

# **SHOULD PHAGE THERAPY BE APPROVED IN THE U.S.?**

An Interactive Qualifying Project Report

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## **ABSTRACT**

Phage therapy is the use of viruses to lyse bacteria. The technique has the potential for treating antibiotic-resistant bacterial infections, an increasing threat to our healthcare system. The goal of this IQP project was to evaluate phage therapy technology by assessing its technical, ethical, and regulatory problems, to help determine whether it should be approved for use in the U.S. Our team performed a review of the current research literature, and conducted interviews with academic researchers, workers in phage companies, regulatory experts, and bioethicists. We conclude that the technology has great potential, but the methods used need to be standardized, precautions need to be enacted to keep the technique safe, and more large-scale blind placebo-controlled human clinical trials need to be performed.

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Mingxin Yu	Problems with Antibiotic-Resistant Bacteria Background on Phage and Early Phage Treatments
Benjamin Cossette	Phage-Related Therapies (Tailocins and Lysins) Phage Safety
Xinyuan Wang	Status of Current Commercial Therapies and Trials Phage Regulatory Issues

# PROJECT GOALS

The overall goal of this IQP project was to document and evaluate the technology of phage therapy (and the related alternatives of tailocins and lysins) for killing antibiotic-resistant bacteria, to determine whether the technique(s) really work, and to assess their ethical and regulatory problems.

**The specific objectives were to:**

- 1 **Develop** a comprehensive assessment of the scientific experiments that led to the development of phage therapy, and discuss the technique's potential applications.
- 2 **Characterize** what key scientific and IVF stakeholders believe are the strengths and weaknesses of this technology, and their ethical and legal concerns.
- 3 **Evaluate** all of the obtained evidence, and prioritize the remaining problems.
- 4 **Recommend** potential solutions to any remaining problems.

## EXECUTIVE SUMMARY

Bacteriophages (phage) are viruses that infect bacteria. Some types of phage are relatively harmless to their host, while other types kill it. Phage therapy is the use of viruses to kill bacteria. It is of recent interest for killing antibiotic-resistant bacteria, especially “superbug” strains that no longer respond to any known current treatment. Bacterial infections resistant to antibiotics have become a top priority in the health care industry as the ability to resist antibiotics spreads. Medically and economically important cases of antibiotic resistance include *Pseudomonas aeruginosa*, *Staphylococcus aureus* (especially methicillin-resistant staph aureus, MRSA), *Clostridium difficile*, and foodborne pathogens *Escherichia coli* strain O157:H7 and *Listeria monocytogenes*. The advantages of phage treatments are 1) their high specificity for a particular bacterial host (due to a specific virus/receptor interaction), 2) the lack of host resistance (the genes that confer antibiotic resistance, and that spread easily from host to host, do not help bacteria resist phage), and 3) the phage can replicate (amplify) within the host releasing more phage to infect other bacteria. Phage therapy has a rich history in Russia and Eastern Europe, especially in countries that cannot afford expensive last-resort antibiotics.

But phage therapy has been used less in Europe and the U.S., producing few large well-controlled clinical trials. Little has been published on the technique in Western journals, especially during the height of antibiotic discoveries, when the world thought that all bacterial diseases could be cured with antibiotics. And our knowledge of phage often involved lab strains, like lambda and T4, so we know relatively little about phage strains that would be used for therapies. Phage treatments are sometimes performed with mixtures of phage isolated from sewerage based solely on their ability to lyse bacteria, with little attention paid to their specificity. Experiments for effectiveness are usually performed in petri dishes, not in animal models or humans, where access to the resistant bacteria can become hindered. Some labs have observed problems with the phage treatments, including side-effects from bacterial debris or toxins released by the bacteria, and inflammation from the patient’s immune response to the phage. Do these techniques really work? Are phage mixtures better than using single species of phage? And what about the newer versions of phage therapy, such as tailocins that use only the phage tails to make a hole in the bacterial membrane, or phage lysins that are enzymes used by phage to help penetrate or exit the host, do they work as well as phage? Are they safer than using a phage that can replicate inside the host?

The overall goal of this IQP project was to document and evaluate the technology of phage therapy (and the related alternatives of tailocins and lysins) for killing antibiotic-resistant bacteria, to determine whether the technique(s) really work, and to assess their ethical and regulatory problems. The specific objectives were to: 1) Develop a comprehensive assessment of the scientific experiments that led to the development of phage therapy, and discuss the technique’s potential applications. 2) Characterize what key scientific and IVF stakeholders believe are the strengths and weaknesses of this technology, and their ethical and legal concerns. 3) Evaluate all of the obtained evidence, and prioritize the remaining problems. 4) Recommend potential solutions to any remaining problems.



To accomplish objective-1, we performed a review of the current literature, including reputable academic journal articles, relevant books, scholarly websites, and other pertinent materials. To accomplish objective-2, we conducted a set of interviews with various academic researchers. The interviewees included individuals working with phage therapy in both academia and industry. The purpose of the interviews was to determine the interviewees full range of opinions on phage therapy, and to solicit their help gauging the strengths and weaknesses of this new technology. After performing the Literature Review and interviews, the team synthesized all of the information collected to ascertain the strength of the evidence for and against phage therapy, and created recommendations for moving forward in the U.S.

## **Problems with Antibiotic-Resistant Bacteria**

Antibiotics are a type of antimicrobial drug used to treat or prevent bacterial infections. With their ability to block bacterial infections, antibiotics have revolutionized medicine in the 20<sup>th</sup> century. In the developed world, antibiotics have helped lead to the near eradication of some types of bacterial diseases. Unfortunately, the overuse of antibiotics (especially in the livestock industry), and the passage of antibiotic resistant genes between bacteria, have led to widespread problems with antibiotic resistance, a situation where the bacteria are no longer killed by the antibiotic. This resistance is typically an adaptation of the microorganism to its environment: in this case either the bacteria adapt to the antibiotic or they die.

The main drivers for antimicrobial resistance are 1) the overuse of antibiotics (especially in the animal husbandry industry), which exerts an ecological pressure on microorganisms killing the sensitive bacteria and leaving the antibiotic-resistant bacteria to survive, 2) the spread of genes between bacteria that confer antibiotic resistance, and 3) the increasing spread of antimicrobial-resistant microorganisms (between humans, between animals, and between humans and animals and the environment). Genes encoding antibiotic resistance proteins (such as enzymes that degrade antibiotics) often occur on plasmid DNAs that are mobile and easily transmitted between bacteria.

Antibiotic-resistant bacteria survive in the presence of the antibiotic, and continue to grow and divide, increasing the length of the illness or even causing death. Infections caused by these bacteria may require more intensive care, may require intravenous antibiotics delivered in a hospital (instead of oral antibiotics taken at home), or can require more expensive antibiotics which can have severe side-effects. Once these bacteria become established in a person, they can spread to another person.

Some bacteria have become resistant to several classes of antibiotics (multi-drug resistance). And even worse, some superbugs are resistant to all known classes of antibiotics. The acquisition of multi-drug resistance is important for all types of microorganisms, but is especially important for the bacteria responsible for healthcare-associated infections, microorganisms responsible for food- and waterborne infections, tuberculosis, and microorganisms responsible for sexually-transmitted diseases. Examples of the most medically important multidrug-resistant bacteria in the U.S. are: Methicillin-resistant *Staphylococcus*

*aureus* (MRSA), Vancomycin-resistant enterococci (VRE), Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae* (such as *Escherichia coli* and *Klebsiella pneumoniae*), Multidrug-resistant *Pseudomonas aeruginosa*, and *Clostridium difficile*. According to the CDC, the organisms classified as urgent threats include: *Clostridium difficile* (CDIFF), Carbapenem-Resistant *Enterobacteriaceae* (CRE), and *Neisseria gonorrhoeae*.

According to the Centers for Disease Control, bacterial antibiotic resistance causes hundreds of thousands of deaths globally annually (CDC, 2013). In the U.S., the CDC estimates that each year at least two million illnesses and 23,000 deaths are caused by antibiotic-resistant bacteria. The increase of antibiotic-resistant bacteria has prompted the World Health Organization to state this resistance is a serious threat, no longer a prediction for the future (WHO, 2014). On January 27, 2015, President Barack Obama issued a Fact Sheet on his fiscal year 2016 budget, which proposed a historic investment to combat antibiotic-resistant bacteria to protect the public health (President Obama, 2015). The Fact Sheet indicated that “antibiotic resistance is one of the most pressing public health issues facing the world today”...it affects our ability to “perform a range of modern medical procedures from joint replacements to organ transplants, the safety of which depends on our ability to treat bacterial infections that can arise as post-surgical complications”. The report states that “the CDC reports that antibiotic-resistant infections account for at least \$20 billion in excess direct health care costs, and up to \$35 billion in lost productivity due to hospitalizations and sick days each year”. In September 2014, President Obama signed an Executive Order initiating federal efforts to combat the rise in antibiotic-resistant bacteria. The President’s FY 2016 budget nearly doubles the amount of federal funding to attack this problem by approving \$1.2 billion to perform risk assessment, surveillance, and reporting capabilities, and fund research innovation (President Obama, 2015).

The challenge with multidrug-resistant microorganisms is the limited number of remaining options, so new therapies must be devised.

## **Phage and Early Phage Treatments**

An alternative option for eliminating antibiotic-resistant bacteria is phage therapy, which relies on the use of bacteriophages, viruses that specifically kill bacterial cells. These viruses occur naturally, and are not thought to attack mammalian cells, so scientists are interested in developing phage therapies to treat bacterial diseases.

A bacteriophage (phage) is a virus that infects and replicates within a bacterium. Phage are among the most common and diverse bio-entities on earth. As expected, they are usually located in the same places as bacteria, such as soil and animal intestines. Structurally, phage are composed of proteins, and a genome of either DNA or RNA. The proteins form the main structures, including the head, collar, and tail. The head contains the genomic material, while the tail usually helps recognize and attach to the surface of the bacterial host cell. Phage can have simple or elaborate structures, and their genomes may encode as few as four genes, and as many as hundreds of genes. Following attachment of the phage to the bacterial surface, the genome is injected into the cytoplasm through the hollow tail structure, and the genome replicates using host enzymes.

To enter a host bacterial cell, phage attach to specific receptors on the bacterial surface. These phage receptors can include lipopolysaccharides (LPS), teichoic acids, proteins, or even flagella. With respect to phage therapy, the important point about the interaction of phage with receptors, is that the phage can infect only bacteria containing the appropriate receptor. This determines the phage's host range and specificity. Phage are not capable of independent movement, so they rely on random encounters with the appropriate bacterium in the blood, lymphatic circulation, irrigation water, soil water, etc. Once bound to its receptor, phage inject their genomic material into the host cell where it replicates and makes more phage particles (lytic stage) or integrates into the host DNA (lysogenic stage).

Phage therapy is the use of phage to kill bacteria. Due to the drastic rise in antibiotic-resistant bacteria, and their burden to patients and the healthcare industry, phage therapy is viewed by some scientists as an alternative method for killing the bacteria. But the development of phage therapy has not been straightforward. In 2012, William C. Summers in his article titled "The Strange History of Phage Therapy (Summers, 2012), concluded that the phage therapy field contains conflicting observations, misinterpretations, and incomplete understanding, while at the same time it is rich with politics, personal feuds, and unrecognized conflicts.

Phage were discovered by Frederick Twort (Twort, 1915) and Felix d'Herelle (d'Herelle, 1917). Only two years later, in France in 1919, d'Herelle performed the world's first human phage therapy experiments (summarized in Pirnay et al., 2011) on patients suffering from severe dysentery at the Hospital des Enfants Malades in Paris. In the 1920's and 1930's, phage therapy continued to be developed in the former Soviet Republic of Georgia (pioneered by Giorgi (George) Eliava and co-discoverer Felix d'Herelle) where they were used to treat soldiers in the Red Army suffering from bacterial infections. Although phage also had some early use in the U.S, by the 1940's they were abandoned in the West, mostly because of the discovery of antibiotics, which were easier to make, store, and prescribe. Subsequently, phage research continued in the former Soviet Union and Eastern European countries, which tended to lack antibiotics, but their scientific publications were not always translated for Western use.

Most of the early phage trials were not well controlled, but a few were well done studies. The first randomized, double-blind, placebo-controlled clinical trial was performed at the UCL Ear Institute and Royal National Throat, Nose and Ear Hospital, Grays Inn Road, London. This trial evaluated the safety and efficacy of a phage cocktail (Biophage-PA) to treat antibiotic-resistant *Pseudomonas aeruginosa* ear infections in 24 patients (Wright et al., 2009). Their results showed that both the patient-reported and physician-reported clinical events improved for the phage treated group relative to the placebo group, and the former group showed significantly lower *P. aeruginosa* counts. No treatment related adverse event was reported. The study concludes that bacteriophage preparations were safe and effective for treatment of chronic ear infections in humans.

However, another controlled human clinical trial showed no benefits. A study done in 2009 in the Department of Psychiatry at the University of Minnesota (Minneapolis, USA) (Rhoads et al., 2009) examined the safety of phage therapy for difficult to treat wounds. They used a mixture of phage against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* to treat 42 patients with chronic venous leg ulcers (VLUs) for 12 weeks. Although they found no adverse events associated with the phage treatment, there was no

difference between the controls and phage groups with respect to rate of healing. The authors speculated that the use of chemicals that are part of standard wound care (e.g. lactoferrin or silver) may have interfered with the survival of the phage.

Overall for human phage therapy trials, the authors usually concluded that their phage therapy worked; but unfortunately the studies were small and not well controlled. Because most of the trials were done on patients that had not previously responded to antibiotics, any hint of an improvement was often touted as a success. The trials were usually not blinded, not all the early cases were presented in detail, and sometimes the phage therapy was also accompanied by antibiotic treatments, so it is hard to separate the two techniques. We concluded that more rigorous studies on larger patients samples is needed to help move the field forward.

To help move the field forward, several commercial companies have become involved in phage research or therapies, including: Companies that focus on pre-clinical R&D: AmpliPhi Biosciences (US), Enbiotix (US), Fixed Phage (UK), InnoPhage (PT), Intralytix (US), Novolytics (UK), Pherecydes Pharma (FR), Sarum Biosciences (UK), Synthetic Genomics (US), Technophage (PT); Companies that do not employ replication-competent phages: AvidBiotics (US), Enbiotix (US), Phico (UK), Companies primarily involved in phage product distribution: Biochimpharm (GE), Imbio (RU), Microgen (RU); Companies that emphasize phage-mediated biocontrol (not "therapy"): APS Biocontrol (UK), Epibiome (US), InnoPhage (PT), Intralytix (US), Micros Food Safety (NL), Omnilytics (US), Phage Biotech (IL), Phagelux (CN), Technophage (PT); Companies that market phage lysates: Delmont (US); Companies involved in enzybiotics: GangaGen (US/IN), Lysando GmbH (DE), Micros Food Safety (NL), New Horizons Diagnostics (US); Companies that emphasize phage-based bacterial detection technologies: Sample6 (US); Companies that focus on phage-associated industrial contamination: Phage Consultants (PL); Companies that emphasize phages in biotechnology products: Versatile BioSciences (US); and Companies that facilitate patient phage therapy treatment: Center for Phage Therapy (PL), Eliava Phage Therapy Center (GE), Globalyz Biotech (US), Novomed (GE), Phage Therapy Center (GE), Phage International (US).

## **Phage-Related Therapies**

Although phage therapy appears to offer a particularly promising solution to the growing problem of antibiotic-resistant bacteria, this does not necessarily ensure the adoption of phage therapy in Western medical practice. Phage therapy relies on the introduction of live replication-competent virus into the patient. This can hinder public acceptance. Phage are live viruses that can mutate and evolve when replicated and manufactured; mutations can give rise to unforeseen and undesirable effects. And it is labor intensive to identify and characterize specific phage species appropriate for treating a specific infection. Although it remains uncertain whether these obstacles will prevent the acceptance of phage therapy in Western medicine, it is clear that they could prolong the adoption process.

In an attempt to circumnavigate some of these obstacles, some scientists are investigating alternative methods of antibacterial therapy using portions of phage, rather than using the whole live virus. If these alternative phage-related therapies can be optimized, they might serve as a safer replacement for phage therapies since there is no phage genomic material that enters the

host cells and no chance of altering the bacterial host cell. The two main alternative therapies discussed in this project are tailocins (pyocins) and lysins.

A tailocin is a term used to describe a bacteriocin that resembles a phage particle consisting of the tail and tail fibers, but missing its head and genomic material. The tailocin is functionally able to attach to the host bacterial cell and depolarize the plasma membrane to kill the bacterium (Ghequire and DeMot, 2015). Tailocins appear to kill bacteria by the same hole-forming process that is used for injection of the phage genomic material. In nature, tailocins serve different ecological uses, such as to kill competing bacteria. One tailocin can kill one bacterial cell, and the bacteria cannot easily evolve resistance, so these agents may have applications for killing antibiotic-resistant bacteria. The tailocin is thought to attach to a bacterium like a mature phage would, with the tail fibers attaching to specific lipopolysaccharide (LPS) residues on the host cell. The tailocin attaches in its extended form, and (for the contractile types) the outer sheath contracts to expose a non-flexible inner tail tube and spike that act like a syringe to insert through the cell membrane. The insertion depolarizes the membrane, and releases cytoplasmic contents, killing the cell.

Although the origin of tailocins is unknown, some scientists believe that during evolution, some bacteria mutated (altered) the phage genes integrated within their chromosomes to suit the bacterium. Some bacteria co-opted the capsid structures, others the tail structures, and others co-opted both. The genes encoding phage tails are especially beneficial for bacteria to use because they are complex nano-machines with moving parts, so there are many functional areas to alter to suit their purposes. If the bacteria can mutate these genes to be under the control of their own secretory systems (type VI secretory system, T6SS), the tail structures are secreted outside the bacterial cell to bind to and affect other bacteria. The gene structure of the core components of all contractile tail-like systems appears to be highly conserved, but have diverged considerably to where ancestry can no longer be easily detected (Leiman and Shneider, 2012).

For use in therapy, some scientists have begun creating recombinant tailocins for antibacterial applications. For example, AvidBiotics Corporation (South San Francisco, CA) has created a recombinant R-type tailocin targeted to *Escherichia coli* O157:H7, a pathogenic *E. coli* strain often seen as a food contaminant (Scholl et al., 2009). The group used tail fiber genes of phage AVR2-V10 (that naturally infects *E. coli*), and fused the genes with the potent catalytic tail spike of the *P. aeruginosa* R-type tailocin, to create a recombinant tailocin capable of targeting and disrupting the LPS layer of the *E. coli* target. Another team at the Channing Laboratory of Brigham and Women's Hospital (Boston) engineered an R-type (contractile) pyocin (termed AvR2-V10.3) to specifically kill enteric pathogen *Escherichia coli* O157 (Ritchie et al., 2011). The team began with the naturally occurring structure of a contractile type R2 tailocin, and altered the gene encoding the tail fibers (which attach to the bacterial cell) to specifically bind *E. coli* O157. In a rabbit model of infection, the team showed that oral administration of AvR2-V10.3 prevented *E. coli* O157:H7-induced diarrhea and intestinal inflammation. The tailocin was effective when delivered either prior to or post-infection. In addition, a team at AvidBiotics Corp. (South San Francisco, CA) and the Microbial Pathogenesis Laboratory of the Wellcome Trust Sanger Institute (Hinxton, United Kingdom) engineered a modified R-type tailocin to target *Clostridium difficile*, a major cause of hospital acquired infections (Gebhart et al., 2015). The team genetically modified the contractile R-type bacteriocin "diffocin" (isolated from *C. difficile* strain CD4) to kill virulent O27-type strains by replacing the natural receptor binding protein (RBP) of diffocin with a newly discovered RBP

that binds virulent 027. The engineered diffocins (termed Avidocin-CDs Av-CD291.1 and Av-CD291.2) were stable and killed all 16 tested 027 strains. Orally administered Av-CD291.2 survived passage through the GI tract, did not detectably alter the mouse gut microbiota, and prevented antibiotic-induced colonization of mice inoculated with 027 spores (Gebhart et al., 2015).

Tailocins, including recombinant tailocins, may represent an alternative approach for lysing antibiotic-resistant bacteria, but without controlled clinical trials, it is impossible to judge its therapeutic potential.

Another type of phage-related therapy being developed are enzymes encoded by phage that act to help the phage particles exit from the bacterium during the lytic stage by degrading the cell wall (Hermoso et al., 2007). These enzymes are termed lysins (endolysins or murein hydrolases). Lysins are highly specific enzymes that degrade a key bond in peptidoglycan, the main component of bacterial cell walls. As is the case with phage tails, the lysins appear to be highly specific and effective killers of bacteria, and so may have applications for killing antibiotic-resistant bacteria (reviewed in Fischetti, 2008). Lysins are usually composed of a cell-binding domain (CBD) and a catalytic domain. The CBD, as its name implies, binds to a specific substrate in the bacterial cell wall, usually a carbohydrate component of the peptidoglycan. The sequence of the CBD is highly variable, allowing great specificity for attaching to specific bacteria (García et al., 1988). This specificity (as with phage and tailocins) is important, as it would leave the beneficial bacteria in the patient unharmed.

Lysins usually degrade the cell wall of the same species of bacteria that produced the phage. So when trying to kill a specific species of antibiotic-resistant bacteria, the lysin would likely need to be manufactured (or originally isolated) from that same species, although some widely acting lysins have also been discovered. The discovery of heat-stable lysins may facilitate their use in medicine because it makes their purification easier (Plotka et al., 2014). Most lysins discovered to date are active against “Gram-positive bacteria” (Fischetti, 2008) because “Gram-negative bacteria” contain an outer membrane that blocks lysin access to the peptidoglycan layer. However, some lysins have been engineered to be active against Gram-negative bacteria (Briers et al., 2014). When naturally utilized by a bacteriophage, lysins act from within the cell (endolysin), but if it is to be used to kill bacteria the lysin must be able to act from outside the cell (exolysin). This is no problem with gram-positive cells because their PG layer is directly accessible from the cell exterior. The PG layer of gram-negative cells can be accessed if the exterior lipopolysaccharide layer is disrupted.

Lysins were first used therapeutically in animals in 2001 in the Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University (New York, NY) (Nelson et al., 2001). The Rockefeller team investigated the ability of the murein hydrolase isolated from the Streptococcal bacteriophage C1 to prevent bacterial colonization in mice. *In vitro* experiments showed that the purified lysin killed group A, C, and E *Streptococci*, leaving the other tested strains alone. Amazingly, 1,000 units of lysin (10 ng) was sufficient for lysing approximately  $10^7$  group-A *Streptococci* within 5 seconds. A single dose of 250 units of lysin applied orally to the mice significantly reduced strep oral colonization. The same Rockefeller team isolated lysin Cpl-1 from a lytic pneumococcal bacteriophage, and tested it in a mouse model by intravenous therapy against a pneumococcal challenge (Loeffler et al., 2003) and found it increased mouse survival to 100% compared to 20% for untreated mice. Other scientists have used lysin CPL-1

to treat *Streptococcus pneumoniae* infections in a mouse sepsis model (Jado, 2003), used topical treatments with Lysin PlyGBS to effectively reduce Group B Streptococci (GBS) vaginal infections in mice (Cheng et al., 2004).

Lysins are not perfect treatments. As with normal phage or phage tails, lysins are foreign proteins that can stimulate an immune response in the patient. An immune response from the patient directed against the phage component could lessen its effectiveness, or could become dangerous to the patient if it induces a cytokine storm response. But as a protein therapeutic, lysin therapy may be more readily accepted by Western medicine, because protein therapeutics are already in use. Additionally, single proteins are much easier to characterize than whole phage particles. The highly desirable specificity that is characteristic of phage therapy is not sacrificed by lysin therapy, potentially allowing for specific bacterial species eradication *in-vivo*. Additionally, like phage therapy, lysin therapy leaves little-to-no potential for the rise of bacterial-resistant strains, as the components lysed by the lysin are required by bacteria for survival. In fact, lysins have been approved and are already being employed by the food industry. Cows have been genetically engineered to secrete a “lysin-like” protein in their milk to kill *Staphylococcus aureus* (Fischetti et al., 2006). Lysin genes have also been engineered into the genomes of different produce products such as potatoes and pears to protect them from specific bacterial infections. As more lysins are characterized, and treatment regimens are optimized for maximum effectiveness, the adoption of lysins as protein therapeutics for bacterial infection in Western medicine grows more feasible.

