification of infectious bacterial agents).
6. Lysis of bacteria by phages leads to the destruction of bacterial cells inducing massive release of antigens and various toxins from them. Such effect is of double meaning. In case of serious bacterial semination of the organism, this release of compounds from bacterial cells may invoke local and generalized reactions of the patient (body temperature increase, for instance). From the other side, release of bacterial antigens may indirectly boost patient's immunity.

The last two issues are plainly related to treatment of the diseases and to the strategies of preparations' improvement; they should be taken into account for advancement of phage therapy efficiency, and for diminishing the risks of side effects.
Hence, the success of phage therapy purely depends on our knowledge of bacterial pathogen' properties, regularities of disease course, characteristics of viruses used, and on correctly chosen scheme of treatment.
Phage selection for introduction into preparation should be based on such its properties as virulence, lytic spectrum, biological and physical/chemical features. In addition, every preparation must contain viruses differing in their receptor specificity. The latter is especially vital due to next reasons. The probability of appearance of bacteria resistant to a given phage in the preparation varies in the limits 10^{-6}-10^{-7}. At the same time, the probability of appearance of microorganism being resistant simultaneously to two phages with different receptor specificity is about 10^{-12}-10^{-14}. Such microbial populations do not exist in the focuses of infectious suppurative inflammation. Thus, for instance, use of preparation composed of 8 different phages guarantees reliable lysis of pathogenic microflora both in case of monoinfections and associated (complex) ones. This conclusion is confirmed by the outcomes of numerous clinic tests.

Conclusions:
I deem that there is nothing better than combination ('cocktail') of virulent natural phages for treating infectious diseases of bacterial etiology. The main task is to compose such a cocktail. Genetic engineering in phage construction is quite tempting but hard-hitting, at least in the nearest future.

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