## Phage Safety

Although phage (and their components) may be excellent candidates for treating antibiotic-resistant bacterial infections, some scientists worry that the treatments may have safety issues. Such worries accompany all new drugs and vaccines, and experiments should be (and in some cases already have been) designed to address the concerns. We identified four main concerns about phage therapy.

The first area of safety concern is the potential for genetic alterations to the phage, bacterium, or human patient. This is the main area of concern to most scientists. A normal replication-competent phage as it replicates to high titers in the patient could mutate and evolve into a pathogen with new undesirable effects. Or using temperate (lysogenic) phage (which are capable of integrating their DNA into the bacterial host chromosome as part of their life cycle) could alter a host gene to create a different pathogen. The large-scale production of phage has been shown to produce a small percentage of that have become mutated (Krylov et al., 1993). However, the mutation process usually makes the phage inactive, not more potent. The most frequent type of mutation observed renders the phage unable to infect the bacterium. Such mutations occur in about 10% of the phage particles in a large-scale batch preparation, which slightly lessens the effectiveness of the batch for therapy, but this process typically has no effect on safety (Krylov et al., 1993). Some labs are developing procedures for preparing and monitoring phage batches with high activity for therapy uses. However, high titer production in bacterial culture is not the same as high titer replication inside humans, so scientists should continue to assay for gene alterations during human clinical trials, even if such alterations are unlikely.

Temperate (lysogenic) phage integrate their DNA into the host chromosome or plasmid as part of their life cycle (Krylov et al., 2014). As an example of a bacterium becoming highly toxic after integrating phage genes, the genes encoding cholera toxin (the CTX-element) move between cells of *Vibrio cholera* using a filamentous phage (Waldor and Mekalanos, 1996). However, temperate phage are not generally used for therapeutic experiments, because lytic phage (that only lyse the host) are more useful for killing the host cell. The vast majority of phage that have been characterized to date are lytic; only a small percentage have the ability to integrate their DNA into the host chromosome (Summers, 2001). From a safety perspective, to avoid the chance of alterations to the bacterial DNA (or in a worst case the human chromosome) it is easy to simply ban the use of any temperate phage for therapy. In addition, the entire problem of genetic alterations could be avoided by simply using tailocins or lysins, which lack genetic material.

The second area of safety concern is the potential release of toxic proteins from the lysing bacteria, which could harm the patient. This is a valid safety concern; in some cases, lysing bacteria inside a patient are known to release endotoxins that cause fever, and in some cases toxic shock. But in the few clinical trials published to date (discussed and cited in Lit Review Section-2) the fever is usually manageable, and is a minor concern relative to the patient's life threatening antibiotic-resistant infection. In addition, the use of tailocins that kill bacteria without lysis would help minimize this problem. With tailocins, the inactivated (non-lysed) bacteria are cleared from the system by the patient's own phagocytic cells.

The third area of concern is the use of ill-defined phage or phage mixtures. Phage genomes remain largely uncharacterized. Even the most studied phages have not yet been fully characterized. Some scientists argue, that before phage therapy will be accepted as safe and effective, the therapeutic phage must be fully characterized and screened to eliminate phages that encode toxic proteins or proteins that allow temperate (integrative) phage behavior. However, other scientists, including those interviewed in this project, argue that scientists use partially characterized viruses all the time in vaccines, and it is not a requirement for use in humans.

Phage samples used for therapies are often cocktails (mixtures of several phage species). Such cocktails, often used in Russia and Eastern Europe, are ill-defined mixtures of phage screened for their ability to lyse bacteria from sewerage etc. Although such mixtures are usually uncharacterized, they are not necessarily unsafe. DNA sequencing is now relatively easy and cheap to perform, and can provide data on which phage are present in a cocktail, and whether known toxic genes are present. Such quality control DNA sequencing could be done on each large scale phage batch prior to therapy. The sequencing could exclude the use of phage whose genomes encode known undesirable products as toxins, transposases, or repressor proteins, although unknown products would not be identified by this process (Krylov et al., 2014).

The fourth area of safety concern are immune responses induced by the phage. The use of phage (or lysins or tailocins), all of which are foreign proteins to humans, could induce an immune response in the patient that could either reduce the effectiveness of (inactivate) the therapy, or could kill the patient. Fortunately, although a patient immune response sometimes forms, it is usually mild and there is little risk of toxic shock. Indeed, most clinical trials of various phage therapies report no adverse immune effects (Wright et al., 2009; Kutter et al., 2010). In support of these findings, it is useful to note that humans have been naturally exposed to phage for our entire existence. We are constantly exposed to phage in our air and drinking



water, and millions of phage can be found in a single mouthful of seawater (Omnilytics Inc., 2016). Many of the phage therapy trials have seen patient successes using a therapeutic load much lower than that found in a mouthful of seawater (Kutter et al., 2010).

Overall with respect to safety, we recommend the following: 1) not using lysogenic phage (no DNA integration), 2) switching to non-lysing tailocins if toxic protein release from the bacteria become a problem, 3) using rapid DNA sequencing to characterize as much as possible any phage sample to be used for therapy to screen for mutations that may have occurred during phage amplification, or to screen for known harmful genes that may be present, and 4) pre-screening patients for hyper-immune reactions to the specific phage sample prior to injecting large quantities into the patient. If a phage passes these screening checkpoints, it is likely that it is safe and suitable for therapeutic use. Most scientists argue that the risks associated with phage therapy are relatively minor and can easily be circumnavigated with proper precautions.

## Status of Commercial Therapies and Clinical Trials

In spite of the fact that there are relatively few scientific studies on phage therapy in the Western literature, several companies are currently performing pre-clinical experiments or are in Phase-I clinical testing. At least three companies have already started phage therapy clinical trials: AmpliPhi (Richmond, Virginia), ContraFect Corp. (Yonkers, NY), and Pherecydes Pharma (Romainville, France).

AmpliPhi Biosciences Corporation (NYSEMKT: APHB, Richmond, Virginia) is a biotechnology company focused on the development and commercialization of novel bacteriophage-based therapeutics ([www.ampliphi.com](http://www.ampliphi.com)). AmpliPhi is currently conducting a Phase-I clinical trial of their lead product AB-SA01 for killing *Staphylococcus aureus* in chronic rhinosinusitis patients, the first phage therapy trial for this condition. The company is also developing bacteriophage therapeutics against *Pseudomonas aeruginosa* and *Clostridium difficile*. The results of AmpliPhi's phase-I clinical trial are not expected until the second half of 2016, but an update was provided on April 20, 2016, which stated that the treatment appears to be well tolerated.

ContraFect Corp. (Yonkers, NY) is a phage company that is currently in Phase-I testing of their lead drug CF-301, a lysin enzyme that is active against *Staph aureus* (<http://www.contrafect.com/>). CF-301 is an enzyme that targets a conserved region of the cell wall that is vital to the bacteria, so resistance is less likely to develop. Experiments performed *in vitro* and *in vivo* have shown that CF-301 degrades biofilms, so the drug likely can interact with its bacterial target *in vivo*. CF-301 was licensed from The Rockefeller University, and was developed at ContraFect. In 2014, the company published their pre-clinical findings of the effectiveness of CF-301 against *S. aureus* and MRSA in mice, showing that CF-301 lysin has potent, specific, and rapid bacteriolytic effects against *Staphylococcus aureus* (Schuch et al., 2014). On December 15, 2015, the company completed the Phase-I portion of their clinical testing of CF-301, and reported no adverse side effects of the drug.

Pherecydes Pharma is located in Romainville, France, and produces phage cocktails to combat *E. coli* and *P. aeruginosa* burn and skin infections, *P. aeruginosa* respiratory infections, and *S. aureus* bone, joint, and prosthetic infections (Pherecydes, 2016). Their lead products are

PhagoBurn, PneumoPhage, and Phosa. PhagoBurn is the world's first phage therapy to be tested in an international multi-center clinical study. PhagoBurn, as its name implies, is a phage cocktail designed to treat burn patients. The phage mixture was collected from bacteria-rich sewerage flowing underground from Parisian hospitals, and is designed to lyse *E. coli* and *P. aeruginosa* bacteria found in burn infections. Their randomized, controlled, single-blind Phase-I/II clinical study was approved in June 2013, and began in July 2015, for a period of 36 months ([www.phagoburn.eu](http://www.phagoburn.eu)). The trial is currently underway at 11 burn unit centers in France, Switzerland and Belgium. An update on the PhagoBurn ongoing clinical trial was recently published ([Servick, 2016](#)), and indicates that it has had a few challenges to overcome, including several delays and a decreased patient numbers. The delays resulted from the increasing burden of validating and documenting the various production steps in preparing the phage, which was supposed to take only 12 months, but took 20 months. And another delay occurred when France's National Agency for the Safety of Medicines and Health Products required the company to prove the stability of the phage product. But very recently (June, 2016) the agency accepted the company's data showing the product was stable and non-contaminated ([Servick, 2016](#)). The trial was supposed to have enrolled 220 patients from 11 hospitals, but in 6 months of recruitment only 15 patients have so far been found to be eligible. Patients infected with more than one bacterial species are not eligible for the trial, and unfortunately this pertains to most burn victims. However, other scientists remain optimistic that much will be learned from this trial and its approval and enrollments, even if it does not work well.

Pneumophage is Pherecydes' phage cocktail designed to treat *Pseudomonas aeruginosa* acute respiratory infections. The product was launched in June 2015, and was designed for inhalation. The project is a collaboration between the French Technology Diffusion (Saint-Etienne, [www.dtf.fr](http://www.dtf.fr)) (who specializes in developing new aerosol dispensers adapted to drugs), Pherecydes Pharma (who develops and prepares the phage cocktail), and pharmaceutical regulatory agencies (to aid the evaluation in humans). Phosa is a phage cocktail designed to treat major bone and joint infections, and diabetic foot ulcers, caused by antibiotic-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. The product was launched in January 2015, and its testing will continue for 24 months, which will include designing the composition of the phage cocktail, testing the prepared phage in two animal models, and then launching a human clinical trial to evaluate safety and efficacy.

## Phage Regulations

Why is phage therapy not in wider use in the U.S. today? Part of the answer, as discussed in other sections, is that much of the early phage literature appeared in Russian, Polish, and French literature, with few English translations. And the early phage experiments performed in the U.S. were discontinued after the discovery of antibiotics, which at the time appeared to be cure-all drugs. In addition, phage therapy is more expensive than antibiotics because each therapy is personally tailored for a specific type of antibiotic-resistant strain infecting that particular patient. The Western paradigm of "one size fits all" does not apply to phage therapy, and it might take "leaps of time and technology to turn them into prescription drugs" ([Wetmore, 2015](#)). So, phage therapy has not been widely tested in the U.S. Especially lacking are large, controlled, blinded, clinical trials. And in the few controlled trials performed in the U.S. the data was not impressive.

With respect to moving the phage therapy field forward, we identified 5 areas that need improvement. First, we need to improve phage quality control and phage production standards. Phage and phage-related products are regulated by the U.S. FDA as “biologics, biologicals, or biotechnology products” (Withington, 2001). Although they are regulated in a manner similar to conventional drugs, the FDA has its own division for this class of biologics: The Center for Biologics Evaluation and Research (CBER). Drugs are evaluated by the Center for Drug Evaluation and Research, CDER. Withington concluded that there is no significant difference between CBER and CDER with respect to the amount of toxicological characterization, clinical testing, and manufacturing data required for submission of approval. Ian Humphery-Smith of the Skolkovo SusInnovations (Moscow) also argues that the production processes used for phage production do not meet the same rigorous standards as used for drug products, so they need refining (Humphery-Smith, 2014). Similar to the conclusions of Withington in 2001, Humphery-Smith concluded that the phage production batch-to-batch reproducibility must be improved, the molecular characterization and definition of the phage and target bacteria must be improved, and the storage conditions of each phage batch be standardized, before their clinical use can become widespread. The production quality of various phage batches could be a problem (each batch is currently grown individually for each patient or each lab), but this problem could be minimized by requiring phage banks that contain large stored supplies of pre-screened phage batches. As part of the quality control process, the FDA needs to require that the materials used to grow the phage batches (cells, culture media, supplements, etc.) be standardized and quality controlled. The phage batches need to be stored in a standard way, and characterized over time to show lack of genetic alteration, retained efficacy, and lack of contamination. With respect to biologic structure, the average phage structure is more complex than the average drug structure, so the FDA needs to decide which components are most important to characterize.

Ry Young and Jason Gill of the Center for Phage Technology at Texas A&M University (College Station, TX) argue that phage therapy will receive increasing attention as antibiotic-resistant bacteria continue to become more prevalent (Young and Gill, 2015). They argue that building a through regulatory environment for phage therapies is important, including requiring that lysogenic phage be prohibited (discussed previously), and similar to the two previous studies cited, they argue that standardized phage collections and protocols should be required to improve quality control.

Second, we need to ensure the high quality of phage materials and products. The cells and materials used to grow viruses must be chosen wisely because the final phage used for therapy can be contaminated with cell products lysed from the cells used to grow the phage. During growth, the phage batches could also become contaminated with bacteria or pathogens accidentally introduced by the technician manufacturing the phage. Establishing standard protocols for producing the phage, and performing quality control tests, is a good way to monitor the material from the very beginning of production. Using state-of-the-art techniques for growing phage with little carry-over of lysed cell products should improve phage purity and help gain approval by regulatory authorities.

Third, we need to improve the design and number of clinical trials. The point was previously mentioned that few large-scale blind placebo-controlled clinical trials have been performed in the U.S., so this needs to be remedied. And in their design, not only do they need to be well controlled, but some scientists like the idea of combining phage treatments with antibiotic treatments to improve faster FDA acceptance and improve efficacy (Nobrega et al.,

2015). Combining phage therapy with antibiotics will “plug more easily into the current way we in the U.S. practice medicine” (Wetmore, 2015). In addition, allowing “compassionate use” trials would be a fast way to obtain clinical data. Compassionate use protocols could be approved at specific U.S. hospitals for patients already near death whose infections have not responded to previous treatments. The patient would be provided information upon which to provide his/her informed consent, and then phage therapy would be administered in an attempt to save the patient’s life (Wetmore, 2015). The clinical trials should also be designed to provide key information that is lacking in the U.S. literature, such as testing phage against non-laboratory strains of bacteria, increasing our understanding of which parts of the immune system become activated by the phage, and determining whether some phage samples induce cytokine storm responses that increase the likelihood of patient death.

Fourth, we need to increase the number of phage development programs in the U.S. For example, the National Institute of Allergy and Infectious Diseases (NIAID) in its strategic plan (NIAID, 2013) listed drug-resistant microbes as one of their top priorities for funding. And the US Army has initiated a large program to develop phage cocktails to fight one of the deadliest bacteria, *Staphylococcus aureus*, and hopes to expand the program to other deadly infections caused by *E. coli* and *Pseudomonas aeruginosa*.

Fifth, we need to increase the number of phage patents using engineered or modified phage. Because naturally occurring phage cannot be patented, some biotech companies are reluctant to get into the phage therapy business because their product is not patent protected. In this case, either academic institutions or medical centers could lead the way, or the companies could use engineered phage altered from their natural counterparts. Altered phage could be a “patent of composition” because they are new. The engineered phage preparations would be more uniform than the undefined phage mixtures isolated from sewerage that were selected solely on the basis of their ability to lyse bacteria, not on their specificity. And engineered phage could be given properties superior to natural phage, such as higher binding specificity.

## CONCLUSIONS

Based on the research performed for this IQP project, our team made several conclusions and recommendations. With respect to human phage therapy trials, we conclude that relatively few trials have been performed in the U.S., and worldwide the trials have not been well controlled. So, moving forward with new phage therapy clinical trials, we conclude that we recommend improving the design and number of trials. The trials need to be well controlled, blinded, and strong attention paid to any observed side-effects. Since antibiotics are well researched and used in the U.S., we agree with the approach being used by some biotech companies to design the clinical trials with a combination treatment of both antibiotic and phage therapy to facilitate FDA approval. In addition, allowing “compassionate use” trials would be a fast way to gain near term approval, and to obtain more clinical data. The clinical trials should also be designed to provide key information currently lacking in the U.S. literature, such as testing phage against non-laboratory strains of bacteria, increasing our understanding of patient immune system activation by the phage, and determining whether some phage samples induce cytokine storm responses that increase the likelihood of patient death. We must also develop better technologies for rapidly determining the species of infecting bacteria, because the species must be known before an appropriate phage can be selected.

With respect to phage safety, we identified several potential issues but believe they are controllable. We recommend: 1) Using only lytic phage, not lysogenic (temperate) phage. Lytic phage are more efficient at killing the bacteria, and do not integrate their DNA into the bacterial or patient DNAs. 2) Switching to tailocin proteins if toxic proteins are released by a particular strain of bacteria. Tailocins attach to and depolarize the bacterial cell membrane, but do not lyse the cell. 3) Using state-of-the-art phage purification protocols to minimize contamination of phage stocks with toxic proteins lysed from bacteria used to grow the phage. Because phage infections are specific to bacteria that contain the right receptors (usually one specific species), that species must be used to grow the large-scale batches of the phage. Infection of a patient with a highly toxic species of bacteria could lyse toxins from the bacteria as they become lysed with the phage. 4) Using rapid DNA sequencing to characterize phage or phage mixtures prior to use. Sequencing will allow the detection of any mutations that may have occurred during phage amplification and purification, and will allow the phage gene sequences to be compared to known toxin genes as much as possible. 5) Pre-screening patients for hyper-immune reactions to the specific phage sample prior to injecting large quantities into the patient.

If problems arise in a patient from phage therapy, alternative procedures could be performed, including the use of tailocins or lysins. These might serve as a safer replacement for phage therapies since there is no phage genomic material that enters the host cells, and the chance of altering the bacterial host cell is greatly diminished. But tailocins and lysins have not yet been investigated in human clinical trials, so it is impossible to judge their therapeutic potential. Tailocins and lysins are foreign proteins that can stimulate an immune response in the patient. An immune response from the patient directed against the phage component could lessen its effectiveness, or could become dangerous to the patient if it induces a cytokine storm response. But a strong case can be made for using tailocins or lysins, especially for patients receiving phage therapy where host cell lysis has become a problem. The use of proteins may be more acceptable to Western medicine, because protein therapeutics are already widely in use. And single proteins are much easier to characterize than phage particles. They retain the highly desirable specificity of phage therapy, while leaving no potential for the rise of bacterial-resistant strains. In fact, lysins have been approved and are already being employed by the food industry.

With respect to regulations, we need to improve phage quality control and phage production standards. Phage production batch-to-batch reproducibility must be improved, and the storage conditions of each phage batch must be standardized before their clinical use can become widespread. We also need to ensure the high quality of phage materials and products. The cells and materials used to grow viruses must be chosen wisely because the final phage used for therapy can be contaminated with cell products lysed from the cells used to grow the phage. During growth, the phage batches could also become contaminated with bacteria or pathogens accidentally introduced by the technician manufacturing the phage. New state-of-the-art procedures exist for minimizing contamination during phage production, and we recommend these be required by the FDA. We also need to increase the number of phage patents allowed in the U.S. by increasing the patents for engineered phage. Because naturally occurring phage cannot be patented, some biotech companies are reluctant to get into the phage therapy business because their product is not patent protected. Because phage therapy has been more widely researched and performed outside the U.S., increasing international cooperation for exchanging characterized phage mixtures and standardized protocols would help. In hospitals, creating special hospital wards or rooms that specialize in performing phage therapy treatments would

help to control phage contamination and spread, and to control the spread of the antibiotic-resistant bacteria. These rooms should be monitored for the spread of phage. And last, we recommend increasing the number of phage development programs in the U.S. The army has led the way with its program to develop phage cocktails to fight *Staphylococcus aureus*, and hopes to expand to other deadly infections. On January 27, 2015, President Barack Obama issued a Fact Sheet on his fiscal year 2016 budget, which proposed a historic investment to combat antibiotic-resistant bacteria to protect the public health ([President Obama, 2015](#)). The government should move forward with these programs.

# LITERATURE REVIEW

## Section-1: Problems with Antibiotic-Resistant Bacteria

*Mingxin Yu*

### Antibiotics

Antibiotics are a type of antimicrobial drug used to treat or prevent bacterial infection (European Center for Disease Prevention and Control, 2016). With their ability to block bacterial infections, antibiotics have revolutionized medicine in the 20<sup>th</sup> century, and in the developed world have helped lead to the near eradication of some types of bacterial diseases, such as tuberculosis.

People often confuse antibiotics with other types of antimicrobial compounds, and mistakenly think they can be used to treat viral infections. Antibiotics belong to a broad family of antimicrobial compounds that kill or stop the growth of living microorganisms, but are usually classified as being specific for bacteria. The antimicrobials include:

**Antibiotics** (Anti-bacterials) (active against bacterial infections)

**Anti-mycobacterial drugs** (active against mycobacteria, such as tuberculosis)

**Anti-virals** (active against viral infections, such as influenza, HIV, herpes)

**Anti-fungals** (active against fungal infections)

**Anti-parasital drugs** (active parasites, such as malaria)

There are hundreds of different types of antibiotics, but most of them can be broadly classified into six groups:

**Penicillins** (such as penicillin and amoxicillin) (widely used to treat skin infections, chest infections and urinary tract infections)

**Cephalosporins** (such as cephalexin) (can treat a wide range of infections, but are often used for serious infections, such as septicemia and meningitis)

**Aminoglycosides** (such as gentamicin and tobramycin) (can cause serious side effects like hearing loss and kidney damage, so are used only when needed to treat very serious illnesses such as septicemia)

**Tetracyclines** (such as tetracycline and doxycycline) (can treat a wide range of infections but commonly used to treat moderate to severe acne and rosacea)

**Macrolides** (such as erythromycin and clarithromycin) (particularly useful for treating lung and chest infections, or as an alternative for people with a penicillin allergy or to treat penicillin-resistant strains of bacteria)

**Fluoroquinolones** (such as ciprofloxacin and levofloxacin) (broad-spectrum antibiotics that can be used to treat a wide range of infections)

## Antibiotic History

We have been using chemicals to fight bacterial infections for thousands of years. Early historical treatments for bacterial infections were based mostly on medicinal folklore, and often used extracts from molds and plants to treat infections (Forrest, 1982; Wainwright, 1989). The use of synthetic compounds to kill bacteria began in the late 1880's in Germany when Paul Ehrlich noticed that certain dyes could stain some types of cells but leave others alone, so he proposed screening for compounds that would bind and kill bacteria, leaving human cells alone. After screening hundreds of compounds, in 1907 Ehrlich discovered that a compound synthesized by Alfred Bertheim (Salvarsan, now called Arsphenamine) could kill bacteria, and it was used to treat syphilis (Bosch and Rosich, 2008; Williams, 2009). The ability of molds to inhibit bacteria had been noticed over history, but in 1928 Alexander Fleming noticed that several types of bacteria were killed in a petri dish by a fungus of the genus *Penicillium*. He thought that the effect might be due to an antibacterial compound, and he named it penicillin. Taking the experiments further, Fleming attempted to use crude preparations of the *Penicillium* fungus to treat wound infections, but he lacked the chemical skills needed to purify penicillin (Fleming, 1980). Penicillin was later purified in 1942.

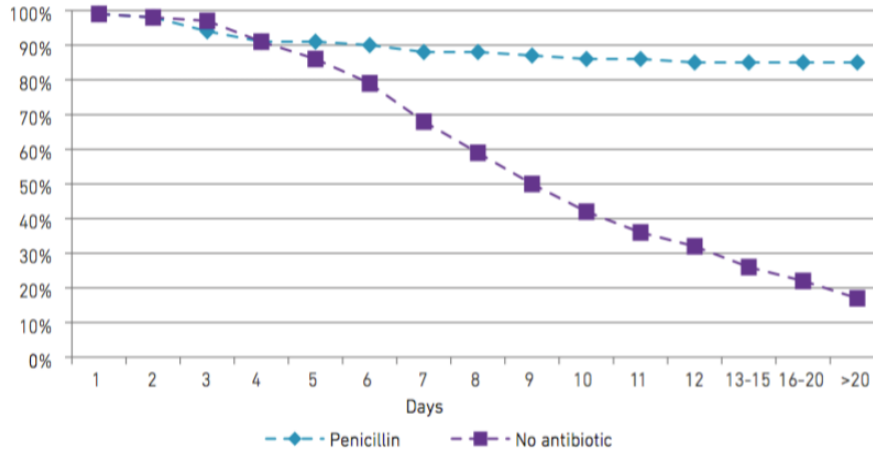
The first commercially available antibacterial drug (and the first systemically active) was Prontosil, developed in 1932 by Gerhard Domagk at the Bayer Laboratories in Germany (Aminov, 2010). Domagk received the 1939 Nobel Prize for Medicine for this achievement, and its development initiated the golden age of new antibacterial discoveries (and the demise in Western countries of phage therapy, discussed later).

The first naturally derived antibiotic was reported in 1939 by Rene Dubos for Tyrothricin (20% Gramicidin and 80% Tyrocidine), isolated from *Bacillus brevis* (Van Epps, 2006). It was very effective in treating surface wounds and ulcers during World War II, but due to the toxicity of both components, it could not be used systemically.

The first purified penicillin-type drug (Penicillin-G) was purified by Florey and Chain in 1942, but it did not become widely available outside the Allied military before 1945 (Florey, 1945). For this discovery, Florey and Chain shared the 1945 Nobel Prize in Medicine (also with Fleming). Later, Norman Heatley developed a back-extraction technique for purifying penicillin in bulk. Because Penicillin-G showed powerful antibacterial activity against a wide range of bacteria, and had low toxicity in humans, its discovery greatly stimulated the search for new antibiotics and a continuance of their golden age. These drugs were named "antibiotics" in 1942 by American microbiologist Selman Waksman (Waksman, 1947).

When penicillin was first introduced, it dramatically changed the outcome for patients with bacterial diseases. As an example, patients with pneumococcal pneumonia with an accompanying bloodstream infection went from an average fatality rate of about 80% prior to the discovery of penicillin, to a survival rate of about 85% when treated with penicillin (Figure-1).





**Figure-1: Example of the Benefits of Penicillin.** Shown is the average percent survival (Y-axis) versus time in days (X-axis) for patients with pneumococcal pneumonia, treated with no antibiotic (purple line) (20% survival after 20 days), or treated with penicillin (turquoise line) (85% survival after 20 days). Figure is from [World Health Organization, 2014](#).

## Antibiotic Resistance

Unfortunately, the overuse of antibiotics (especially in the livestock industry), and the passage of antibiotic resistant genes between bacteria, have led to widespread problems with antibiotic resistance, a situation where the bacteria are no longer killed by the antibiotic. This resistance is typically an adaptation of the microorganism to its environment: in this case either the bacteria adapt to the antibiotic or they die. Some bacteria are naturally resistant to specific antibiotics (intrinsic or inherent resistance), so in this case a different antibiotic is sometimes used to treat the disease. But the more serious problem medically is the adaptation of bacteria to an antibiotic for which they are normally susceptible.

The main drivers for antimicrobial resistance are 1) the overuse of antibiotics (especially in the animal husbandry industry), which exerts an ecological pressure on microorganisms (it kills the sensitive bacteria, leaving the antibiotic resistant bacteria to survive), 2) the spread of genes between bacteria that confer antibiotic resistance, and 3) the increasing spread of antimicrobial-resistant microorganisms (between humans, between animals, and between humans and animals and the environment). Genes encoding antibiotic resistance proteins (such as enzymes that degrade antibiotics) often occur on plasmid DNAs that are mobile and easily transmitted between bacteria.

Antibiotic-resistant bacteria survive in the presence of the antibiotic, and continue to grow and divide, increasing the length of the illness or even causing death. Infections caused by these bacteria may require more intensive care, may require intravenous antibiotics delivered in a hospital instead of oral antibiotics taken at home, or can require more expensive antibiotics

which can have severe side-effects. Once these bacteria become established in a person, they can spread to another person.

## Superbugs

Some bacteria have become resistant to several classes of antibiotics (multi-drug resistance). Worse, some superbugs are resistant to all known classes of antibiotics. The acquisition of multi-drug resistance is important for all types of microorganisms, but is especially important for the bacteria responsible for healthcare-associated infections, microorganisms responsible for food- and waterborne infections, the *Mycobacterium* that causes tuberculosis, and microorganisms responsible for sexually-transmitted diseases. Thus, the challenge with multidrug-resistant microorganisms is the limited number of remaining options, if any, for therapy. Examples of common and medically important multidrug-resistant bacteria are:

Methicillin-resistant *Staphylococcus aureus* (MRSA)

Vancomycin-resistant enterococci (VRE)

Extended-spectrum beta-lactamase (ESBL)-producing Enterobacteriaceae (such as *Escherichia coli* and *Klebsiella pneumoniae*)

Multidrug-resistant *Pseudomonas aeruginosa*

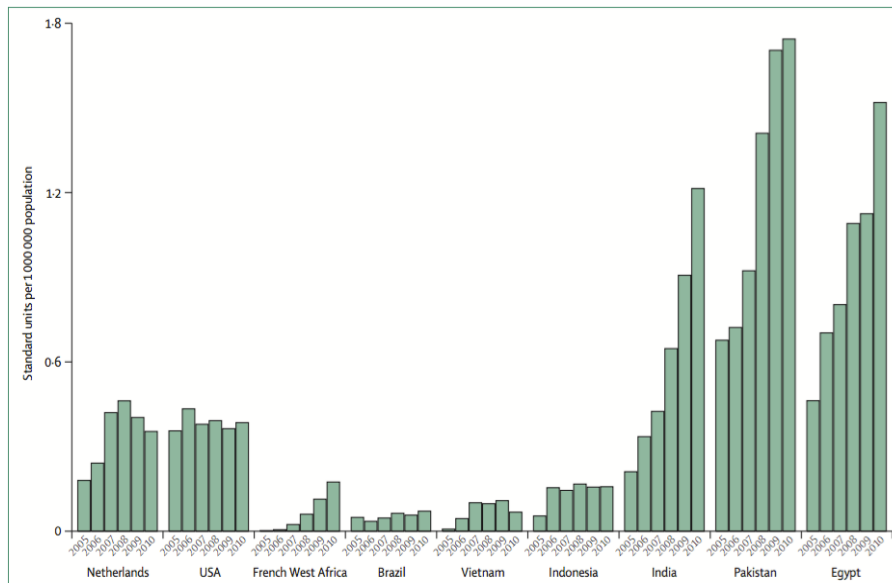
*Clostridium difficile*

## Medical Importance of Antibiotic Resistance

Bacterial antibiotic resistance causes hundreds of thousands of deaths globally annually (CDC, 2013). In the U.S., the CDC estimates that each year at least two million illnesses and 23,000 deaths are caused by antibiotic-resistant bacteria. The increase of antibiotic-resistant bacteria has prompted the World Health Organization to state this resistance is a "serious threat...no longer a prediction for the future, it is happening right now in every region of the world and has the potential to affect anyone, of any age, in any country" (WHO, 2014).

The spread of antibiotic-resistant bacteria occurs by direct contact of humans with the bacteria, and by interactions between bacteria themselves (which spreads the genes for antibiotic resistance) (Allen et al., 2010). The environment's reservoir of antibiotic-resistance genes (termed the resistome), from which naïve bacteria can become resistant, is ancient, diverse, and widespread (D'Costa et al., 2011; Forsberg et al., 2014). So, the driver of the spread of antibiotic resistance genes was initially likely the natural presence of antibiotics in the environment, and the selective pressure later grew exponentially after the widespread use of antibiotics.

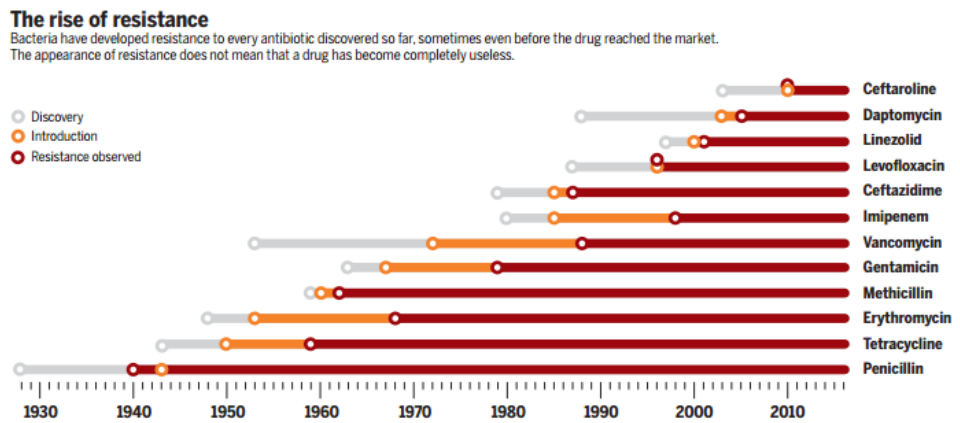
The worldwide consumption of antibiotics is on the rise. As an example, although carbapenem antibiotics are expensive, their sales in Egypt, India, and Pakistan have drastically increased from 2005 to 2010 due to the increased over-the-counter availability in those countries (Figure-2).



**Figure-2: Increase in Retail Sales of Carbapenem Antibiotics Over Time.** Shown is a plot of the standard units of carbapenem antibiotics per  $10^6$  population (Y-axis) versus different countries from the years 2005 to 2010 (X-axis). Easily seen is the strong increase in carbapenem sales in India, Pakistan and Egypt (figure right side), due to their over-the-counter availability in those countries. Figure is from [Laxminarayan et al., 2013](#).

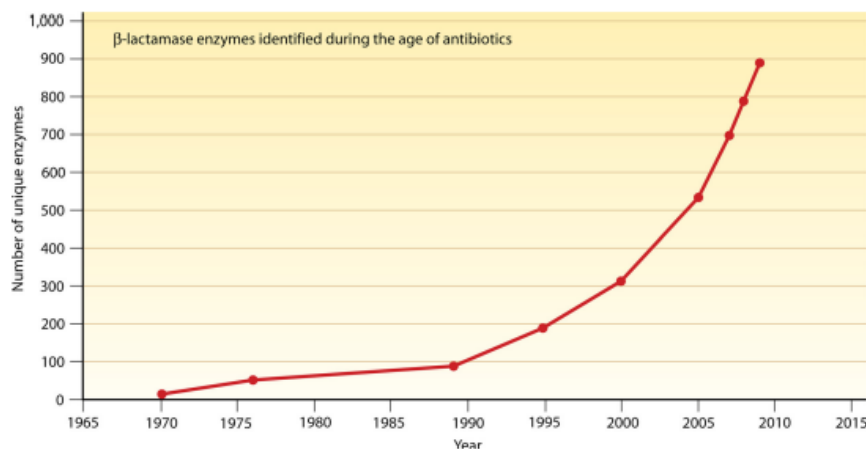
In China, one factor driving an increase in antibiotic sales is that hospitals rely on pharmaceutical sales for income, so they have an incentive to over-prescribe; one study estimated that a quarter of revenue in two hospitals came from antibiotic sales ([Sweidan et al., 2005](#)). In India, doctors routinely receive compensation from drug sellers in exchange for prescribing their drugs. And antibiotic sales increase with patient insurance coverage; patients with insurance are likely to worry less about drug prices ([Dong et al., 1999](#)). In other studies, antibiotic prescriptions increase with competition between health-care providers, and the distribution of antibiotics from unsanctioned providers also increased their use.

A recent review article on antibiotic-resistance ([Kupferschmidt, 2016](#)) showed a time-line of when various antibiotics were first discovered (or synthesized) versus when antibiotic resistance was first observed for that antibiotic (**Figure-3**). The figure shows that for each of the 12 antibiotics listed, resistance occurred (red color) within 1-10 years of the compound's first introduction (yellow color). In one case, Penicillin (lowest row in the figure), antibiotic-resistance was observed even before Penicillin was widely marketed.



**Figure-3: Timeline of Antibiotic-Resistance.** Shown is a timeline of the introduction of various antibiotic compounds (yellow color) versus when resistance was first observed for each compound (red color). Note that for penicillin (lowest row) antibiotic resistant strains existed even prior to the isolation and production of penicillin. Figure is from [Kupferschmidt, 2016](#).

Another way to visualize the exponential increase in antibiotic resistance is to plot the increase over time for a specific type of antibiotic resistance gene.  $\beta$ -lactamases are enzymes produced by bacteria that provide resistance to  $\beta$ -lactam type antibiotics (such as the various penicillins, cephamycins, and carbapenems). **Figure-4** shows the exponential increase in the number of  $\beta$ -lactamase-type enzymes discovered over time. The genes encoding the different type of  $\beta$ -lactamase-type enzymes has evolved over time to produce a greater variety of sequences.



**Figure-4: The Increase in  $\beta$ -lactamase Type Antibiotic Resistance Enzymes Over Time.** Shown is the exponential increase in the number of different  $\beta$ -lactamase-type enzymes discovered over time. Figure from [Davies and Davies, 2010](#).

In 2013, the Centers for Disease Control (CDC) published a report outlining the top 18 drug-resistant threats to the United States (CDC, 2013). These threats were categorized based on the level of concern as “urgent” (of highest importance), “serious”, or “concerning” (see list below). The organisms in the highest categories require more monitoring and prevention strategies.

#### **Urgent Threats (of highest importance)**

*Clostridium difficile* (CDIFF)  
Carbapenem-Resistant Enterobacteriaceae (CRE)  
*Neisseria gonorrhoeae*

#### **Serious Threats**

Multidrug-Resistant Acinetobacter  
Drug-Resistant Campylobacter  
Fluconazole-Resistant Candida  
Extended Spectrum Enterobacteriaceae (ESBL)  
Vancomycin-Resistant Enterococcus (VRE)  
Multidrug-Resistant Pseudomonas Aeruginosa  
Drug-Resistant Non-Typhoidal Salmonella  
Drug-Resistant Salmonella Serotype Typhi  
Drug-Resistant Shigella  
Methicillin-Resistant Staphylococcus Aureus (MRSA)  
Drug-Resistant Streptococcus Pneumoniae  
Drug-Resistant Tuberculosis

#### **Concerning Threats**

Vancomycin-Resistant Staphylococcus aureus  
Erythromycin-Resistant Group-A Streptococcus  
Clindamycin-Resistant Group-B Streptococcus

In 2014, The New York Times wrote an opinion article titled “The Rise of Antibiotic Resistance”, which summarized the findings of the World Health Organization’s (WHO) global survey of antibiotic resistant bacteria, the first such survey conducted. The survey found that antibiotic resistance “is a serious threat in every part of the world”, and is “a problem so serious that it threatens the achievements of modern medicine”. Because the standard treatments no longer work, infections are harder (or impossible) to control, infection spread increases, and illnesses and hospital stays are prolonged. All of these drive up the costs of illnesses and the risk of death. And the problem exists “both for antibiotics used routinely and for those deemed “last resort” treatments when all else has failed” (New York Times, 2014). The article concludes that “the most urgent need is to minimize the overuse of antibiotics in medicine and agriculture, which accelerates the development of resistant strains”. In the U.S., the FDA has issued voluntary guidelines calling on drug companies, animal producers, and veterinarians, and physicians to stop indiscriminately using antibiotics.

On January 27, 2015, President Barack Obama issued a Fact Sheet on his fiscal year 2016 budget, which proposed a historic investment to combat antibiotic-resistant bacteria to protect the public health ([President Obama, 2015](#)). The Fact Sheet indicated that “antibiotic resistance is one of the most pressing public health issues facing the world today”...it affects our ability to “perform a range of modern medical procedures from joint replacements to organ transplants, the safety of which depends on our ability to treat bacterial infections that can arise as post-surgical complications”. The report states that “the CDC reports that antibiotic-resistant infections account for at least \$20 billion in excess direct health care costs, and up to \$35 billion in lost productivity due to hospitalizations and sick days each year”. In September 2014, President Obama signed an Executive Order initiating federal efforts to combat the rise in antibiotic-resistant bacteria. The President’s FY 2016 budget nearly doubles the amount of federal funding to attack this problem by approving \$1.2 billion to perform risk assessment, surveillance, and reporting capabilities, and fund research innovation ([President Obama, 2015](#)).

In 2014, the President’s Council of Advisors on Science and Technology (PCAST) released their report “Report to the President on Antibiotic Resistance” ([PCAST, 2014](#)). The report stated that “The evolution of antibiotic resistance is now occurring at an alarming rate and is outpacing the development of new counter-measures capable of thwarting infections in humans.” The report identified four problem areas related to antibiotic-resistance:

- 1. Human Health Care:** 50% percent of all the antibiotics prescribed for patients in the U.S. are not needed, or are not optimally prescribed. Their overuse further spreads antibiotic resistance.
- 2. Animal Agriculture:** Medically important antibiotics are extensively used in animal agriculture, not only to treat sick animals, but also to promote animal growth and to prevent infections. The large-scale use of antibiotics in animal agriculture is a major source of antibiotic resistance.
- 3. Drug Development:** The world lacks a robust pipeline of new antibiotics to replace those being steadily lost to antibiotic resistance. This leaves few new classes of antibiotic compounds as last resort treatments.
- 4. Surveillance and Response:** The U.S. currently lacks a comprehensive monitoring program for antibiotic-resistance, either strains emerging domestically or those being imported.

The PCAST report also recommends 3 steps for getting antibiotic resistance under control:

- 1. Improving our surveillance of the rise of antibiotic-resistant bacteria** to enable an effective response, help stop outbreaks, and limit the spread of antibiotic-resistant organisms. They also recommend acting on the surveillance data to implement appropriate infection control.
- 2. Increasing the longevity of current antibiotics,** by controlling their over-use, preventing the spread of antibiotic-resistant bacteria, and scaling up proven interventions to decrease the rate at which microbes develop resistance to current antibiotics.

**3. Increasing the rate of discovery of new antibiotics and new therapies, including phage therapy.**

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## Section-2: Background on Phage and Early Phage Treatments

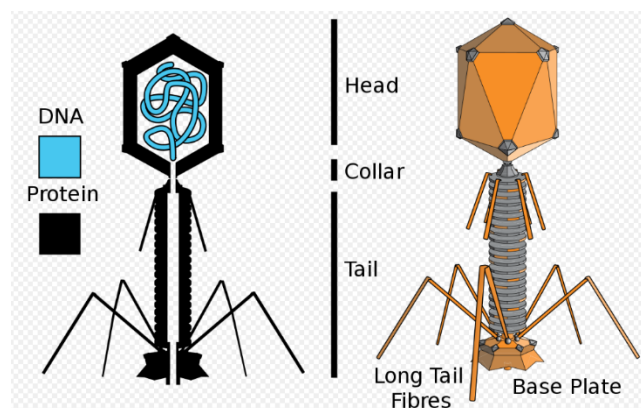
Mingxin Yu

As discussed in the previous section, the increasing presence of antibiotic-resistant bacteria on a global scale is currently considered one of the greatest therapeutic challenges facing mankind. This problem is exacerbated by a crisis in the search for new classes of antibiotics that are effective against the resistant strains. An alternative option for eliminating resistant bacteria is phage therapy, which relies on the use of bacteriophages, viruses that specifically kill bacterial cells. These viruses occur naturally, and are not thought to attack mammalian cells, so scientists are interested in developing phage therapies to treat bacterial diseases.

### Phage Introduction

A bacteriophage (phage) is a virus that infects and replicates within a bacterium. Phage are among the most common and diverse bio-entities on earth. As expected, they are usually located in the same places as bacteria, such as soil and animal intestines. Surprisingly, one of the densest natural locations for phage is sea water, where up to  $9 \times 10^8$  virions per milliliter have been found in “microbial mats” at the surface (Wommack and Colwell, 2000).

Structurally, phage are composed of proteins and a genome of either DNA or RNA. The proteins form the main structures, including the head, collar, and tail (Figure-1). The head contains the genomic material, while the tail usually helps recognize and attach to the surface of the bacterial host cell. Phage can have simple or elaborate structures, and their genomes may encode as few as four genes, and as many as hundreds of genes. Following attachment of the phage to the bacterial surface, the genome is injected into the cytoplasm through the hollow tail structure, and the genome replicates using host enzymes.



**Figure-1: Diagram of a Typical Phage.** Shown are the main structural features of phage, including the head, collar, and tail structures. The head structure contains the DNA or RNA genomic material. The tails typically facilitate recognition and attachment to the bacterial surface. The figure is from: <https://en.wikipedia.org/wiki/Bacteriophage>

## Phage Classification

Phage are classified by the International Committee on Taxonomy of Viruses (ICTV) according to their morphology and genome type (DNA or RNA). The ICTV currently recognizes about 19 phage families (**Table-I**). The vast majority (17 of 19) have DNA genomes. Only 5 phage families are enveloped, where the virion is enclosed by a lipid membrane.

**Table-I: Classification of Bacteriophages**

Order	Family	Morphology	Genome	Examples
<i>Caudovirales</i>	<i>Myoviridae</i>	Non-enveloped. Contractile tail.	Linear dsDNA	T4 phage, Mu, PBSX, P1, P2, I3, Bcep-1, 43, 78
	<i>Siphoviridae</i>	Non-enveloped. Non-contractile tail (long)	Linear dsDNA	$\lambda$ phage, T5 phage, Phi, C2, L5, HK97, N15
	<i>Podoviridae</i>	Non-enveloped. Non-contractile tail (short)	Linear dsDNA	T7 phage, T3 phage, $\Phi$ 29, P22, P37
<i>Ligamenvirales</i>	<i>Lipothrixviridae</i>	Enveloped, rod-shaped	Linear dsDNA	Acidianus filamentous virus-1
	<i>Rudiviridae</i>	Non-enveloped, rod-shaped	Linear dsDNA	Sulfolobus islandicus rod-shaped virus 1
Unassigned	<i>Ampullaviridae</i>	Enveloped, bottle-shaped	Linear dsDNA	
	<i>Bicaudaviridae</i>	Non-enveloped, lemon-shaped	Circular dsDNA	
	<i>Clavaviridae</i>	Non-enveloped, rod-shaped	Circular dsDNA	
	<i>Corticoviridae</i>	Non-enveloped, isometric	Circular dsDNA	
	<i>Cystoviridae</i>	Enveloped, spherical	Segmented dsRNA	
	<i>Fuselloviridae</i>	Non-enveloped, lemon-shaped	Circular dsDNA	
	<i>Globuloviridae</i>	Enveloped, isometric	Linear dsDNA	
	<i>Guttaviridae</i>	Non-enveloped, ovoid	Circular dsDNA	
	<i>Inoviridae</i>	Non-enveloped, filamentous	Circular ssDNA	M13
	<i>Leviviridae</i>	Non-enveloped, isometric	Linear ssRNA	MS2, Q $\beta$
	<i>Microviridae</i>	Non-enveloped, isometric	Circular ssDNA	$\Phi$ X174
	<i>Plasmaviridae</i>	Enveloped, pleomorphic	Circular dsDNA	
	<i>Tectiviridae</i>	Nonenveloped, isometric	Linear dsDNA	

Table downloaded on 6-14-16 from <https://en.wikipedia.org/wiki/Bacteriophage>

## Phage Attachment and Penetration

To enter a host bacterial cell, phage attach to specific receptors on the bacterial surface. These phage receptors can include lipopolysaccharides, teichoic acids, proteins, or even flagella. With respect to phage therapy, the important point about the interaction of phage with receptors, is that the phage can infect only bacteria containing the appropriate receptor. This determines the phage's host range and specificity. Phage are not capable of independent movement, so they rely on random encounters with the appropriate bacterium in the blood, lymphatic circulation, irrigation water, soil water, etc.

Once bound to its receptor, phage inject their genomic material into the host cell where it replicates and makes more phage particles (lytic stage) or integrates into the host DNA (lysogenic stage). Much about viral attachment and entry has been learned from Myoviral phage, such as T4. The long tail fibers first make contact with the receptor, and then flex upwards to bring the baseplate closer to the bacterial surface. The tail then contracts, and in a syringe-like motion, injects the viruses' linear dsDNA through the bacterial membrane. Other viruses, such as Podoviruses T3 and T7, lack an elongated tail, so they use their small tail fibers to enzymatically degrade a portion of the cell membrane.

## Phage Discovery

Entire articles have been written solely on the topic of phage discovery ([Duckworth, 1976](#); [Summers, 2012](#)). For our purposes, we can say that phage discovery occurred over 100 years ago now, and is usually credited to two individuals: the Englishman, Frederick Twort ([Twort, 1915](#)) and the French Canadian microbiologist, Felix d'Herelle ([d'Herelle, 1917](#)). Most scientists claim that the latter individual more accurately recognized the entities he was investigating, their clinical significance, and he named them. Others argue that d'Herelle may not have been honest in stating he was unaware of Twort's discovery two years earlier ([Duckworth, 1976](#)). For example, Gunther Stent in 1966 wrote that "Bacterial viruses were discovered in 1915 by the English microbiologist F. W. Twort, and two years later-perhaps independently, perhaps not-by the French-Canadian F. d'Herelle" ([Stent, 1966](#)). However, most scientists appear to agree that d'Herelle is credited with introducing the concept of phage therapy and using it (see below).

Phage were first seen in 1940, in electron micrographs taken in Germany ([reviewed in Ackermann, 2011](#)). The phage particles were initially viewed with no staining by the Germans, then U.S. scientists introduced shadowing and freeze-drying EM techniques that greatly increased contrast, allowing greater details to be seen.

## Phage Therapy

Phage therapy is the use of phage to kill bacteria. Due to the drastic rise in antibiotic-resistant bacteria, and their burden to patients and the healthcare industry (discussed in the

previous section), phage therapy is viewed by some scientists as an alternative method for killing the bacteria. But the phage therapy field is not straightforward. In 2012, William C. Summers in his article titled “The Strange History of Phage Therapy (Summers, 2012), concluded that the phage therapy field has been fraught with conflicting observations, misinterpretations, and incomplete understanding, while at the same time it is rich with politics, personal feuds, and unrecognized conflicts.

As mentioned above, phage were co-discovered in 1915 by Frederick Twort (Twort, 1915) and in 1917 by Felix d’Herelle (d’Herelle, 1917). Only two years later, in 1919, d’Herelle had performed the world’s first phage therapy experiments (summarized in Pirnay et al., 2011) on patients suffering from severe dysentery at the Hospital des Enfants Malades in Paris. In the 1920’s and 1930’s, phage therapy continued to be developed in the former **Soviet Republic of Georgia** (pioneered by **Giorgi (George) Eliava** and co-discoverer **Felix d’Herelle**) where they were used to treat soldiers in the Red Army suffering from bacterial infections. Although phage also had some early use in the U.S, by the 1940’s they were abandoned in the West, mostly because of the discovery of antibiotics, which were easier to make, store, and prescribe. Phage research continued in the former Soviet Union and Eastern European countries, which tended to lack antibiotics, but their scientific publications were not translated for Western use.

### *Disease Candidates for Phage Therapy*

In a world in which antibiotics represent the standard first-line therapy against bacterial infections, phage therapy is thought to be best suited for: 1) combating infections involving antibiotic-resistant bacteria, 2) combating infections that appear to be antibiotic-resistant in vivo, although they are sensitive in vitro, 3) and targeting bacteria under circumstances where antibiotic use would harm the patient (for example where antibiotics could cause a patient allergy, or an irritable bowel problem), and 4) targeting bacteria in food and agricultural applications to lower antibiotic use. Example diseases include: skin ulcers, purulent infections, methicillin-resistant *Staphylococcus aureus* (MRSA), wound prophylaxis, burns, poorly accessible infections, eye infections, gastrointestinal ailments, respiratory tract infections, chronic otitis, urogenital tract infections, and sepsis.

### *Human Phage Therapy Experiments*

#### *France*

Human phage therapy was first practiced in France in 1919, when **Felix d’Hérelle** successfully treated several children at the Hospital des Enfants Malades in Paris suffering from severe dysentery (summarized in Pirnay et al., 2011; Abedon et al., 2011). For those treatments, he used phage samples isolated from the stools of soldiers treated at the Pasteur Institute. That work does not directly appear in the literature, d’Herelle delayed publishing until he had further characterized the properties of phage in experiments with fowl typhoid, but he describes the experiments later in several books, some of which were translated to English (d’Herelle and Smith, 1930). While d’Hérelle actually performed the first human therapeutic phage

experiments, the first *publication* of such research was in 1921 in Belgium ([Bruynoghe and Maisin, 1921](#)) who reported their experiments injecting phage into Staphylococcus skin boils in 6 patients, who within 2 days showed less swelling and pain. Many other experiments with human phage therapy continued in France. d'Herelle established his own "Laboratoire du Bacteriophage, which produced the world's first commercial phage cocktails: Bacté-Coli-Phage, Bacté-Intesti-Phage, Bacté-Dysentérie-Phage, Bacté-Pyo-Phage and Bacté-Rhino-Phage, which were commercially available until 1978 ([Abedon et al., 2011](#)). In 1976, the Pasteur Institute of Lyon appears to have produced over 60 therapeutic phages, including 20 phages for enterobacteria, 30 for Pseudomonas, and over 10 for Staphylococcus.

Other phage therapy experiments continued in France through 1979. These included key findings about the requirements for phage purification, especially the use of cesium chloride gradient centrifugation to rid the phage samples from bacterial pyrogens released from the lysing bacteria, not using media components isolated from animal cells, and improving lysing efficiency by repeated passage through the bacteria to be killed. They also found that the therapy should work within a few days, as prolonged phage use can stimulate an immune response against the phage. The Pasteur Institute stopped making therapeutic phage cocktails in the mid-1990s, and scientists now mostly obtain their phage samples from Russia or Georgia ([Abedon et al., 2011](#)).

### *Soviet Republic of Georgia*

In 1923, the **Eliava Institute** opened in the former Soviet Republic of Georgia, in Tbilisi, to research the then new science of phage and to put phage therapy into practice. According to their website ([Eliava Institute, 2010](#)), the institute was founded by distinguished Georgian physician and phage researcher Prof. **Giorgi (George) Eliava**, together with phage co-discoverer French-Canadian scientist **Felix D'Herelle**. These two men founded the center, and then in 1937 under Stalin's rule, Eliava was executed and D'Herelle never came back to Georgia. Throughout its long history, the institute has been known by a variety of names, the best of which is "Scientific-Industrial Union (SIU) Bacteriophage". The center focused on receiving pathogenic strains of bacteria from across the Soviet Union, and isolating and adapting phage from the bacteria. They routinely tested virulence and host range of each phage. At its peak it was a large operation, employing 1,200 people, most performing phage production. The majority of their products were shipped to the Soviet military for treating diarrhea and wounds ([Abedon et al., 2011](#)). "Interstiphage" is a phage product made by Biochimpharm that is directly available to the Georgian public without a prescription; it contains 20 different types of phage against pathogenic GI bacteria. "Pyophage" contains phage against Staphylococcus, Streptococcus, Pseudomonas, Proteus, and E. coli, and is used for skin and wound infections. The Pyophage cocktail has also been incorporated into a biodegradable bandage "PhagoBioDerm" ([Kutter et al., 2010](#)) providing a timed release of the phage for treating wounds.

Although Georgia has apparently had a long literature of successful studies with phage, few of their studies were translated into English, in part due to the secrecy of Russian military sciences. There has been little transfer of phage samples to the West, with the exception of Pyophage that apparently successfully treated several patients at the Lubbock Wound Center in Texas. However, a well-controlled FDA-approved clinical trial of Pyophage ([Rhoads et al., 2009](#)) failed to see any difference with saline treated patients.

## *Poland*

Thousands of patients have undergone phage therapy in Poland, especially at the Hirszfeld Institute of Immunology and Experimental Therapy in Wroclaw. The institute was founded in 1954, and is associated with the Polish Academy of Sciences (<https://www.iitd.pan.wroc.pl/en>). **Stefan Slopek's** group was especially productive at the institute, providing the most documentation on phage therapy in the English language (Cislo et al., 1987; Kucharewicz-Krukowska and Slopek, 1987; Mulczyk and Slopek, 1974; Slopek et al., 1983a; 1983b; 1984; 1985a; 1985b; 1985c; 1987; Weber-Dabrowska et al., 1987; 2000). From 1981 to 1986, that team alone used phage therapy on approximately 550 patients, most of them resistant to antibiotic treatment, obtaining “positive results” in 508 cases (92.4%), transient improvement in 38 (6.9%), and no improvement in 4 (0.7%) (Slopek et al., 1987). Since 2005, the institute has had a phage therapy center devoted especially to treating antibiotic resistant infections. Other well published practitioners from Poland were Beata Weber-Dabrowska and Andrzej Gorski.

## *North America*

Interest in phage therapy began in the U.S. in the 1920's and 1930's. Interestingly, the subject of phage therapy was part of the plot in Sinclair Lewis's book *Arrowsmith* (1925). The book follows the career of a fictitious Dr. Martin Arrowsmith, who as a part of the plot discovers a phage that destroys bacteria. So, he is sent to a Caribbean island to help quell an outbreak of the bubonic plague; the epidemic also took the life of his wife Leora. *Arrowsmith* is arguably the earliest major novel to deal with the culture of science (<https://en.wikipedia.org/wiki/Arrowsmith>).

One of the earliest phage therapy experiments in the U.S. was done in 1929 at the Michigan Department of Health (Larkum, 1929). The team treated 208 patients suffering from chronic skin boils caused by staphylococci or streptococci (furunculosis). Their data showed that 78% of the patients treated with phage had no recurring infections at least 6 months after therapy, while only 3% showed no improvement.

Two other U.S. studies showed remarkable success with septicemia and meningitis (Schultz, 1929; Schless, 1932, respectively). Several U.S. companies also got into the phage business, including Eli Lilly (producing Staphylo-lysate, Colo-lysate, Ento-lysate, and Neiso-lysate), ER Squibb and Sons, and Abbott Labs. Unfortunately, the companies had problems with quality control, phage instability, and lack of efficacy, perhaps due to the chemicals added to the phage batches (Abedon et al., 2011). In 1934, a negative review of the entire phage industry by Eaton and Bayne-Jones (1934) set back progress of this technology in the U.S. This study was a year-long review by the American Medical Association of phage therapy, analyzing over 100 publications in the field. They raised many serious problems with the procedure, and found consistent positive data only for treating localized staph infections and inflammation of the bladder. Other North American phage therapy trials include: Bryant et al., 1965; Wittig et al., 1966; Reynaud et al., 1992.

### *Other Countries*

Other human phage therapy trials have been published from Britain (Shera, 1970; Corbel and Morris, 1980), Romania (Zilisteanu et al., 1971; 1973; Meitert et al., 1987), France (Grimont et al., 1978; Lang et al., 1979; Vieu et al., 1979), and Czechoslovakia (Pillich et al., 1978).

### *Controlled Human Studies*

Although most of the early phage trials were not well controlled, a few were well done studies. The first randomized, double-blind, placebo-controlled clinical trial was performed at the UCL Ear Institute and Royal National Throat, Nose and Ear Hospital, Grays Inn Road, London. This trial evaluated the safety and efficacy of a phage cocktail (Biophage-PA) to treat antibiotic-resistant *Pseudomonas aeruginosa* ear infections (Wright et al., 2009). The 24 patients were randomly assigned to two groups, 12 treated with placebo and 12 treated with phage. Each patient was followed up at 7, 21 and 42 days post-treatment. Their results showed that both the patient-reported and physician-reported clinical events improved for the phage treated group relative to the placebo group, and the former group showed significantly lower *P. aeruginosa* counts. No treatment related adverse event was reported. The study concludes that bacteriophage preparations were safe and effective for treatment of chronic ear infections in humans.

However, another well controlled trial showed no benefits. A study done in 2009 in the Department of Psychiatry at the University of Minnesota (Minneapolis, USA) (Rhoads et al., 2009) examined the safety of phage therapy for difficult to treat wounds. They used a mixture of phage against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Escherichia coli* to treat 42 patients with chronic venous leg ulcers (VLUs) for 12 weeks. Patients treated with saline acted as the control. Although they found no adverse events associated with the phage treatment, there was no difference between the controls and phage groups with respect to rate of healing. The authors speculated that the use of chemicals that are part of standard wound care (e.g. lactoferrin or silver) may have interfered with the survival of the phage. They are continuing this study into phase-II for efficacy.

Overall, the authors in most of the human phage therapy trials concluded that their phage therapy worked, but unfortunately the studies were small and not well controlled. Because most of the trials were done on patients that had not previously responded to antibiotics, any hint of an improvement was often touted as a success. The trials were usually not blinded, not all the early cases were presented in detail, and sometimes the phage therapy was accompanied by antibiotic treatments, so it is hard to separate the two techniques. It appears that more rigorous studies on larger patients samples is needed to determine whether phage therapy will truly work.

### *Phage Therapy in Animals*

Much has been learned about phage therapy from animal experiments, and the work described below helped start up phage therapy again in the West following a long period of lack of interest when antibiotics were discovered. At the Institute for Animal Disease Research, Houghton Laboratory (Huntingdon, Cambridgeshire, UK) calves, piglets, and lambs have all

been used as a model for phage therapy against *E. coli* induced diarrhea (Smith and Huggins, 1983). In the calf experiments, phage therapy with a mixture of phages B44/1 and B44/2 administered prior to infection protected calves against a potentially lethal oral infection with *E. coli* strain O9:K30.99. Therapy with phage B44/3 was effective even after the onset of diarrhea. Calves responding to phage treatment showed greatly reduced numbers of *E. coli* in their alimentary tract than untreated calves. Calves that died from the *E. coli* infection showed high numbers of *E. coli* in the small intestine that were resistant to phage. Calves inoculated orally with fecal samples from phage-treated calves (containing *E. coli* infected with B44 phage) remained healthy. They showed similar data with piglets, in this case allowing their survival against an *E. coli* strain O20:K101.987P when treated either with phage P433/1 alone or a mixture phages P433/1 and P433/2. In lambs, phage S13 delivered 8 hours after infection protected them against a challenge with *E. coli* strain O8:K85.99 (Smith and Huggins, 1983). The observance of *E. coli* resistant to phage infection is a potential problem with phage therapy and should be researched further.

This same research group published their results of a follow-up study in calves in 1987 (Smith et al., 1987). The team isolated 7 phages from sewerage showing high activity in vitro and in vivo against 6 different serotypes of bovine entero-pathogenic *E. coli*. Their data showed that severe experimentally induced diarrhea in calves could be cured (post infection) by a single oral dose of  $10^5$  phage particles, and could be prevented by a dose as low as  $10^2$  sprayed on the litter in the housing room, or by simply housing the calves in un-cleaned rooms previously occupied by phage-treated calves. The phage quickly reduced the number of *E. coli* to harmless numbers. Challenge of the calves with a mixture of six *E. coli* strains could be controlled by therapy with a pool of six phages, although the control was less complete than with the single strain infections.

A team at the Puy-de-Dôme Departmental Veterinary Laboratory, Lempdes, France, isolated a phage with activity against *Escherichia coli* strain 0103, which is common in rabbits with diarrhea (Reynaud et al., 1992). The phage resembled members of the Podoviridae, with a narrow host range. The team investigated its efficacy in rabbits against 0103. When administered orally, although the phage persisted for about 12 days in the spleen, it was ineffective in preventing disease in rabbits inoculated with 0103.

A team at the Department of Infection, Birmingham Medical School, UK, investigated phage against several types of important bacteria *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* in experimental infections of mice (Soothill, 1992). Their data showed that as few as  $10^2$  particles of an acinetobacter phage protected mice against a 5X LD-50 challenge of  $1 \times 10^8$  *A. baumannii*. A pseudomonas phage protected mice against a 5X LD-50 challenge of  $1.2 \times 10^7$  *P. aeruginosa*. However, their staphylococcal phage failed to protect mice infected with *S. aureus*. These studies support the view that bacteriophages can sometimes be useful in the treatment of human infections caused by antibiotic-resistant strains of bacteria.

The same team later used guinea pigs to investigate whether phage therapy could work against skin grafts infected with *Pseudomonas aeruginosa* (Soothill, 1994). Their data showed that phage BS24, lytic for *P. aeruginosa* strain 3719, protected the grafts. This work could be expanded to help support human burn patients.



Another team in the Department of Microbiology at Kochi Medical School (Kochi, Japan) tested phage therapy in mice against a *Staphylococcus aureus* challenge (Matsuzaki et al., 2003). Several *S. aureus* infecting phage were isolated in the study, and phage phi MR11 was used for therapy because of its broad *S. aureus* host range, and it carries no known genes for toxins or antibiotic resistance. The model involved injecting mice IP with  $8 \times 10^8$  cells of *S. aureus*, including methicillin-resistant bacteria (MRSA), which caused death in the mice. In contrast, therapy with IP injection of purified phi MR11 ( $\text{MOI} \geq 0.1$ ) suppressed the *S. aureus*-induced lethality. Survival correlated with rapid appearance of phi MR11 in the circulation. Safety tests with high doses of phage showed no adverse effects.

In 2011, a team at Kansas State University investigated whether phage therapy might work in a hamster model for *Clostridium difficile* infection, a pathogen associated with hospital acquired diarrhea and colitis (Revathi et al., 2011). Their data showed that following treatment of the hamsters with ΦCD119 phage, integration of the phage DNA (lysogeny) occurred, as measured by PCR. ELISA tests showed that *C. difficile* toxin production decreased. So, their data showed that phage infection of *C. difficile* can occur in vivo.

### *Phage Therapy Institutes and Companies*

The website <http://companies.phage.org/> lists various companies involved in phage research or phage therapy:

**Companies that focus on pre-clinical R&D:** AmpliPhi Biosciences (US), Enbiotix (US), Fixed Phage (UK), InnoPhage (PT), Intralytix (US), Novolytics (UK), Pherecydes Pharma (FR), Sarum Biosciences (UK), Synthetic Genomics (US), Technophage (PT)

**Companies that do not employ replication-competent phages:** AvidBiotics (US), Enbiotix (US), Phico (UK)

**Companies primarily involved in phage product distribution:** Biochimpharm (GE), Imbio (RU), Microgen (RU)

**Companies that emphasize phage-mediated biocontrol (not "therapy"):** APS Biocontrol (UK), Epibiome (US), InnoPhage (PT), Intralytix (US), Micros Food Safety (NL), Omnilytics (US), Phage Biotech (IL), Phagelux (CN), Technophage (PT)

**Companies that market phage lysates:** Delmont (US)

**Companies involved in enzybiotics:** GangaGen (US/IN), Lysando GmbH (DE), Micros Food Safety (NL), New Horizons Diagnostics (US)

**Companies that emphasize phage-based bacterial detection technologies:** Sample6 (US)

**Companies that focus on phage-associated industrial contamination:** Phage Consultants (PL)

**Companies that emphasize phages in biotechnology products:** Versatile BioSciences (US)

**Companies that facilitate patient phage therapy treatment:** Center for Phage Therapy (PL), Eliava Phage Therapy Center (GE), Globalyz Biotech (US), Novomed (GE), Phage Therapy Center (GE), Phage International (US)

In 1923, the **Eliava Institute** opened in the former Soviet Republic of Georgia, in Tbilisi, to research the then new science of phage and to put phage therapy into practice. According to their website ([Eliava Institute, 2010](#)), the institute was founded by distinguished Georgian physician and phage researcher Prof. George Eliava, together with phage co-discoverer French-Canadian scientist Felix D’Herelle. These two men founded the center, and then in 1937 under Stalin’s rule, Eliava was executed and D’Herelle never came back to Georgia. Throughout its long history, the institute has been known by a variety of names, the best of which is “Scientific-Industrial Union (SIU) Bacteriophage”. The center included two major divisions, a Research Institute, and an Industrial Department (the latter contained 10 manufacturing units). At its peak, approximately 800 people worked at the center. After the collapse of the Soviet Union, the Industrial Department was partly privatized. The center has participated in the preparation and manufacture of products against most of the major bacterial and viral diseases, including anthrax, rubies, tuberculosis, brucellosis, salmonellosis, and dysentery.

In 2009, Naaman, Inc. was founded in Panama City Florida by Michelle Nicholson, an MRSA patient who was successfully treated with phage in the Republic of Georgia. **Globalyz Biotech** (<https://twitter.com/globalyz>) is an international joint venture between Naaman, Inc. and ProLab S.A.S. of Medellin, Colombia. Their goal is to commercialize bacteriophage science globally. The company has successfully administered phage therapy to patients suffering from bacterial infections, including: *Staphylococcus* (including MRSA), *Streptococcus*, *Pseudomonas*, *Salmonella*, skin and soft tissue, gastrointestinal, respiratory, and orthopedic infections.

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## Section-3: Phage-Related Therapies

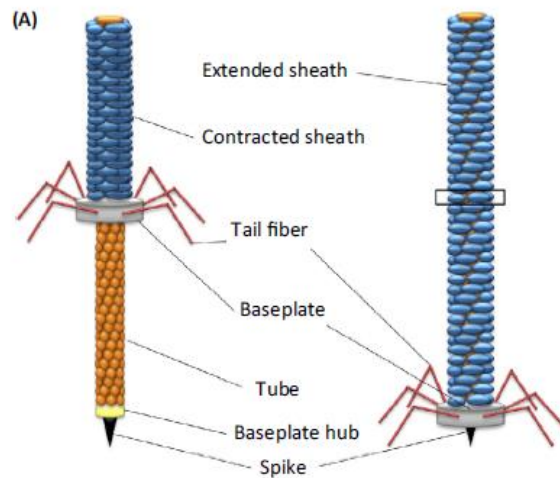
*Benjamin Cossette*

As discussed in the previous section, bacteriophage therapy appears to offer a particularly promising solution to the growing problem of antibiotic-resistant bacteria, however this does not necessarily ensure the adoption of phage therapy in Western medical practice. In spite of the promising evidence showing phage therapy works in both animal models and in some human clinical trials, phage therapy nonetheless relies on the introduction of live replication-competent virus into the patient, a fact that can hinder public acceptance. The concept of treating a bacterial infection with a viral infection, understandably, may seem foreign and dangerous to the layman (Loc-Carrillo and Abedon, 2011). Phage are live viruses, and will mutate and evolve when replicated and manufactured. Mutations can give rise to unforeseen and undesirable effects. Another obstacle to the adoption of phage therapy resides in the labor intensive search and characterization of specific phage species appropriate for treating a specific infection. Phage are astoundingly specific in which bacterial species they infect, which is a benefit by not infecting the human host cells, but is a negative in terms of requiring much work to derive a new therapy. Thousands of phage must be sorted through and thoroughly characterized before selected for use in the treatment of any given bacterial infection. In addition, the genomes must be fully characterized to screen for potential toxins (Loc-Carrillo and Abedon, 2011). While it remains uncertain whether or not these obstacles will prevent the acceptance of phage therapy in Western medicine, it is clear that they will at least prolong the adoption process.

In an attempt to circumnavigate some of the aforementioned obstacles, alternative methods of antibacterial therapy using portions of phage are being investigated by a number of groups, rather than using the whole live virus. If these alternative phage-related therapies can be optimized, they might serve as a safer replacement for phage therapies since there is no phage genomic material that enters the host cells; the chance of altering the bacterial host cell is greatly diminished. The two main alternative therapies discussed in this project are tailocins (pyocins) and lysins.

### Tailocins and Pyocins

A tailocin is a relatively new term used to describe a bacteriocin that resembles a phage particle consisting of the tail and tail fibers, but missing its head and genomic material (**Figure-1**). The tailocin is functionally able to attach to the host bacterial cell and depolarize the plasma membrane to kill the bacterium (Ghequire and DeMot, 2015). Tailocins appear to kill bacteria by the same hole-forming process that is used for injection of the phage genomic material. In nature, tailocins serve different ecological uses, such as to kill competing bacteria. One tailocin can kill one bacterial cell, and the bacteria cannot easily evolve resistance, so these agents may have applications for killing antibiotic-resistant bacteria.



**Figure-1: General Structure of a Bacteriocidal Tailocin.** The structure consists of an extended sheath tube (blue), an inner rigid tail tube (orange), lipopolysaccharide (LPS)-targeting tail fibers (red), and a baseplate (gray). The tailocin attaches to a host bacterium while in its extended form (**diagram right**), and then contracts (**diagram left**) to insert the rigid spike through the cell membrane, causing membrane depolarization and cytoplasmic leakage. From: [Ghequire and deMot, 2015](#).

The tailocin is thought to attach to a bacterium like a mature phage would, with the tail fibers attaching to specific lipopolysaccharide (LPS) residues on the host cell. The tailocin attaches in its extended form (**Figure-1, right side**), and (for the contractile types) the outer sheath contracts (**Figure-1, left side**) to expose a non-flexible inner tail tube and spike that act like a syringe to insert into through the cell membrane. The insertion depolarizes the membrane, and releases cytoplasmic contents, killing the cell. Much is known about the contractile-type T4 phage tails whose structures have been studied at 15-17 angstrom resolution ([Kanamaru et al., 2002](#); [Kostyuchenko et al., 2005](#)). The baseplate has a hexagonal structure containing 6 tail fibers. Tail contraction is caused by a substantial rearrangement of the tail sheath proteins to cause about a one-third shortening. When inserted through the membrane, the inner tube tail extends about half the length beyond the baseplate, which is sufficient for crossing the host cell's periplasmic space ([Kostyuchenko et al., 2005](#)). The crystalline structure of the T4 tail indicates the sheath is composed of 138 copies of the tail sheath protein, which surrounds the non-contractile tube, and that during contraction the sheath proteins slide over each other ([Aksyuk et al., 2009](#)). Recent research indicates the T4 tail sheath resembles a stretched, coiled spring, wound around a rigid tube that has a spike-shaped protein at its tip that penetrates the bacterium ([Taylor et al., 2016](#)). The triggering mechanism appears to be highly conserved among various bacteria and phage. The structure of the Phi-29 phage at 2.0 Angstrom resolution also shows a hexameric tube structure that forms a channel that spans the bacterial bilayer in a pore-forming mechanism similar to non-enveloped eukaryotic viruses ([Xu et al., 2016](#)).



Bacteria typically produce specialized antimicrobial compounds called bacteriocins that act upon organisms of the same or closely related species (Nakayama et al., 2000). While these bacteriocins are usually encoded on plasmid DNA, several chromosomally-encoded bacteriocins were initially discovered in *Pseudomonas aeruginosa* and were termed pyocins (reviewed in Shinomiya et al., 1975; Michel-Briand and Baysse, 2002). Evolutionarily, pyocin genes most likely evolved from phage genes that had inserted into the bacterial chromosome. Although they were initially discovered in *P. aeruginosa*, other bacteria also produce pyocins, including both gram-positive and gram-negative species (Ghequire and De Mot, 2015). The pyocins found in *P. aeruginosa*, however, remain the most studied and best characterized. These *P. aeruginosa* pyocins have since been divided into three sub-classes:

**R-Type Pyocins:** resemble non-flexible and contractile tails of bacteriophages, and induce depolarization of the cytoplasmic membrane. These genes are carried in the *Pseudomonas* chromosome, and almost certainly evolved from integrated phage genes of the *Myoviridae* family.

**F-Type Pyocins:** also resemble phage tails, but have a flexible and non-contractile rod-like structure. These pyocins may have evolved from *Siphoviridae* (non-contractile) phage genes. The killing mechanism of F-type tailocins are similar to that of R-type tailocins, however the targeting mechanism is unlike that of the R-type tailocins (Nakayama et al., 2000).

**S-Type Pyocins:** colicin-like, protease-sensitive proteins, containing DNase and RNase activity.

Bacteriocins that resemble phage tail structures, like the R and F-type pyocins of *P. aeruginosa*, are now being termed tailocins.

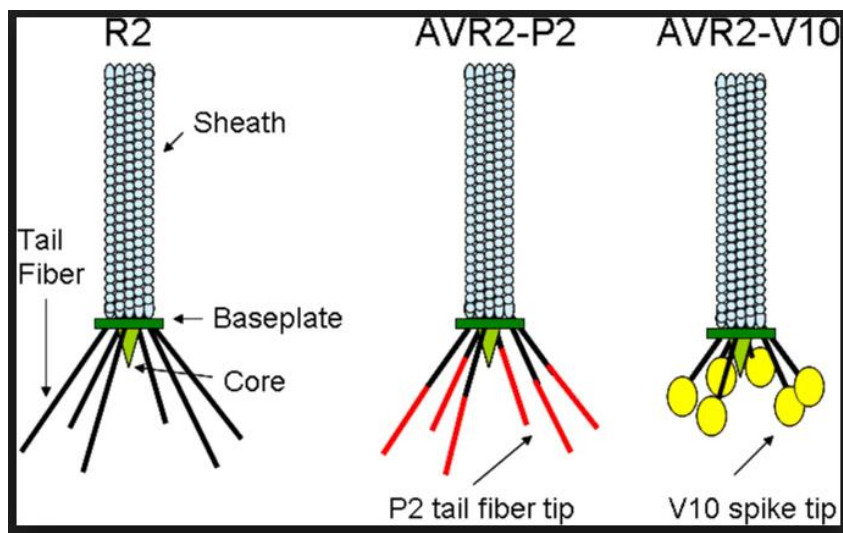
During evolution, some types of bacteria mutated (altered) the phage genes integrated within their chromosomes to suit the bacterium. Some bacteria co-opted the capsid structures, others the tail structures, and others co-opted both. The genes encoding phage tails are especially beneficial for bacteria to co-opt because they are complex nano-machines with moving parts, so there are many functional areas to alter to suit their purposes. If the bacteria can mutate these genes to be under the control of their own secretory systems (type VI secretory system, T6SS), the tail structures are secreted outside the bacterial cell to bind to and affect other bacteria. The genes encoding tail fibers can also be mutated to bind to different species of bacteria. The gene structure of the core components of all contractile tail-like systems appears to be highly conserved, but have diverged considerably to where ancestry can no longer be easily detected (Leiman and Shneider, 2012).

In 2013, scientists at the College of Life Sciences at Wuhan University (Wuhan, China) identified the first tailocin structure from *Stenotrophomonas maltophilia*, an important global opportunistic pathogen with multidrug-resistant strains (Liu et al., 2013). Electron microscopy revealed that the tailocin, termed maltocin P28, resembles a contractile but nonflexible phage tail structure. It is composed of two major proteins, 43 and 20 kDa in size, and their N-termini have been sequenced. The gene encoding P28 was identified, and is located within the *S. maltophilia* genome in an organization that is similar to that of the P2 phage genome and the R2 pyocin. *In vitro*, P28 showed bactericidal activity against 38 of 81 tested *S. maltophilia* strains.

## Engineered Tailocins and Pyocins

While the therapeutic potential of tailocins remains largely unexplored, some groups have begun creating recombinant tailocins for other antibacterial applications. In 2009, one group at AvidBiotics Corporation (South San Francisco, CA) created a recombinant R-type tailocin targeted to *Escherichia coli* O157:H7, a pathogenic *E. coli* strain often seen as a food contaminant (Scholl et al., 2009). Tailocins typically only target a specific bacterial species, often one closely related to the tailocin-encoding bacterial species. In order to create an *E. coli*-targeting tailocin from a *P. aeruginosa* tailocin, the group utilized tail fibers encoded by AVR2-V10, a bacteriophage that naturally infects *E. coli*, and fused the tail fiber genes onto the potent catalytic tail spike of the *P. aeruginosa* R-type tailocin. The resulting recombinant tailocin was capable of targeting and disrupting the LPS layer of the *E. coli* target, resulting in cell death. The therapeutic implications of this remarkable ability to create custom recombinant tailocins from bacteriophage tail fibers and bacterial-encoded tailocins are immense, and yet largely unexplored.

This same idea was expanded in 2011 by scientists at the Channing Laboratory of Brigham and Women's Hospital (Boston), who engineered an R-type (contractile) pyocin (termed AvR2-V10.3) to specifically kill enteric pathogen *Escherichia coli* O157 (Ritchie et al., 2011). The team began with the naturally occurring structure of contractile type R2 tailocin (Figure-2, left side), and altered the gene encoding the tail fibers (which attach to the bacterial cell) to specifically bind *E. coli* O157 (diagram right). In a rabbit model of infection, the team showed that oral administration of AvR2-V10.3 can prevent or ameliorate *E. coli* O157:H7-induced diarrhea and intestinal inflammation. The tailocin was effective when delivered either prior to or post-infection. AvR2-V10.3 also reduced the amount of fecal shedding of O157.



**Figure-2: Diagram of the Genetically Engineered Tailocin AvR2-V10.** The team began with the structure of the naturally occurring R2 contractile tailocin (diagram left) and altered the genes encoding the tail fibers (which attach to bacteria) to bind *E. coli* O157 (diagram right). Diagram from: Ritchie et al., 2011.

In 2015, a team at AvidBiotics Corp. (South San Francisco, CA) and the Microbial Pathogenesis Laboratory of the Wellcome Trust Sanger Institute (Hinxton, United Kingdom) engineered a modified R-type tailocin to target *Clostridium difficile*, a major cause of hospital acquired infections (Gebhart et al., 2015). The shed spores are impervious to most interventions, including antibiotics. The team genetically modified the contractile R-type bacteriocin "diffocin" (isolated from *C. difficile* strain CD4) to kill 027-type strains. The natural receptor binding protein (RBP) of diffocin was replaced with a newly discovered RBP that binds 027. The engineered diffocins (termed Avidocin-CDs Av-CD291.1 and Av-CD291.2) were stable and killed all 16 tested 027 strains. Orally administered Av-CD291.2 survived passage through the GI tract, did not detectably alter the mouse gut microbiota, and prevented antibiotic-induced colonization of mice inoculated with 027 spores (Gebhart et al., 2015).

Overall, tailocins, including recombinant tailocins, represent a pool of thus-far untapped therapeutic potential. Without further studies, it is impossible to judge the therapeutic potential of tailocins as an alternative to whole-phage therapy.

## Lysins

Tail structures are not the only phage components being investigated for potential anti-bacterial activity. Also being investigated are enzymes encoded by phage that act to help the phage particles exit from the bacterium during the lytic stage by degrading the cell wall (Hermoso et al., 2007). These enzymes are termed **lysins** (or **endolysins** or murein hydrolases). Lysins are highly specific enzymes that are able to break a key bond in peptidoglycan (murein), consisting of sugars and amino acids, and is the main component of bacterial cell walls. As is the case with phage tails, the lysins appear to be highly specific and effective killers of bacteria, and so may have applications for killing antibiotic-resistant bacteria (reviewed in Fischetti, 2008).

Lysins are usually composed of a cell-binding domain (CBD) and a catalytic domain. The CBD, as its name implies, binds to a specific substrate in the bacterial cell wall, usually a carbohydrate component of the peptidoglycan. The sequence of the CBD is highly variable, allowing great specificity for attaching to specific bacteria (García et al., 1988). This specificity (as with phage and tailocins) is important, as it would leave the beneficial bacteria in the patient unharmed. Lysins usually degrade the cell wall of the same species of bacteria that produced the phage. So when trying to kill a specific species of antibiotic-resistant bacteria, the lysin would likely need to be manufactured (or originally isolated) from that same species, although some widely acting lysins have also been discovered. The discovery of heat-stable lysins may facilitate their use in medicine because it makes their purification easier (Plotka et al., 2014). Most lysins discovered to date are active against "Gram-positive bacteria" (Fischetti, 2008) because "Gram-negative bacteria" contain an outer membrane that blocks lysin access to the peptidoglycan layer. However, some lysins have been engineered to be active against Gram-negative bacteria (Briers et al., 2014).

During bacteriophage infection, these lysins must pass through the lipid bilayer in order to gain access to the peptidoglycan (PG) layer. This is achieved through cooperation with another class of phage-encoded proteins called holins (Loessner, 2005). These holins bind to the inner lipid layer of the bacterial membrane and create pores through which the lysins can travel.

The C-terminus of these lytic enzymes contains the targeting domain, which allows specific targeting of PG-associated carbohydrate motifs. It has been suggested that due to the targeting of lysins to these carbohydrate motifs, the emergence of lysin-resistant bacteria strains may be impossible due to the necessity of these peptidoglycan motifs for bacterial survival (Loessner, 2005). The catalytic domain of lysins is located at the N-terminus, and is capable of efficiently cleaving bonds in the PG network, compromising structural integrity and triggering the subsequent lysis of the bacteria cell (Hermoso et al., 2007).

When naturally utilized by a bacteriophage, these lysins act from within the cell (**endolysin**). But to be considered for use as an antibacterial therapeutic, the lysin must be able to act from without (**exolysin**). But the therapeutic potential of lysins does not appear to be lost, because the PG layer of gram-positive cells is directly accessible from the exterior of the cell, and the PG layer of gram-negative cells can be accessed if the exterior lipopolysaccharide layer is disrupted. It has also been suggested that lysins that can act alone on gram-negative cells are likely to exist but have not yet been discovered (Loessner, 2005).

Most excitingly, the unique advantages of phage therapy are conserved by lysin therapy, with some advantages being enhanced. For example, phage and lysins both have in common their high specificity. Most phage and lysins are only capable of infecting a specific species of bacteria, a desirable trait that can be exploited in order to eradicate a pathogenic bacterial species while leaving beneficial bacterial populations intact. However, some lysins target PG motifs that are common to a wide range of bacteria, creating the potential for an “emergency widespread eradication” similar to the effect of current antibiotics (Yoong et al., 2004; Loessner, 2005). The killing efficacy of bacteriophage is also preserved by lysins as demonstrated by several *in-vitro* and *in-vivo* studies (Jado, 2003; Loeffler et al., 2003; Cheng et al., 2004).

## Examples of Lysin Treatments

Lysins were first used therapeutically in animals in 2001 in the Laboratory of Bacterial Pathogenesis and Immunology, The Rockefeller University (New York, NY) (Nelson et al., 2001). The Rockefeller team investigated the ability of the murein hydrolase isolated from the streptococcal bacteriophage C1 to prevent colonization in mice. *In vitro* experiments showed that the purified lysin lysed group A, C, and E *Streptococci*, leaving the other tested strains alone. Amazingly, 1,000 units of lysin (10 ng) was sufficient for lysing approximately  $10^7$  group-A *Streptococci* within 5 seconds. A single dose of 250 units of lysin applied orally to mice reduced strep oral colonization to 28.5% (n=21) compared to 70.5% (n=16) for untreated controls ( $P < 0.03$ ). In mice already orally colonized by strep, treatment with 500 units of lysin produced no detectable streptococci after 2 hours.

In 2003, the same team at Rockefeller University isolated lysin Cpl-1 from a lytic pneumococcal bacteriophage, and tested it in a mouse model by intravenous therapy against a pneumococcal challenge (Loeffler et al., 2003). They found that a 2,000  $\mu\text{g}$  dose of Cpl-1 given 1 hour after iv infection, reduced pneumococcal titers from  $10^4$  to undetectable levels ( $<10^2$  CFU/ml) within 15 minutes, and increased mouse survival to 100% compared to 20% for untreated mice. Although they found that the enzyme therapy was immunogenic, its efficacy was not significantly reduced in mice with a previous exposure. They also found that Cpl-1 was

effective as a topical nasal treatment against *S. pneumoniae* colonization. Cpl-1 was active *in vitro* against many *S. pneumoniae*, and was independent of penicillin resistance.

In a similar 2003 study, the lysin CPL-1 was used to treat *Streptococcus pneumoniae* infections in a mouse sepsis model (Jado, 2003). Various doses of *S. pneumoniae* were introduced to mice via IP injection. The mortality rate for untreated animals was 100% after 72 hours, while animals treated with CPL-1 at one-hour post-infection were rescued from the *S. pneumoniae* infection. These results clearly demonstrate the ability of locally administered lysins to effectively treat localized infections *in-vivo*.

In a 2004 study, a topical lysin treatment of Group B Streptococci (GBS) vaginal infections in mice was also explored (Cheng et al., 2004). Topical treatment with Lysin PlyGBS effectively reduced vaginal GBS colonization in mice after a single treatment, a promising result with potential implications in the reduction of neonatal meningitis and sepsis.

In 2013, a team of scientists at the Instituto de Productos Lácteos de Asturias (Villaviciosa Asturias, Spain) summarized the work on the therapeutic potential of a different class of enzymes, the virion-associated peptidoglycan hydrolases (VAPGHs), which, in contrast to endolysins, are enzymes that create a small hole through which the phage tail tube crosses the cell envelope to eject the phage genetic material at the beginning of the infection cycle (Rodríguez-Rubio et al., 2013). The VAPGHs have several features that make them excellent candidates for therapy, including high specificity for the target cell, heat stability, and a modular organization that facilitates subsequent engineering. The authors suggested that these enzymes may have applications for treating antibiotic-resistant bacteria in human therapy and veterinary applications, as well as bio-preservatives in food safety, and as biocontrol agents in agriculture.

Although most lysins are active against Gram-positive bacteria (lacking an outer cell wall), lysins active against Gram-negative bacteria were engineered in 2014 by a team at the Laboratory of Gene Technology in Leuven, Belgium (Briers et al., 2014). The team developed and optimized an approach to engineer the enzymes to penetrate the outer membrane of gram-negative bacteria, termed Artilysins, and tested their effectiveness against *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. The design of the Artilysins included combining a polycationic nona-peptide with a modular endolysin. The drug was able to reduce infection *in vitro* by 4 to 5 log orders of several strains of multi-drug resistant gram-negative bacteria. The activity was further increased by adding a linker between the two domains of the drug, or by creating a mixture of polyanionic and polycationic domains. Time-lapse microscopy showed that the drug worked as hypothesized, by passing the outer bacterial membrane to degrade the peptidoglycan layer, followed by cell lysis. The drug was effective *in vitro* to protect human keratinocytes, and *in vivo* to protect *Caenorhabditis elegans* against a bacterial challenge.

Lysins are not perfect treatments. As with phage or their tail structures, lysins are foreign proteins that can stimulate an immune response in the patient. An immune response from the patient directed against the phage component could lessen its effectiveness, or could become dangerous to the patient if it induces a cytokine storm response.

In conclusion, antibacterial lysin therapy may provide a promising and exciting alternative to full phage therapy. As a protein therapeutic, lysin therapy may be more readily accepted by Western medicine, because protein therapeutics are already in use. Additionally,

single proteins are much easier to characterize than whole phage particles. The highly desirable specificity that is characteristic of phage therapy is not sacrificed by lysin therapy, potentially allowing for specific bacterial species eradication *in-vivo*. Additionally, like phage therapy, lysin therapy leaves little-to-no potential for the rise of bacterial-resistant strains, as the components lysed by the lysin are required by bacteria for survival. In fact, lysins have been approved and are already being employed by the food industry. Cows have been genetically engineered to secrete a “lysin-like” protein in their milk to kill *Staphylococcus aureus* (Fischetti et al., 2006). Lysin genes have also been engineered into the genomes of different produce products such as potatoes and pears to protect them from specific bacterial infections. As more lysins are characterized, and treatment regimens are optimized for maximum effectiveness, the adoption of lysins as protein therapeutics for bacterial infection in Western medicine grows more feasible.

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## Section-4: Phage Safety

*Benjamin Cossette*

Although phage (and their components) may be excellent candidates for treating antibiotic-resistant bacterial infections, some scientists worry that the treatments could produce unwanted side-effects. Such worries accompany all new drugs and vaccines, and experiments should be (and in some cases already have been) designed to address the concerns.

The following are four main concerns about phage therapy (discussed below in more detail):

- 1. Genetic Alteration of Phage, Bacterium, or Human Chromosomes:** A replication-competent phage as it replicates to high titers in the patient could mutate and evolve into a pathogen with new undesirable effects. The use of temperate (lysogenic) phage which are capable of integrating their DNA into the bacterial host chromosome as part of their life cycle, could alter a host gene to create a different pathogen. Could such phage mutate to allow integration into human chromosomes?
- 2. Release of Toxic Proteins:** As the phage (or tailocin or lysin) lyse the bacteria, it could release toxic bacterial proteins which harm the patient.
- 3. Undefined Phage:** The use of ill-defined phage or phage mixtures adds an unknown variable to their use.
- 4. Immune Responses:** The use of phage (or lysins or tailocins), all of which are foreign proteins to humans, could induce an immune response in the patient that could either reduce the effectiveness of (inactivate) the therapy, or kill the patient.

### Safety Point-1: Genetic Alterations

Genetic alterations are one of the chief concerns for phage therapy. These alterations can occur either during phage amplification or by DNA integration/exchange. With respect to phage mutations occurring during the large-scale production of specific batches, the phage are replicated to high titers using *in vitro* bacterial cultures. This production process has been shown to produce a small percentage of phage that have become mutated (Krylov et al., 1993), but the mutation process usually makes the phage inactive, not more potent. The most frequent type of mutation observed renders the phage unable to infect the bacterium. Such mutations occur in about 10% of the phage particles in a large-scale batch preparation, which slightly lessens the effectiveness of the batch for therapy, but this process typically has no effect on safety (Krylov et al., 1993). Some labs are developing procedures for preparing and monitoring phage batches with high activity for therapy uses. However, high titer production in bacterial culture is not the same as high titer replication inside humans, so scientists should continue to assay for gene alterations during human clinical trials, even if such alterations are unlikely.

Temperate (lysogenic) phage integrate their DNA into the host chromosome or plasmid as part of their life cycle (Krylov et al., 2014). As an example of a bacterium becoming highly toxic after integrating phage genes, this is known to occur in the case of *Vibrio cholera*, where the genes encoding the cholera toxin (CTX element) move from bacterium to bacterium via a

filamentous phage (Waldor and Mekalanos, 1996). However, temperate phages are not generally used for therapeutic experiments, because lytic phages (that only lyse the host) are more useful for killing the host cell. The vast majority of phages that have been characterized are lytic; only a small percentage have the ability to integrate their DNA into the host chromosome (Summers, 2001).

In 1977, scientists investigated the safety of a live phage vaccine in rhesus monkeys by assaying for the presence of phage DNA integrated into the rhesus chromosomes (Milstien et al., 1977). The team isolated bacteriophage phiV1 from a live virus vaccine designed against *E. coli*, and inoculated 4 young rhesus monkeys with  $10^{12}$  plaque-forming units (PFU). After the phage had been cleared from the blood, DNA was isolated from the monkey's livers and kidneys, and analyzed for the presence of bacteriophage DNA by re-association kinetics, and for the ability to produce live phage by plaque forming assays. Their data showed no evidence of the presence of phage DNA integrated in the monkey DNA, nor in the ability of the host DNA to produce detectable phage particles. More experiments of this type should be performed in the future to continue to monitor the ability of new phage therapies to integrate their DNAs.

To avoid the chance of alterations to the bacterial DNA (or in a worst case the human chromosome) from a safety perspective it is easy to simply eliminate the use of any temperate phage for therapy. The use of temperate phages as therapeutics could give rise to even more dangerous or phage resistant bacteria and must therefore be avoided. The use of lytic phage for therapy is likely to be more efficient for lysing bacteria, and thousands of such phage species are available for use. In some cases where a lysogenic phage is known to infect a particular bacterial species of interest for therapy, scientists have screened for phage mutants that have become only lytic and might better be suited for phage therapy. For example, one team identified a new phage strain YMC01 that is lytic against *Pseudomonas aeruginosa* (Jeon et al., 2012). This new strain is closely related to the known temperate phage Phi-297 (Burkal'tseva et al., 2011), so perhaps YMC01 was created from Phi297 by natural mutation.

In addition, the entire problem of genetic alterations could be avoided by simply using tailocins or lysins, which lack genetic material.

## **Safety Point-2: Release of Toxic Proteins**

This is a valid safety concern. In some cases, lysing bacteria inside a patient are known to release endotoxins that cause fever, and in some cases toxic shock. In the few clinical trials published to date (discussed and cited in Section-2) the fever is easily manageable, and is a minor concern relative to the patient's antibiotic-resistant infection.

To prevent the release of toxic proteins, some scientists have proposed using genetic engineering to remove the phage genes that cause lysis, so the phage inserts itself into the bacterium to kill it, without lysing the bacterium. A similar result would be obtained simply by using tailocins, which kill without lysis. Inactivated (non-lysed) bacteria are cleared from the system by the patient's phagocytic cells. However, the removal of lysing genes would also prevent the beneficial exponential expansion of the phage population *in vivo* which helps lyse more bacteria in the patient. So, perhaps this gene removal process should be used only for the rare phage known to cause toxic shock problems.

### **Safety Point-3: Undefined Phage**

A widespread concern about phage therapy revolves around the fact that phage genomes remain largely uncharacterized. Even the most studied phages have not yet been fully characterized. Take for example the *Pseudomonas aeruginosa* phage PaP1, a virulent phage whose genome encodes 157 open reading frames (Krylov et al., 2015). 144 of the encoded proteins have homologs with known functions, however evidence supporting a known function exists for only 38 of these 144 proteins. 14 protein products encoded by this phage remain completely uncharacterized (Krylov et al., 2015). Many argue, that before phage therapy will be accepted as a safe and effective, the therapeutic phage must be fully characterized and screened to eliminate phages that encode toxic proteins or proteins that allow temperate (integrative) phage behavior.

In addition to individual phage species being uncharacterized, a problem with phage therapy is the frequent use of phage cocktails (mixtures of several phage species). Such cocktails, often used in Russia and Eastern Europe, are ill-defined mixtures of phage screened for their ability to lyse bacteria from sewerage etc. Although such mixtures are usually uncharacterized, they are not necessarily unsafe. In 2013, a group at the Nestlé Research Centre, Nestec Ltd, Vers-chez-les-Blanc, in Lausanne, Switzerland, investigated the safety of a complex phage cocktail prepared at the Russian pharmaceutical company Microgen (McCallin et al., 2013). The team began by sequencing the genomes of the phage present in the cocktail and analyzing the sequences by bioinformatics. The cocktail was designed to target *Escherichia coli* infections. Using electron microscopy, the team identified six phage types present in the cocktail, with T7-like phages dominating over T4-like phages. Their meta-genomic sequencing approach followed by taxonomical classification identified 18 distinct phage types, of which 7 genera were *Podoviridae*, 2 were established genera of *Myoviridae*, 2 were proposed genera of *Myoviridae*, 2 were genera of *Siphoviridae*, and one was a new phage. Bioinformatic analysis of the sequences revealed no undesired genes. A small trial with several volunteers found no adverse side-effects following oral exposure to the cocktail. So, DNA sequencing can help provide data on which phage are present in a cocktail, and whether known toxic genes are present. Such quality control DNA sequencing could be done on each large scale phage batch prior to therapy. The sequencing could exclude the use of phage whose genomes encode such undesirable products as toxins, transposases, or repressor proteins (Krylov et al., 2014). But, DNA sequencing alone does not guarantee the safety of phage. In some cases, toxic genes might not be characterized, so novel unknown toxic genes would not be identified by the analysis of existing databases.

### **Safety Point-4: Immune Responses**

Another concern surrounding phage therapy is that phage (or lysins or tailocins) are viewed as foreign molecules to our immune systems, so these molecules are capable of triggering an immune response. Fortunately, although a patient immune response sometimes forms, it is usually mild and there is little risk of toxic shock. Indeed, most clinical trials of various phage therapies report no adverse health effects (Wright et al., 2009; Kutter et al., 2010). In support of these findings, it is useful to note that humans have been naturally exposed to phage for our entire existence. We are constantly exposed to phage in our air and drinking water, and millions of phage can be found in a single mouthful of seawater (Omnilytics Inc., 2016).

Many of the phage therapy trials have seen patient successes using a therapeutic load much lower than that found in a mouthful of seawater (Kutter et al., 2010).

## Section-4 Conclusion

It is concluded that if proper precautions are taken, phage therapy represents a safe option for treating bacterial infections. With respect to genetic alterations, lysogenic phage should not be used for therapy, and any lytic phage used should be pre-screened for lysogenic potential. This step alone negates the possibility of genetic alterations by integrations. DNA mutations occurring during the amplification of phage batches could easily be detected by quality control DNA sequencing of the batch prior to use. Genetic alterations could also be avoided by using tailocins or lysins, which lack genetic material. With respect to uncharacterized phage, the phage should be fully sequenced to provide plausible evidence for the functionality of all protein products. *In vitro* data should also be provided demonstrating infection specificity to show no off-target effects. With respect to the release of toxic proteins, this does not appear to be a major problem in most cases, and is likely to be manageable if it occurs. If a particular bacterial species is known to cause problems upon lysis, the therapy for that species could be switched to non-lysing tailocins. With respect to immune responses, switching to tailocins or lysins will not solve this problem, but significant immune responses appear to be rare and most are easy to manage. Altogether, if a phage passes these screening checkpoints, it is more than likely that it is safe and suitable for therapeutic use. Most scientists argue that the risks associated with phage therapy are relatively minor and can easily be circumnavigated with proper precautions.

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## Section-5: Status of Current Commercial Therapies and Clinical Trials

*Xinyuan Wang*

In spite of the fact that there are relatively few scientific studies on phage therapy in the Western literature, several companies are currently performing pre-clinical experiments or are in Phase-I clinical testing (Table-I).

**Table-I: List of Commercial Companies Performing Pre-Clinical or Clinical Phage Trials**

Company	Location	Product(s)	Applications	Trials
AmpliPhi	Richmond, Virginia	Natural phage cocktails	<i>P. aeruginosa</i> lung infections in cystic fibrosis, <i>S. aureus</i> wound and skin infections, <i>C. difficile</i> GI infections.	<b>Phase-I approved Nov. 2015</b>
ContraFect Corp.	Yonkers, NY	Phage lysins	<i>S. aureus</i>	<b>Phase-I started April 2015</b>
EnBiotix	Cambridge, MA	Engineered Phage	Staphylococcal infection of prosthetic joints	Pre-clinical
EpiBiome	San Francisco, CA	Natural phage cocktails	<i>E. coli</i> and Shigella dysenteriae infections in children	Pre-clinical
Fixed-Phage	Glasgow, UK	Natural phage fixed to surfaces	MRSA wound infections	Pre-clinical
Intralytix	Baltimore, MD	Natural phage cocktails	<i>S. aureus</i> , <i>P. aeruginosa</i> , <i>E. coli</i> wound infections. Irritable bowel disease.	Pre-clinical
Micreos	Wageningen, Netherlands	Phage lysins	<i>S. aureus</i> and MRSA skin infections	Pre-clinical
Novolytics	Warrington, UK	Natural phage cocktails	MRSA skin infections	Pre-clinical
Pherecydes Pharma	Romainville, France	Natural phage cocktails	<i>E. coli</i> and <i>P. aeruginosa</i> burn and skin infections; <i>P. aeruginosa</i> respiratory infections; <i>S. aureus</i> bone, joint, prosthetic infections	<b>Phase-I trial started Sept. 2015</b>
Synthetic Genomics	San Diego, CA	Engineered Phage	Infections in burn wounds, skin, and cystic fibrosis	Pre-clinical
TechnoPhage	Lisbon, Portugal	Natural phage cocktails	Chronic ulcers, respiratory and skin infections.	Pre-clinical

Table Adapted from: Madhusoodanan, 2016.

From the Table above, it can be seen that at least three companies have already started phage clinical trials: AmpliPhi (Richmond, Virginia), ContraFect Corp. (Yonkers, NY), and Pherecydes Pharma (Romainville, France). Each of these 3 companies will be discussed below.

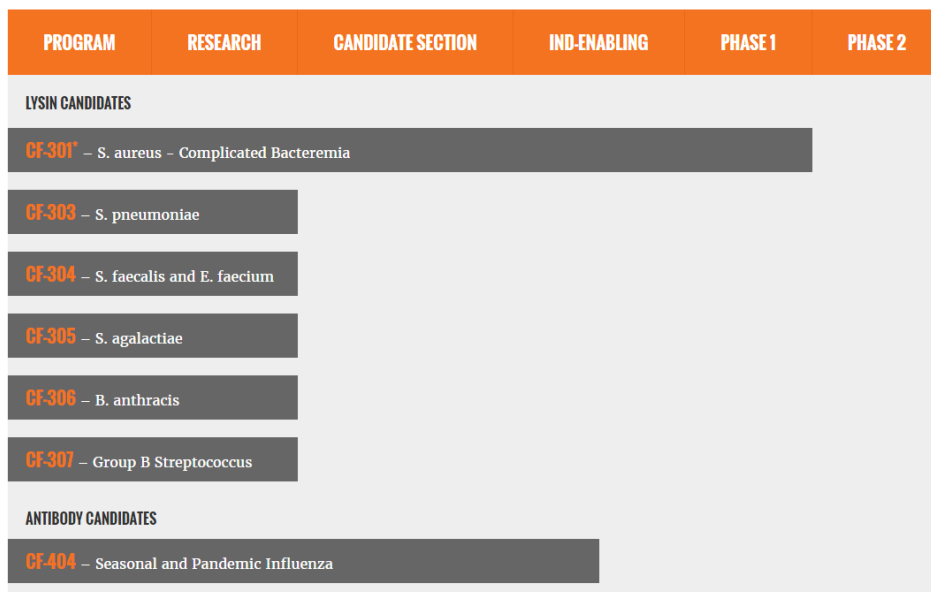
## AmpliPhi Biosciences

AmpliPhi Biosciences Corporation (NYSEMKT: APHB, Richmond, Virginia) is a biotechnology company focused on the development and commercialization of novel bacteriophage-based antibacterial therapeutics ([www.ampliphi.com](http://www.ampliphi.com)). AmpliPhi's product development programs mostly target antibiotic-resistant bacteria. AmpliPhi is currently conducting a Phase-I clinical trial of their product AB-SA01 for the treatment of *Staphylococcus aureus* in chronic rhinosinusitis patients, the first phage therapy trial for this condition, but the company is also developing bacteriophage therapeutics against *Pseudomonas aeruginosa* and *Clostridium difficile*.

The full results of AmpliPhi's clinical trial are expected in the second half of 2016, but an update was provided on April 20, 2016, which stated that the treatment appears to be well tolerated. The first cohort of 3 patients was administered phage AB-SA01 twice daily for seven days. The treatment appeared to be well tolerated and there were no apparent drug-related adverse events. They have also enrolled the first patient in cohort-2 who has been treated with the same dose but longer (14 days). According to the CEO M. Scott Salka, "We are encouraged that the preliminary data indicate decreased bacterial loads and an easing of symptoms following treatment.....We expect to complete the trial mid-year and report final data later in 2016."

## ContraFect Corporation

ContraFect Corp. (Yonkers, NY) is a phage company that is currently in Phase-I testing of their lead drug CF-301, a lysin enzyme that is active against *Staph aureus* (**Figure-1**) (<http://www.contrafect.com/>). CF-301 is an enzyme that targets a conserved region of the cell wall that is vital to bacteria, so resistance is less likely to develop. *In vitro* and *in vivo* experiments have shown that CF-301 clears biofilms, so the drug likely can interact with its bacterial target *in vivo*. CF-301 was licensed from The Rockefeller University, and developed at ContraFect.



**Figure-1: Chart of the Products Being Developed at ContraFect Corp.** CF-301, a lysin treatment for *Staph aureus*, is their lead drug, having just completed Phase-I clinical trials that showed safety. (<http://www.contrafect.com/>)

In 2014, the company published their pre-clinical findings of the effectiveness of CF-301 against *S. aureus* and MRSA in mice (Schuch et al., 2014). The authors showed that CF-301 lysin has potent, specific, and rapid bacteriolytic effects against *Staphylococcus aureus*. It also shows activity against drug-resistant strains, has a low resistance profile, eradicates biofilms, and acts synergistically with antibiotics. With respect to in vitro lysis ability, CF-301 could lyse 250 of the *S. aureus* strains tested, including 120 methicillin-resistant *S. aureus* (MRSA) isolates. And in time-course experiments with 62 strains, CF-301 reduced *S. aureus* by 1000-fold within 30 minutes, compared to 6-12 hours required by antibiotics. In mice infected with *S. aureus*, CF-301 increased survival by reducing blood MRSA 100-fold within 1 hour.

On December 15, 2015, the company completed the Phase-I portion of their clinical testing of CF-301, and reported no adverse side effects of the drug. The CF-301 Phase 1 study was a randomized, double-blind, placebo-controlled, single escalating-dose study in healthy volunteers in the United States to evaluate safety, tolerability, and pharmacokinetics. An independent DSMB was established to review the safety, tolerability, and pharmacokinetic data at each dose level. The DSMB observed no clinical adverse side effects. According to Julia P. Gregory, ContraFect's CEO, "This is a major milestone for CF-301, a first-in-class, first-in-field biologic agent targeting Staph infections, including MRSA," "We are excited to have achieved our objectives for this Phase-I study, and we will now continue preparations and discussions with regulatory agencies for our next study of CF-301 which is anticipated to be conducted in patients with Staph bloodstream infections including endocarditis."



## Pherecydes Pharma

Pherecydes Pharma is a biotech company located in Romainville, France, that produces phage cocktails to combat *E. coli* and *P. aeruginosa* burn and skin infections, *P. aeruginosa* respiratory infections, and *S. aureus* bone, joint, and prosthetic infections (Pherecydes, 2016). Their lead products are PhagoBurn, PneumoPhage, and Phosa.

**PhagoBurn**, as its name implies, is a phage cocktail (12-13 phage species) designed to treat burn patients. The phage mixture was collected from bacteria-rich sewerage flowing underground from Parisian hospitals, and is designed to lyse *E. coli* and *P. aeruginosa* bacteria found in burn infections. In burn patients, bacterial infections are the first cause of mortality, especially as the bacteria acquire high levels of antibiotic resistance. PhagoBurn is the world's first phage therapy to be tested in an international multi-center clinical study. Their randomized, controlled, single-blind Phase-I/II clinical study was approved in June 2013, and began in July 2015, for a period of 36 months ([www.phagoburn.eu](http://www.phagoburn.eu)). The trial is underway at 11 burn unit centers in France, Switzerland and Belgium. All of the necessary clinical authorizations have been obtained from the appropriate regulatory agencies and ethical committees in these three countries.

An update on the PhagoBurn ongoing clinical trial was recently published (Servick, 2016), and indicates that it has had a few challenges to overcome, including several delays and a decreased patient numbers. The delays have resulted from the increasing burden of validating and documenting the various production steps in preparing the phage, which was supposed to take only 12 months but took 20 months. And another delay occurred when France's National Agency for the Safety of Medicines and Health Products required the company to prove the stability of the product. This month (June, 2016) the agency accepted the company's data showing the product was stable and non-contaminated (Servick, 2016). And the trial was supposed to have enrolled 220 patients from 11 hospitals, but in 6 months of recruitment only 15 patients were found to be eligible. Patients infected with more than one bacterial species are not eligible for the trial, and recently this pertains to most burn victims. However, other scientists remain optimistic that much will be learned from this trial and its approval and enrollments, even if it does not work well.

**Pneumophage** is a cocktail designed to treat specific types of (*Pseudomonas aeruginosa*) acute respiratory infections. The product was launched in June 2015, and was designed for inhalation. *P. aeruginosa* is largely responsible for ventilator-acquired pneumonia (VAP), a serious and common complication of mechanical ventilation in intensive care units (ICU). The project is a collaboration between the French Technology Diffusion (Saint-Etienne, [www.dtf.fr](http://www.dtf.fr)) (who specializes in developing new aerosol dispensers adapted to drugs), Pherecydes Pharma (who develops and prepares the phage cocktail), and pharmaceutical regulatory agencies (to aid the evaluation in humans).

**Phosa** is a phage cocktail designed to treat major bone and joint infections, and diabetic foot ulcers, caused by antibiotic-resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. The product was launched in January 2015, and its testing will continue for 24 months. These severe conditions are often associated with heavy disability and mortality. The project includes designing the composition of the phage cocktail, testing the prepared phage in two animal models, and then launching a human clinical trial to evaluate safety and efficacy.

The project is highly collaborative, led by Pherecydes Pharma, and including BioFilm Control (Clermont-Ferrand, France) ([www.biofilmcontrol.com](http://www.biofilmcontrol.com)) (specializing in the rapid analysis of the effectiveness of anti-infective products on biofilms), Vivexia (Dijon, France) ([www.vivexia.fr](http://www.vivexia.fr)) (specializing in the development of animal models for anti-infectives), the Centre Hospitalier Intercommunal de Villeneuve-Saint-Georges (a pioneer in the field of phage therapy), and the Hospices Civils de Lyon (home to the national Reference Center for Staphylococci).

## Other Companies and Phage Centers

In addition to Table-I discussed above, the website: <http://companies.phage.org/> provides a list of commercial companies and centers that use phage in various capacities:

- 1. Companies and centers that facilitate patient phage therapy treatments:** Center for Phage Therapy (PL), Eliava Phage Therapy Center (GE), Globalyz Biotech (US), Novomed (GE), Phage Therapy Center (GE), Phage International (US).
- 2. Companies that emphasize pre-clinical phage therapy R&D:** AmpliPhi Biosciences (US), Enbiotix (US), Fixed Phage (UK), InnoPhage (PT), Intralytix (US), Novolytics (UK), Pherecydes Pharma (FR), Sarum Biosciences (UK), Synthetic Genomics (US), Technophage (PT).
- 3. Companies that emphasize R&D without replication-competent phages:** AvidBiotics (US), Enbiotix (US), Phico (UK).
- 4. Companies that are involved primarily in phage product distribution:** Biochimpharm (GE), Imbio (RU), Microgen (RU).
- 5. Companies that emphasize phage-mediated biocontrol:** APS Biocontrol (UK), Epibiome (US), InnoPhage (PT), Intralytix (US), Micros Food Safety (NL), Omnilytics (US), Phage Biotech (IL), Phagelux (CN), Technophage (PT).
- 6. Companies that market phage lysates:** Delmont (US).
- 7. Companies that use or develop enzybiotics:** GangaGen (US/IN), Lysando GmbH (DE), Micros Food Safety (NL), New Horizons Diagnostics (US).
- 8. Companies that emphasize phage-based bacterial detection technologies:** Sample6 Corp (US).
- 9. Companies that address issues of phage-associated industrial contamination:** Phage Consultants (PL).
- 10. Companies that emphasize phage in biotechnology products:** Versatile BioSciences (US).

Of the companies listed above from the phage.org website, those that best pertain to this project are those that facilitate human phage treatments: Center for Phage Therapy (PL), Eliava

Phage Therapy Center (GE), Globalyz Biotech (US), Novomed (GE), Phage Therapy Center (GE), Phage International (US). The Eliava Phage Therapy Center was discussed in detail in the section on phage history.

## Other Phage Studies

Recent phage studies have also been performed in China. For example, in 2015 a team of scientists at the School of Life Science and Biotechnology, Dalian University of Technology, (Dalian, China) performed an evaluation of the ability of phage therapy in mice to treat pneumonia induced by multi-drug resistant *Klebsiella pneumoniae* (Cao et al., 2015). They isolated and characterized a new lytic bacteriophage, phage 1513, from a clinical strain of multi-drug resistant *K. pneumoniae* (MRKP). During *in vitro* infection studies, the new phage produced a clear plaque with a halo, had a short latent period of 30 min, a burst size of 264 phage, and could inhibit bacterial growth in a dose-dependent manner. By electron microscopy, the new phage was classified as a *Siphoviridae*. In mice *in vivo*, intranasal administration of a single dose of  $2 \times 10^9$  PFU two hours post-infection with bacteria could protect against lethal pneumonia. With a sub-lethal challenge, phage-treated mice showed a lower level of bacterial burden in the lungs compared to untreated control. The treated mice lost less body weight and exhibited lower levels of inflammatory cytokines in their lungs.

Also in 2015, a multidisciplinary team of scientists headed by the Department of Electrical Engineering & Computer Science, and Department of Biological Engineering, Synthetic Biology Center, Massachusetts Institute of Technology (Cambridge, MA) developed a new synthetic biology strategy for modulating phage host target ranges (Ando et al., 2015). The technique engineers the phage genomes while based in yeast. The strategy was used to redirect *E. coli* phage to new targets including *Klebsiella* and *Yersinia* by swapping the *E. coli* phage tail genes with the tail genes of known phage against the two targets. The team showed using *in vitro* infection experiments that the synthetic phage efficiently killed their new target bacteria, and could efficiently and specifically remove bacteria from multi-species bacterial communities.

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## Section-6: Phage Regulations

*Xinyuan Wang*

The previous sections of the Lit Review described numerous experiments conducted on both animals and humans, where phage therapy has worked to reduce bacterial load, including bacteria resistant to antibiotics. And at least 3 different Phase-I clinical trials have shown that phage therapy can be applied safely to humans when using approved protocols. So, why is phage therapy not in wider use in the U.S. today?

Part of the answer lies in the fact, as discussed in previous sections, that much of the phage literature has appeared in Russian, Polish, or French literature with no English translation. And the early phage experiments performed in the U.S. were discontinued after the discovery of antibiotics that, at the time, appeared to be cure-all drugs. So, phage therapy has not been widely tested in the U.S. Especially lacking are large, controlled, blinded, clinical trials. And in the few controlled trials performed in the U.S. the data was not impressive.

Another reason that phage therapy is not more widely used in the U.S. has to do with the technique itself. Phage therapy is more expensive than antibiotics because the each therapy is personally tailored for a specific type of antibiotic-resistant strain infecting that particular patient. The Western paradigm of “one size fits all” does not apply to phage therapy, and it might take “leaps of time and technology to turn them into prescription drugs” (Wetmore, 2015). To get phage therapy approved in the US, a drug company might need to test not only each individual phage, but also each combination of phage cocktails to prove their safety and efficacy in clinical trials. The phase-II and III studies need hundreds of volunteers and patients, and take years to perform.

### Improving Phage Quality Control and Production Standards

In 2001, Roger Withington of the Centre for Applied Microbiology and Research, Porton Down (Wiltshire, UK) published a review of the regulatory issues for phage-based clinical products (Withington, 2001). His conclusion was that phage-based products are growing, and they are regulated by the U.S. FDA as “biologics, biologicals, or biotechnology products”. Although they are regulated in a manner similar to conventional drugs, the FDA has its own division for this class of biologics: The Center for Biologics Evaluation and Research (CBER) (drugs are evaluated by the Center for Drug Evaluation and Research, CDER). He concluded that there is no significant difference between CBER and CDER with respect to the amount of toxicological characterization, clinical testing, and manufacturing data required for submission of approval.

With respect to specific regulatory approval problems, Withington indicated that public acceptance of phage therapy could be a problem (Withington, 2001). The public is most worried about the possibility of genetic changes occurring to the host bacterium by integrating phage, but (as we discussed in the Safety Section) this worry could be eliminated by using only lytic phage which do not integrate, and agreeing to screen the phage batches to prove a lack of lysogenic

capacity. The production quality of various phage batches could also be a problem (each batch is currently grown individually for each patient or each lab), but this problem could be minimized by requiring phage banks that contain large stored supplies of pre-screened phage batches. As part of the quality control process, the FDA needs to require that the materials used to grow the phage batches (cells, culture media, supplements, etc.) be standardized and quality controlled. The phage batches need to be stored in a standard way, and characterized over time to show lack of genetic alteration, retained efficacy, and lack of contamination. With respect to biologic structure, the average phage structure is more complex than the average drug structure, so the FDA needs to decide which components are most important to characterize. With the advent of inexpensive DNA sequencing, requiring a full phage genomic sequence would help identify potentially toxic proteins in advance of their use. Clinical trials should be designed in double-blind mode with full placebo usage. The author concluded that with these regulations put in place before hand, subsequent approval of phage therapies would become easier ([Withington, 2001](#)).

In 2014, Ian Humphery-Smith of the Skolkovo Suslnnovations (Moscow) investigated the potential importance of using phage to combat hospital-acquired infections (HAI) ([Humphery-Smith, 2014](#)). His study concluded that phage have an important role in reducing HAIs, and should be combined with efforts to curb the overuse of antibiotics. But with respect to gaining broader approvals for phage treatments, he concluded that the processes used for phage production do not meet the same rigorous standards as used for drug products, so they need refining. Similar to the conclusions of Withington in 2001, Humphery-Smith concluded that the phage production batch-to-batch reproducibility must be improved, the molecular characterization and definition of the phage and target bacteria must be improved, and the storage conditions of each phage batch be standardized, before their clinical use can become widespread. He believes that the published historical data have demonstrated phage therapy safety in humans beyond any doubt.

In 2015, Ry Young and Jason Gill of the Center for Phage Technology at Texas A&M University (College Station, TX) published an article titled “Phage Therapy Redux: What is to be Done?” ([Young and Gill, 2015](#)). The authors discussed various phage therapy products in the pipeline, both clinically and commercially, and argue that phage therapy will receive increasing attention as antibiotic-resistant bacterial strains continue to become more prevalent. They state that building a through regulatory environment for phage therapies is important, including requiring that lysogenic phage be prohibited (because they can genetically integrate their DNA into the host chromosome to alter the properties of the host). And similar to the two previous studies cited, they argue that standardized phage collections and protocols should be required to improve quality control.

Also in 2015, scientists at the Centre of Biological Engineering, University of Minho (Braga, Portugal) published a study concluding that the success of phage therapy depends on developing an adequate regulatory framework, implementing safety protocols, and gaining acceptance by the public ([Nobrega et al., 2015](#)). The authors discuss the major hurdles of phage therapy, and provide some solutions for circumventing them. They especially focused on using genetically modified phage to help overcome some of the problems. Safety issues were discussed in our Lit Review Section-4, but briefly the authors indicate that lysogenic type phage can alter the properties of the host bacterium, the lysing bacteria can release endotoxins which

harm the patient, and some phage can induce strong immune responses. In section-4 we suggested ways of overcoming each of these hurdles. The authors suggest that using phage cocktails with a mixture of phage can reduce the chance of some of the target bacteria becoming resistant to a specific species of phage. They also like the idea of combining phage treatments with antibiotic treatments to improve acceptance and efficacy (Nobrega et al., 2015).

In 2016, Jyoti Madhusoodanan published an article titled “Viral Soldiers” which discussed some of the commercial companies developing phage therapies (Madhusoodanan, 2016). The author reminded us that even in cases where an antibiotic might kill the bacterial infection, sometimes it can’t be used due to side-effects to the patient. This fact, combined with the increasing occurrence of antibiotic-resistant bacteria, places an urgency on developing new bacteriocidal procedures. Phage could also be used to help the gut microbiome maintain homeostasis by selectively killing undesirable bacteria. But due to some lingering questions about phage, the author calls them “viral dark matter”. The author reminds us that phage were actually used for therapy prior to understanding what phage were, especially using bacterial lysates (containing phage) to treat cholera in India and streptococcal infections in France. Nowadays, more people are willing to accept phage treatments in view of increasing antibiotic-resistance, but carefully designed regulations need to be in place. The best methods for delivering the phage need to be established for each disorder, including oral, IV or IP injections. And the clinical trials need to be carefully designed with modern standards.

## **Ensuring the Quality of Phage Materials and Products**

In Roger Withington’s article (2001), he mentioned that phage products can be produced using materials from either animals or humans. These materials must be chosen wisely because the final phage used for therapy can be contaminated with cell products lysed from the cells used to grow the phage. The phage batches can also become contaminated with bacteria or pathogens introduced by the technician manufacturing the phage. Establishing standard protocols for producing the phage, and performing quality control tests, is a good way to monitor the material from the very beginning of production. Doing this will help gain acceptance of phage therapy by regulatory authorities.

Dr. Withington also indicates that the components of a final phage mixture are very complex, which is a different situation than commonly used medicines. The final product is a mixture of live phage plus cell metabolites, cell debris, culture media ingredients, and the reagents used in the extraction and purification processes. This complexity causes problems when characterizing the product. This problem can be minimized by using advanced techniques for increasing phage titers with little carry-over of lysed cell products. Adoption of state-of-the-art phage purification methods should help gain approval by regulatory authorities.

## **Improving Clinical Trial Designs**

Some scientists think that instead of importing or interpreting the data from Eastern European and Russian phage trials, the U.S. should focus on trying to incorporate phage therapy into current antibiotic treatment protocols for faster U.S. approval. MIT's Professor Lu stated, “Because antibiotics are so entrenched here, phages need to be part of the arsenal, accompanying

the drugs and enhancing their effects rather than replacing the meds altogether. In the short term, that will plug more easily into the current way people practice medicine (Wetmore, 2015)."

In addition to using phage therapy protocols that complement current antibiotic treatment protocols, some scientists argue that using "compassionate use" trials is a fast way to obtain human phage therapy data. Compassionate use protocols could be approved at specific U.S. hospitals for patients near death whose infections have not responded to previous treatments. The patient would be provided information upon which to provide his/her informed consent, and then phage therapy administered in an attempt to save the patient's life. Virologist Ryland Young, Director of the Center for Phage Technology at Texas A&M University stated, "Someone who is about to die of a MRSA infection could be given an injection with a phage cocktail that could be a lifesaver. It is within the power of the medical community to do this" (Wetmore, 2015).

The clinical trials should also be designed to provide key information that is lacking in the U.S. literature. For example, most of what we currently know about phage in the U.S. comes from well understood lab model systems such as *E. coli* and its phage, while different phage genera would be used to treat Staph or *Pseudomonas* infections. We also need more information on how phage interact with the human body during treatments, including increasing our understanding of which parts of the immune system become activated by the phage, whether human immune responses lower phage effectiveness, and whether some phage induce cytokine storm responses that increase the likelihood of patient death.

## **Increasing the Number of Phage Development Programs**

One way to stimulate phage therapy in the U.S. is to support it with special programs. For example, the National Institute of Allergy and Infectious Diseases (NIAID) in its 2013 strategic plan (NIAID, 2013) listed drug-resistant microbes as one of their top priorities for funding. The US Army has initiated a large program to develop phage cocktails to fight one of our deadliest bacteria, *Staphylococcus aureus*, and hopes to expand to other deadly infections caused by pathogens such as *E. coli* and *Pseudomonas aeruginosa*.

## **Increasing Phage Patents Using Engineered Phage**

Because naturally occurring phage cannot be patented, some biotech companies are reluctant to get into the phage therapy business. In this case, perhaps academic institutions and medical centers should lead the way.

Alternatively, some scientists recommend working with engineered phage that are altered from their natural counterparts. Altered phage might be patented as a "patent of composition" specifically because they are new. The engineered phage preparations would be more uniform than the undefined phage mixtures isolated from sewerage that were selected on the basis of their ability to lyse bacteria, not on their specificity. And engineered phage could be given properties superior to natural phage, such as higher binding specificity.



## Section-6 Conclusion

Specific recommendations for improving the likelihood of gaining phage approvals, includes: 1) using DNA sequencing to fully sequence the genome of each phage to search for potential harmful proteins, 2) fully characterizing the specificity of each phage relative to other non-target bacterial species, 3) establishing sets of pre-standardized phage batches for rapidly treating specific cases of antibiotic-resistant infections, 4) stop the use of phage mixtures without a prescription, 5) creating special hospital wards or rooms that specialize in performing phage therapy treatments (for controlling phage contamination and spread, and for controlling spread of the antibiotic-resistant bacteria), 6) monitoring for the spread of phage outside these approved application areas, 7) increasing the funding of phage therapy experiments designed to thoroughly test safety, 8) allowing the use of “compassionate use” clinical treatments, as a means for increasing our data on phage usage and efficacy, and 9) establishing international cooperation for exchanging characterized phage mixtures and standardized protocols.

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# METHODS

**To accomplish objective-1**, we performed a review of the current literature, including reputable academic journal articles, relevant books, scholarly websites, and other pertinent materials.

**To accomplish objective-2**, we conducted a set of interviews with various academic and medical researchers, bio-ethicists and legal experts to determine their full range of opinions on the strengths and weaknesses of phage therapy technology, and to determine which obstacles remain for its further expansion in the U.S.

**Who:** The stakeholders included individuals performing phage therapy, bioethics experts, and legal experts. Some of the stakeholders initially were identified by referral from the project advisor, Dr. David Adams, but other interviewees were identified from the literature as authors on key scientific papers, or by referral from the initial interviewees.

**Where and When:** Whenever possible, interviews were conducted in person, but the majority were performed by email, phone, or Skype.

**How:** We developed our interview questions based on our background research. A preliminary set of questions is shown in the Appendix. Based on our background search of each interviewee, we designed a pertinent initial question. Any subsequent questions were based on their response to the initial question. The appendix shows the topics covered in our interviews.

With respect to the method of the interview, after establishing contact with an interviewee, we informed the interviewee about the purpose of our project, and asked for permission to quote them (see interview preamble in the Appendix). If the need arose for confidentiality, we protected it by either not quoting them directly, or by giving them the right to review any quotations used in the final published report, explaining that the interview is voluntary, and explaining that they may stop the interview at any time or refuse to answer any question. At the end of the interview, we sometimes asked the interviewee to recommend other potential stakeholders we might interview, to further increase the number of interviews with key individuals.

With respect to the total number of interviews performed for our project, we discontinued our interviews once we had obtained sufficient information to represent all sides of the phage therapy problem, and when the unclear points had been clarified.

**To accomplish objectives-3 and 4**, the IQP team synthesized all of the information collected in our literature research, interviews, and follow-up interviews to ascertain the strength of the evidence for and against phage therapy, and created recommendations for further research.

# RESULTS / FINDINGS

## Problems with Antibiotic-Resistant Bacteria, and Early Phage Treatments

Mingxin Yu

This area of the IQP focused on introducing the reader to antibiotics and the problems encountered when bacteria become resistant to antibiotics. It also introduced the reader to the history of phage therapy treatments. Our review of the literature in this area identified several specific “superbugs” that represent serious health risks in the United States, identified several labs performing phage therapy against antibiotic-resistant bacteria, and identified some problems encountered during the treatments. To investigate these issues further, interviews were performed with several experts on antibiotic resistance and on phage therapy.

The first interview was conducted with **Dr. Julian Davies**, Professor of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, Vancouver, Canada. Dr. Davies was corresponding author on a 2010 paper published in the journal of *Microbiology and Molecular Biology Reviews* (74: 417-433) entitled “Origins and Evolution of Antibiotic Resistance”. One of the sections in the article is on superbugs and super-resistance. When asked his opinion of which “superbugs” are of highest concern in the U.S., and why, he pointed us towards the list of so-called “ESKAPE” pathogens. The ESKAPE name is derived from six serious pathogens showing increasing multi-drug resistance: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter*, *Pseudomonas aeruginosa* and *Enterobacter*. This group of bacteria was initially identified by the Infectious Diseases Society of America as most important in the U.S. among those bacteria that can escape the biocidal action of antibiotics. The ESKAPE pathogens have resulted in new paradigms in pathogenesis, transmission, and resistance (Pendleton JN, Gorman SP, Gilmore BF, Clinical relevance of the ESKAPE pathogens. *Expert Review of Anti-Infective Therapy*, 2013 Mar; 11(3): 297-308).

The next interview was performed with **Dr. Gerard D. Wright** of the Michael G. DeGroote Institute for Infectious Disease Research, McMaster University, Ontario, Canada. Dr. Wright was corresponding author on a 2011 paper in *Nature* (477: 457-461), entitled “Antibiotic Resistance is Ancient”. This paper interestingly concluded that antibiotic resistance in bacteria evolved prior to the isolation and use of antibiotics by humans. When asked to describe his working hypothesis on this conclusion, he stated that “all the evidence supports the evolution of resistance long before the human use of antibiotics”, and that “antibiotic-resistance genes may have aided microbial survival against chemical environmental challenges prior to our isolating and using antibiotics”. So, antibiotic resistance genes have been benefitting bacteria far longer than our human use of antibiotics, likely to aid bacterial survival against environmental chemicals.

Our search of the literature showed that phage therapy is not restricted for use in patients. Some scientists are currently investigating the use of phage to control bacterial contaminants in aquaculture conditions. To provide information on this type of phage application, we interviewed **Dr. Jiancheng Zhang** of the Dalian University of Technology, Dalian, China. Dr. Zhang was a corresponding author on 3 different papers using phage for aquaculture treatments.

When asked about his initial bacterial targets, he stated: “The initial target is to solve the problem caused by *Vibriosis* infection in aquaculture. And more specifically, trying to use mixed phage samples to replace the use of traditional antibiotics in the modern aquaculture business”. The use of antibiotics in aquaculture is expensive, and its use in aquaculture further increases the presence of antibiotic-resistant bacteria, so hopefully this phage treatment technique can be further developed.

Our search of the literature identified several commercial companies developing phage therapies for a variety of purposes. For example, Phagelux, Inc. is a privately held company based in Shanghai, with laboratory locations in Nanjing, China and Montreal, Canada. The company is advanced and has phage production facilities in Suzhou, China, and in Montreal ([www.phagelux.com](http://www.phagelux.com)). Phagelux develops, manufactures, and markets phage products for a variety of fields, including agriculture, food safety, animal health, and topical and inhaled treatments to treat various human diseases. The company offers its products directly in China, and also sells them internationally through partners. We reasoned that employees of this large well-established phage company might have much experience with potential problems of phage treatments, so we interviewed **Dr. Wenbin Fei** of the Phagelux branch in Nanjing, China. When asked to define his role in the company and potential problems, he stated:

“I am a microbiologist whose job focuses on phage screening and purification, assaying lytic activity against antibiotic-resistant bacteria, and expressing useful phage components. With respect to potential problems with phage treatments, we focus on four main areas: 1) Bacteriophage particles are covered by proteins. When a phage enters the human body, it sometimes causes an immune response which can weaken the therapeutic virus or can harm the patient. 2) During the phage production process, we use pathogenic bacteria as a host to grow the phage. As the host become pyrolyzed (lysed by heat treatment), this can release toxic material like pyrogens and lipopolysaccharides (LPS) into the phage sample which can harm the patient. So, one key problem is to make sure those materials have been removed during the phage production. 3) When amplifying progeny phage in the production system, there is a chance that the new phage offspring will add some pathogenic bacterial gene into the phage genome. If the added gene happens to be part of the bacterium’s drug resistance, then these phage offspring could carry the genes into other bacteria creating super-resistant bacteria. 4) It is still unclear whether some patients are naturally allergic to phage. We need more studies done on this”.

Our IQP team encountered each of the four problems mentioned above by Dr. Fei in our search of the literature, and developed a response to each. With respect to his points 1 and 4, it should be relatively easy to make sure the patient is not hyper-sensitive to the phage species about to be used in therapy by testing a very small quantity of it in the patient prior to large-scale injection. If a patient is hyper-sensitive to one species, a different phage species capable of lysing the same bacterium could be used for therapy. With respect to point-2, modern phage purification protocols help reduce the presence of pyrogens and LPS in the phage samples, so such protocols should become standardized in the industry. With respect to point-3, requiring the use of phage that are not lysogenic (do not incorporate into the bacterial genome) should help minimize this problem.

The next interview was with **Dr. Vaibhav Rastogi** who is an Assistant Professor in the Department of Pharmacy, IFTM University, Moradabad, India. Dr. Rastogi was first author on a recent 2016 article published in *Current Drug Delivery*, 2016 Apr 6. [Epub ahead of print], titled “An Overview on Bacteriophages: A Natural Nanostructured Antibacterial Agent”. The paper reviewed the general biology of bacteriophages, the topic of bacterial receptors which are necessary for the recognition and adsorption of the phage, the pharmacokinetics and therapeutic potential of various modes of phage administration, and problems associated with bacteriophage therapy. The authors concluded that the parenteral route (IM, IV, SC) was found to be the most efficient route for treating systemic infections, oral delivery was most efficient for gastrointestinal infections, and local delivery (skin, nasal, ears) was best for topical infections. When asked his opinion on which disease he believes phage therapy will work best for, and is he aware of any side-effects, he replied:

“I am very glad to know that a paper like this is getting attention from the engineering field too! With respect to the question you asked, phage are ubiquitous in nature and they are very specific to their host bacteria. Hence, for variety of diseases caused by bacteria, a variety of phage will need to be identified, isolated, and characterized. With respect to side-effects, few phage have been found to elicit inflammatory responses as monitored by the formation of inflammatory markers such as interleukins and cytokines, etc. These side-effects need to be carefully monitored for each patient”.

So, Dr. Rastogi pointed out that due to their high specificity, phage will need to be isolated for each strain of bacteria causing problems in patients. He also believes that few phage have been shown to induce problems in patients, but agrees this needs to be carefully monitored.

The next interview was with **Dr. Paul Orndorff** of the Department of Population Health and Pathobiology at the College of Veterinary Medicine, North Carolina State University, Raleigh, NC. Dr. Paul Orndorff was sole (and corresponding) author on 2016 a paper in *Current Genetics* (April 25 online), titled: “Use of bacteriophage to target bacterial surface structures required for virulence: a systematic search for antibiotic alternatives.” When asked his opinion of the biggest challenge for phage therapy, and how it could be made more effective, he responded:

“Many thanks for your note and kind comments on our recent publication. With respect to the biggest challenge [facing phage therapy], it would be a lack of insight as to situations where phage therapy would be the best (or a viable) alternative to antibiotics. Keep in mind that phage therapy is pathogen-specific. This aspect alone makes its application situational. Consequently, it presciently can't replace antibiotics--which are most useful when you don't know what the pathogen is and need to get rid of everything. In most respects, the routine application of phage therapy (or other pathogen-specific therapies) look toward the day when pathogen identification will not be the rate-limiting step in making a diagnosis”.

So, Dr. Orndorff stated that he thinks the biggest challenge to moving forward with phage therapy is identifying the situations in which phage therapy would be a viable alternative to using antibiotics. He points out the important fact that if the bacterial infection is uncharacterized (species not known yet), we will first need to use broadly acting antibiotics to knock down the

uncharacterized bacterium if possible. If the infection is found to be resistant to antibiotics, we then need to determine the bacterial species to be able to determine exactly which phage will kill it. He believes that the rate-limiting step currently is a lack of rapid identification of the pathogen.

## **Results: Phage-Related Therapies: Lysins and Tailocins, and Phage Safety**

*Benjamin Cossette*

Some scientists are currently developing therapies against antibiotic-resistant bacteria that do not use replication-competent phage, but instead use phage components. For example, some scientists are developing techniques using phage tail-like structures (tailocins) that attach to and penetrate the bacterial membrane to depolarize and kill the cell. Other scientists are using enzymes that degrade the bacterial cell wall (lysins). From our review of the literature, we found this area to be less researched than phage therapy, and it was not clear whether their treatments work as well as using phage themselves (the latter can amplify inside the patient).

To provide more information on this interesting topic, we interviewed **Dr. Maarten Ghequire** of the Centre of Microbial and Plant Genetics, Department Microbial and Molecular Systems, KU Leuven, Belgium. Dr. Ghequire was corresponding author on a 2015 paper in *Trends in Microbiology* (23: 587-590) entitled “The Tailocin Tale: Peeling off Phage Tails”. When asked whether he agreed with our finding of relatively few human studies in this area, and why, he responded:

“Interesting question! Indeed, at this moment, most efforts are on tailocins with modified "feet" (to alter the target spectrum) and are directed towards the design of these particles, but not really towards their therapeutic use. They have been tested successfully (mainly/solely) in (a few) murine models. So, I definitely agree with what you say [about the lack of human data].

There are also some pitfalls and drawbacks on the use of these tailocins, which may slow down the enthusiasm for their use: 1) they may be too specific. They only target a very narrow set of bacterial strains. However, diagnostics are advancing very fast, and once you know what pathogen is infecting the patient, you can use the right tailocin to kill it. Alternatively, you could use a cocktail of tailocins with different specificities to help ensure you kill the pathogen of interest. But pharmaceutical companies are not fully ready yet for the development of such patient-specific drugs. 2) The cost of production and purification is difficult to estimate (though this usually is no immediate scientific concern). 3) There is a risk of an immune response against the phage, which can lead to shock. So, these phage particles should never be injected in the bloodstream or other tissues (which limits the options). They may be used by spray applications, for example, to treat burn wounds and lung infections, or to treat gut infections (although one needs to ensure that these tailocins are not broken down in the stomach, so they may need to be encapsulated). 4) Public opinion: A lot of effort has already been paid towards the design of phage (cocktails), which contain genetic information. It is very difficult to convince the crowd that "bacterial viruses" are harmless and pose no risk. Tailocins on the contrary do not contain DNA (so you are treating patients with a non-living drug), but are

indirectly related to phages. The tailocin research is still "young", and it may be one step too far for most people to accept tailocins; they may first need to be convinced of the therapeutic potential of phage themselves, I think. 5) I may also add one other reason [tailocins are not well developed]: the necessity to start using these compounds is not high enough yet. Multi-drug resistance is (becoming) an issue indeed, but, pharmaceutical companies will postpone clinical trials with new compounds as long as possible. These tests are extremely expensive and it is not always worth the effort if they can still make enough money with the current antibiotics”.

Thus, Dr. Ghequire provided 5 different reasons that tailocins are not as developed as phage for therapies. His point-1 should take care of itself if tests are used to identify the exact bacterial species infecting the patient, which would allow a tailocin or phage treatment specific for that species to be used. For point-2, the current cost of phage production might be high, but the cost should come down as the technology is more widely used, including the production of large batches of standardized phage. For point-3, we addressed the potential for a strong immune response previously, which could be minimized by pre-testing the patient against the phage to be used. For point-4, perhaps the public needs to learn more about phage before accepting the use of their components for therapy, but both should be developed. For point-5, our section-1 of the Lit Review addressed the seriousness of the antibiotic-resistance problem, including the call to action by President Obama, and this problem will only grow worse.

The next interview was with **Dr. Gregory R. Govoni** of AvidBiotics Corp., South San Francisco, CA, USA. Dr. Govoni was a corresponding author on a 2015 paper in *MBio* (6: e02368-14) entitled “A Modified R-Type Bacteriocin Specifically Targeting *Clostridium difficile* Prevents Colonization of Mice without Affecting Gut Microbiota Diversity”. Their team genetically modified a contractile R-type bacteriocin (Diffocin) isolated from *Clostridium difficile*, the leading cause of hospital acquired infections worldwide. They replaced the natural receptor-binding domain of the *C. difficile* tailocin with a newly discovered binding domain from a phage that targets the most important clinical strains of *C. difficile* (BI, NAP1, and 027). The resulting modified tailocins (Av-CD291.1 and Av-CD291.2) were stable, and killed all 16 strains of *C. difficile* tested *in vitro*. Importantly, when tailocin Av-CD291.2 was administered to mice in their drinking water, the tailocin survived passage through the GI tract, did not appear to alter the gut microbiota, and prevented *C. difficile* infection. When asked his opinion whether tailocins (such as the modified Av-CD291.2) will provide benefits over other potential treatment options, such as whole phage therapy or phage-encoded lysin therapy, and if so what would be the benefits, he replied:

“YES! Tailocins are better than phage because: 1) there is no DNA. The tailocins can’t mutate like bacteriophages. Agents that undergo genetic changes after administration may have many unintended side effects. Tailocins can’t transfer resistance/virulence genes between hosts like a bacteriophage. And not containing DNA, tailocins avoid many mechanisms that bacteria use to evade bacteriophage foreign DNA (such as the Crispr-Cas and other restriction modification systems). As a result of the bacterial restriction modification systems, phage can bind to, but cannot infect many bacterial cells. Tailocin killing is not restricted by these restriction mechanisms. So, as a result, the tailocin killing spectra are wider and more consistent than the corresponding host range for the complete bacteriophage. 2) Tailocin production can be done in food-grade bacterial hosts (e.g. *E. coli* or *B. subtilis*). Many phage therapies are specific to pathogenic bacteria [so

they must be produced inside cultured pathogens], making production difficult w/o genetic manipulation of the pathogenic production strain”.

So, Dr. Govoni provided very useful information on the rationale for using tailocins instead of whole phage for therapies. The fact that tailocins contain no DNA provides multiple scientific advantages, and as we learned from our Lit Review, may also help gain public acceptance.

Another therapy being researched are phage endolysins. These are enzymes that help dissolve the bacterial cell wall. Our review of the literature identified numerous pre-clinical (animal) studies with endolysins, but no human clinical trials. We also identified several endolysin studies on gram-positive bacteria (they were effective at degrading the single cell wall), but we identified few studies on gram-negative bacteria (organisms that contain two cell walls, and the lysins were unable to penetrate the outer LPS layer). To obtain more information on endolysins, we interviewed **Prof. Martin J. Loessner**, ETH Zurich, Institute of Food, Nutrition and Health, Zürich, Switzerland. Dr. Loessner was corresponding author on a 2005 review article published in *Current Opinions in Microbiology* (Aug; 8(4): 480-487) titled “Bacteriophage endolysins — current state of research and applications”. We asked Dr. Loessner whether we are correct that no endolysin clinical trials have been completed, and also asked whether there are advances to treat gram-negative bacteria. He responded:

“Yes to both questions! Endolysins are currently in clinical trials (by commercial partners of us and others, see websites of Microcos and Contrafect), and there have also been attempts to modify the enzymes to be able to cross membranes (see Artilysin by Lysando)”.

Our IQP team was familiar with the ongoing clinical trial of ContraFect, where their lead drug CF-301 is an endolysin that has completed phase-I testing (Lit Review, Section-5), but the efficacy phase-II trial data is not published yet. So, although endolysins appears to be a promising approach for lysing bacteria, perhaps no clinical trials have been completed yet simply because they have not been researched as long as phage.

The next interview was with **Dr. Victor N. Krylov**, MD, Professor and Head, Laboratory for Genetics of Bacteriophages, Mechnikov Research Institute for Vaccines & Sera, RAMS, Moscow, Russia. Dr. Krylov was corresponding author on a 2015 paper published in *Virologica Sinica* (30: 33-44) entitled “Selection of Phages and Conditions for the Safe Phage Therapy against *Pseudomonas aeruginosa* infections”. Their team optimized conditions for the safe, long-term use of phage to treat *Pseudomonas aeruginosa* infections by selecting the most suitable phage against a variety of *Pseudomonas* targets. They optimized the most effective phage combinations, and developed a test to rapidly recognize which phage are unsuitable for therapy. They also helped outline the conditions required for the safe use of phage in hospitals, and the development of personalized phage therapy. We recognized that personalized phage therapy (to design and produce a phage cocktail specific for an individual’s infection) might be time consuming, so we asked Dr. Krylov how long it takes to create an optimized phage mixture. He responded:

“Yes, it is extremely important to find the right phage to use in a short time. In 1991, our lab in collaboration with the Department of Mucoviscidosis in the Republican Children’s



Hospital in Moscow, prepared phage mixtures for 5 children, 7-12 years old (with compulsory permission from their parents). The mixtures were composed of several phages of different species active on *Pseudomonas aeruginosa* strains isolated from the sputum of the children. The phages were carefully purified and concentrated. The preparations were then given through inhalation using nebulizers. We found evidence of phage resistance in two cases. In one case, it was a phage-resistant mutant of the initial strain. In the other child it was a new bacterial strain resistant to our phage (possibly a cross infection from other children). So, we showed that the new active phage preparation must be prepared and applied after a short time interval to prevent the growth of phage-resistant bacterial variants. Moreover, the new phages must be safe. For instance, transposable phages can convert *P. aeruginosa* strains to a highly pathogenic and epidemic condition”.

Thus, Dr. Krylov pointed out the importance of rapidly preparing the custom phage mixtures when treating patients to prevent the natural formation of phage-resistant strains in the patient. Their team chose to custom tailor their phage from sputum isolated from the children, instead of attempting to use a pre-characterized phage sample active against *P. aeruginosa*, perhaps because tailored isolates are more effective. But this takes time, which can allow resistance to evolve in the patient.

The next interview was with **Dr. Graham F. Hatfull** of the Department of Biological Sciences and the Pittsburgh Bacteriophage Institute, University of Pittsburgh, Pittsburgh, PA. Dr. Hatfull was sole author on a 2008 review article published in *Current Opinion in Microbiology* (11: 447-453), entitled “Bacteriophage Genomics”. One of the conclusions of the article was that phage genomes contain a very high proportion of novel genetic sequences of unknown function. The presence of unknown genetic sequences could hinder the approval and use of such phage in therapy. When asked whether it is necessary to characterize every gene before a phage can be used in therapy, he replied:

“This is a tricky question. It is easy to argue that you need to know the sequence of the phage that might be used for therapy, because it is not only simple to do, but is useful for monitoring the phages that persist in the therapy and whether they are actually the same as the input phage or not. But I am not sure that it follows that you need to know the function of every gene product in order to use a biological in this way. Live viral vaccines have been used for decades without knowing what most or any of the gene products do. You could also argue that no one can ever confidently know what all the genes do, as genes can have multiple functions, and there are likely to be RNA products with functions that are still as yet unknown. You would certainly want to know if any toxin or toxic genes are predicted to be present, and it of course helps to have a thorough understanding of the phage including the functions of many of the gene products. But I doubt that any regulatory agency would demand that you need to know the functions of all of the gene products”.

Thus, Dr. Hatfull believes it is not necessary (or even achievable) to fully characterize the function of every viral gene prior to use in therapy, and indeed we have for a very long time used viruses as vaccines without knowing the functions of all their genes. So, full gene characterization does not appear to be required.

The next interview was with **Dr. Stephen T. Abedon** of the Department of Orthopedics, University of Utah, Salt Lake City, UT. Dr. Abedon was author of a commentary article published in the journal *Bacteriophage* (2011 Mar; 1(2): 111-114) titled “Pros and cons of phage therapy”. In the article, he noted that some scientists believe the public may be hesitant to accept phage therapy because it involves the introduction of live, replication-competent virus into the patient. When asked if he had to make a prediction, whether he thinks phage therapy will be able to overcome this obstacle, or will be displaced by more easily accepted therapeutic approaches such as endolysin therapy, he responded “My sense is that this is not a huge problem. We do, after all, put maggots on wounds. Still, it might in some instances be problematic”. So, Dr. Abedon himself does not believe we will require the use of endolysins to overcome the public’s worries about using replication-competent phage for therapy, although he recognizes using the latter can be problematic in the public’s eye.

## **Results: Status of Current Commercial Therapies and Clinical Trials, and Phage Regulations**

*Xinyuan Wang*

In this section, we identified several commercial companies performing various aspects of phage therapy. We also identified some problems associated with phage regulations. To obtain more information on these topics, we interviewed **Dr. Timothy Lu** of the Synthetic Biology Center, MIT, Cambridge, MA. Dr. Lu was corresponding author on a 2015 paper in *Cell Systems* (1: 187-196) entitled “Engineering Modular Viral Scaffolds for Targeted Bacterial Population Editing”. The paper described their lab’s development of a new synthetic biology approach to alter phage host ranges by engineering phage genomes in yeast. The approach was shown by the authors to produce phage with highly specific targeting to specific bacteria in a complex mixture of bacteria. This approach appears to be promising for preparing phage with altered host ranges using a high through-put method. When asked whether the approach could, in theory, be applied to any DNA-containing phage, he responded: “In theory yes, but the molecular details matter”. So, in general he agreed that his synthetic biology approach (altering phage genomes inside yeast) could likely be applied to any DNA phage (not RNA phage), but that due to molecular variances, some phage would respond better than others.

The next interview was with **Dr. Michael Wittekind**, PhD, Senior Vice President of Research and CSO, ContraFect Corporation, Yonkers, NY. Dr. Wittekind was corresponding author on a 2014 paper in the *Journal of Infectious Diseases* (209: 1469-1478), entitled “Combination Therapy With Lysin CD-301 and Antibiotic is Superior to Antibiotic Alone for Treating Methicillin-Resistant Staphylococcus aureus-Induced Murine Bacteremia”. Lysins are bacteriophage-derived enzymes that degrade peptidoglycans in the bacterial cell wall, and are capable of killing the bacterial cell. Lysin CF-301 (licensed by ContraFect from Rockefeller University) is the company’s lead product that was shown to be safe in Phase-I testing (company press release). It has potent, specific, and rapid bacteriolytic effects against *S. aureus*, has a low bacterial resistance profile, eradicates biofilms, and synergizes with antibiotics. In this study, CF-301 was bacteriolytic against 250 different strains of *S. aureus*, including 120 clinically important MRSA isolates. In time-kill experiments with 62 strains, CF-301 reduced *S. aureus* by

1000-fold within 30 minutes, compared to 6-12 hours for antibiotics. In mouse studies, CF-301 increased survival by reducing blood MRSA 100-fold within 1 hour. Importantly, combining CF-301 with antibiotics Vancomycin or Daptomycin increased survival significantly relative to the antibiotics alone ( $p = 0.0001$ ). The company chose to develop the lysin therapy coupled with antibiotic therapy. When asked whether the combined approach is more likely to gain a broader approval in the U.S. (where antibiotics are already widely used and approved) than using lysin therapy alone, he stated:

“Thanks for your interest in our work. We use the lysin in combination with antibiotics primarily because the synergy between the lysin and antibiotics is so strong. We see this synergy clearly in *in vitro* experiments, and when we test the lysin/antibiotic therapy *in vivo* the efficacy of the combinations are better than those of the single agents. So the increased efficacy is the main reason for using the combinations with antibiotics. This approach also has advantages from the clinical trial design perspective”.

So, Dr. Wittekind believes that combining lysin therapy with existing antibiotic therapy produces a synergizing effect that is superior to either treatment alone, and the combined approach also facilitates approval of the clinical trial.

The next interview was with **Dr. Colin Hill** of the School of Microbiology, University College, Cork, Ireland. Dr. Hill was corresponding author on a recent 2016 article in *PLoS One*, 2016 Jun 9; 11(6): e0156773, titled “Three New *Escherichia coli* Phages from the Human Gut Show Promising Potential for Phage Therapy”. In this article, the authors isolated and characterized three new coliphage species (that lyse coliform bacteria) from human fecal samples:  $\phi$ APCEc01,  $\phi$ APCEc02 and  $\phi$ APCEc03. *In vitro*, all three of the newly discovered phage reduced the growth of *E. coli* strain DPC6051 when used at a MOI between  $10^3$  and  $10^5$ . A cocktail of all three types of phage completely inhibited *E. coli* growth, reduced biofilm formation, and prevented the emergence of phage-resistant mutants, the latter which sometimes occurred when using single phage. Similar results were obtained when combining the phage with the antibiotic Ciprofloxacin. The authors concluded that phage therapy might work well against *E. coli* infections. When asked his opinion about the next steps for moving the phage field forward, he stated: “I think everything is in place, except for the regulatory approval”. So, Dr. Hill thinks that the science is already in place to move phage therapy forward, and we just need the FDA to approve it.

The next interview was with **Dr. Gopal Nath** of the Department of Microbiology, Institute of Medical Sciences, Banaras Hindu University, Uttar Pradesh, India. Dr. Nath was corresponding author on a recent 2016 article published in the *Indian Journal of Medical Research* (Jan; 143(1): 87-94) titled “Phage therapy of staphylococcal chronic osteomyelitis in an experimental animal model”. Methicillin-resistant *Staphylococcus aureus* bacteria (MRSA) are the most common cause of osteomyelitis. The authors investigated phage therapy in a rabbit model of MRSA osteomyelitis. 22 rabbits were used in the study, of which 2 were used to test safety, group-A (n=4) was control without therapy, group-B (n=4) was phage treatment at 6-weeks post-infection, and group-C (n=12) was phage therapy at 3-weeks post-infection. The treatments consisted of four phage doses using a mixture of 7 types of phage. Their results showed that both of the phage-treated groups recovered from the Staph illness, and showed improved appetite, activity, and reduced edema, erythema and induration. X-ray analysis showed no infection in newly forming bone tissue. The authors concluded that phage therapy shows the

potential for treating difficult infections caused by multi-drug resistant bacteria. When asked his opinion of the next steps for moving the phage therapy field forward, he stated: “Thank you for your interest! It is now pertinent to decide the exact phage dosage and duration of therapy”. So, Dr. Nath thinks that we need more scientific studies to determine optimum phage dosage and timing to help move the field forward. This response contrasts with Dr. Hill discussed above who thought that the field was ready for FDA approval.

The last interview was with **Dr. Joana Azeredo** of the Centre of Biological Engineering, University of Minho, Braga, Portugal. Dr. Azeredo was corresponding author on a 2015 paper in *Trends in Microbiology* (23: 185-191) entitled “Revisiting Phage Therapy: New Applications for Old Resources”. This paper reviewed the major hurdles of phage therapy, and described solutions to circumvent them. The authors paid special attention to genetic modification of phage to overcome the remaining problems. When asked her opinion of the most serious hurdle facing phage therapy, public acceptance or technology, she replied: “Public concern is the smallest problem. There are safety and efficacy issues that need to be clarified. Safety is mostly related to the fact that we cannot be assured that 100% of the phage genome does not encode toxins, and the fact that phages are non-stable agents and can suffer mutations very easily. Additionally, there is the concern of bacterial phage-resistance. We have to obtain a deep understanding about phage-host interactions in order to identify and fully disclose safety concerns”. So, Dr. Azeredo believes that we have several key scientific problems remaining to address phage safety, including identifying potential phage toxins and preventing phage mutations, before the field can move forward.

## CONCLUSIONS / RECOMMENDATIONS

Based on the research performed for this project, our team has made several conclusions and recommendations.

### Clinical Trial Designs

With respect to human phage therapy trials, we conclude that relatively few trials have been performed in the U.S., and worldwide the trials have not been well controlled. Much of the early phage literature appeared in Russian, Polish, and French literature, with few English translations. And the early phage experiments performed in the U.S. were discontinued after the discovery of antibiotics, which at the time appeared to be cure-all drugs. Phage therapy is also more expensive than antibiotics because each therapy is personally tailored for a specific type of antibiotic-resistant strain infecting that particular patient. For most of the published data, the phage therapy generally worked, but in most cases the studies were small, not blinded, not placebo controlled, and many of the early cases were not presented in detail. Most of the trials were performed on patients that had not previously responded to antibiotics, so any hint of an improvement was concluded by the authors as a success. And for the few controlled trials that were performed in the U.S., the data were not impressive. We conclude that more rigorous studies on larger patient samples are needed to help move the field forward.

With respect to moving forward with new phage therapy clinical trials, we conclude that we need to improve the design and number of trials. Once the safety phase has been concluded on a small number of patients, the patient size needs to be expanded. The trials need to be well controlled, blinded, and strong attention paid to any observed side-effects. Since antibiotics are well researched and used in the U.S., we agree with the approach being used by some biotech companies to design the clinical trials with a combination treatment of both antibiotic and phage therapy to facilitate FDA approval (Nobrega et al., 2015). Some patient groups would receive placebo, some antibiotic, some phage therapy, and some both. Combining phage therapy with antibiotics will “plug more easily into the current way we in the U.S. practice medicine” (Wetmore, 2015). In addition, allowing “compassionate use” trials would be a fast way to gain near term approval, and to obtain more clinical data. The clinical trials should also be designed to provide key information currently lacking in the U.S. literature, such as testing phage against non-laboratory strains of bacteria, increasing our understanding of patient immune system activation by the phage, and determining whether some phage samples induce cytokine storm responses that increase the likelihood of patient death. And we agree with an important point brought up by one of our interviewees that once a patient has been screened to determine exactly which strain of bacteria they are infected with and a matching phage sample has been chosen, the therapy should proceed quickly to minimize the formation of phage-resistant bacteria. Using phage cocktails instead of individual phage would also minimize the chance of the patient becoming resistant to the phage treatment.

## Phage Safety

With respect to phage safety, we identified several potential issues but believe they are controllable. We recommend the following: 1) Using only lytic phage, not lysogenic (temperate) phage. Lytic phage are more efficient at killing the bacteria, and do not integrate their DNA into the bacterial or patient DNAs. These phage would not move genes between host cells, so would not themselves transmit antibiotic-resistance genes. 2) Switching to tailocin proteins if toxic proteins are released by a particular strain of bacteria. Tailocins attach to and depolarize the bacterial cell membrane, but do not lyse the cell. The depolarized cell is then phagocytosed by the patient's immune system. 3) Using state-of-the-art phage purification protocols to minimize contamination of phage stocks with toxic proteins lysed from bacteria used to grow the phage. Because phage infections are specific to bacteria that contain the right receptors (usually one specific species), that species must be used to grow the large-scale batches of the phage, so with some types of virulent bacteria it is especially important to minimize toxic bacterial proteins in the phage sample. 4) Using rapid DNA sequencing to characterize phage or phage mixtures prior to use. Sequencing will allow the detection of any mutations that may have occurred during phage amplification and purification, and will allow the phage gene sequences to be compared to known toxin genes as much as possible. We agree with some of our interviewees that we do not need to know the function of all phage genes prior to use, because we have used viruses for decades in vaccines without knowing the function of all their genes. 5) Pre-screening patients for hyper-immune reactions to the specific phage sample prior to injecting large quantities into the patient. Injecting a small quantity of phage (like  $10^2$  particles instead of the  $10^{11}$  particles used for a therapy) would allow each patient to be tested in advance for hyper-sensitivity to a specific phage sample.

If a phage passes these screening checkpoints, it is likely that it is safe and suitable for therapeutic use. Most scientists argue that the risks associated with phage therapy are relatively minor and can easily be controlled with proper precautions.

## Alternative Therapies

Although phage therapy appears to offer a particularly promising solution to the growing problem of antibiotic-resistant bacteria, this does not necessarily ensure the adoption of phage therapy in Western medical practice, as it relies on the introduction of live replication-competent virus into a patient, a fact that can hinder public acceptance. Although it remains uncertain whether these obstacles will prevent the acceptance of phage therapy in Western medicine, especially given our recommendations discussed above, it is clear that the use of live phage could prolong the adoption process. In an attempt to circumnavigate some of the aforementioned obstacles, alternative methods of antibacterial therapy could be used, including tailocins or lysins. If these alternative phage-related therapies could be optimized, they might serve as a safer replacement for phage therapies since there is no phage genomic material that enters the host cells, and the chance of altering the bacterial host cell is greatly diminished. But tailocins and lysins have not yet been investigated in human clinical trials. So it is impossible to judge their therapeutic potential. Tailocins and lysins are foreign proteins that can stimulate an immune response in the patient. An immune response from the patient directed against the phage component could lessen its effectiveness, or could become dangerous to the patient if it induces a cytokine storm response.

But a strong case can be made for using tailocins or lysins, especially for patients receiving phage therapy where host cell lysis has become a problem. The use of proteins may be more acceptable to Western medicine, because protein therapeutics are already widely in use. And single proteins are much easier to characterize than phage particles. They retain the highly desirable specificity of phage therapy, while leaving no potential for the rise of bacterial-resistant strains (the components lysed by the lysin are required by bacteria for survival). In fact, lysins have been approved and are already being employed by the food industry. Cows have been genetically engineered to secrete a “lysin-like” protein in their milk to kill *Staphylococcus aureus* (Fischetti et al., 2006). Lysin genes have also been engineered into the genomes of different produce products such as potatoes and pears to protect them from specific bacterial infections. As more lysins are characterized, treatment regimens are optimized for maximum effectiveness, and clinical trial data shows safety and efficacy, the adoption of lysins as protein therapeutics for treating bacterial infections will increase.

## **Regulatory Improvements**

With respect to regulations, we need to improve phage quality control and phage production standards. Phage production batch-to-batch reproducibility must be improved, and the storage conditions of each phage batch must be standardized before their clinical use can become widespread. Quality control is expensive if each patient’s phage batch must be characterized individually, but this could be avoided by producing large standardized pre-screened phage batches for use in hundreds of patients (if the patient has been shown to be infected by a bacterial species that would respond to that standard batch). As part of the quality control process, the FDA needs to require that the materials used to grow the phage batches (cells, culture media, supplements, etc.) be standardized and quality controlled. The phage batches need to be stored in a standard way, and characterized over time to show lack of genetic alteration, retained efficacy, and lack of contamination. We also need to improve the speed at which infecting bacteria can be identified, because the species must be known before the appropriate phage can be chosen for therapy.

We also need to ensure the high quality of phage materials and products. The cells and materials used to grow viruses must be chosen wisely because the final phage used for therapy can be contaminated with cell products lysed from the cells used to grow the phage. During growth, the phage batches could also become contaminated with bacteria or pathogens accidentally introduced by the technician manufacturing the phage. New state-of-the-art procedures exist for minimizing contamination during phage production, and we recommend these be required by the FDA.

We also need to increase the number of phage patents allowed in the U.S. by increasing the patents for engineered phage. Because naturally occurring phage cannot be patented, some biotech companies are reluctant to get into the phage therapy business because their product is not patent protected. Because phage therapy has been more widely researched and performed outside the U.S., increasing international cooperation for exchanging characterized phage mixtures and standardized protocols would help. In hospitals, creating special hospital wards or rooms that specialize in performing phage therapy treatments would help to control phage contamination and spread, and to control the spread of the antibiotic-resistant bacteria. These

rooms should be monitored for the spread of phage. And last, we recommend increasing the number of phage development programs in the U.S. The army has led the way with its program to develop phage cocktails to fight *Staphylococcus aureus*, and hopes to expand to other deadly infections. On January 27, 2015, President Barack Obama issued a Fact Sheet on his fiscal year 2016 budget, which proposed a historic investment to combat antibiotic-resistant bacteria to protect the public health ([President Obama, 2015](#)). The government should move forward with these programs.



# APPENDIX

## Example Questions for Phage Therapy Experts:

1. Clinical Trials: Our search of the literature indicates that relatively few large-scale phage clinical trials have been performed. Do you agree with this assessment, and if so, why haven't they been done?
2. Which Diseases: In your opinion, which diseases are best suited for phage therapy, and why? Where do you think the data are the strongest? Our search of the literature identified several medically and economically important bacteria that seem like excellent candidates for therapy (including *Pseudomonas aeruginosa*, *Staphylococcus aureus* (especially methicillin-resistant staph aureus, MRSA), *Clostridium difficile*, and foodborne pathogens *Escherichia coli* strain O157:H7 and *Listeria monocytogenes*). Do you agree with this list?
3. Phage Mixtures: Are batches of individual phage species as effective as phage “cocktails” (batches containing multiple phage species) for lysing bacteria? Are they safer?
4. Side-Effects: Have you observed any undesirable side-effects in your phage therapy treatments? If so, which side-effects, and were they easily treatable? Do you think the medical benefits of treating the patient's primary bacterial disorder outweigh the side effects?
5. Cost: How expensive are phage treatments? Must individualized batches of phage be prepared for each patient, and if so, does that increase the cost?
6. Alternatives: Do you think that alternatives to working with replication-competent phage (such as tailocins or lysins) are as effective as phage?

## Example Questions for Bioethicists:

1. Safety: How safe do you think phage therapy is? Are you aware of any patient deaths directly caused by phage therapy? Do you think the medical benefits of treating the patient's primary bacterial disorder outweigh the side-effects?
2. Cost: Do you think that it is cheaper for health providers to pay for phage therapy than to treat a patient for an antibiotic-resistant infection?
3. Applications:
  - a. Which Diseases: Do you think that phage therapy should be restricted for treating potentially fatal antibiotic-resistant infections, or could it also be used to “refine” the gut bacteria to make a healthier flora by killing unhealthy bacteria?
  - b. Compassionate Use Protocols: How about “compassionate use” protocols for treating an apparently end-stage patient who has provided patient consent? Do you think this can provide one way of obtaining badly needed data on phage treatments?

- c. Engineered Phage: How about using phage that have been engineered to be more lethal to bacteria than naturally occurring phage?
  - d. Lysogenic Phage: Do you think that lysogenic phage (that can integrate a copy of the phage DNA into the host chromosome) should be banned? Such chromosomal alterations could in theory make a bacterium more harmful.
  - e. Known Receptors: Do you think that we should know exactly which host receptor is being used before we test a particular phage treatment? In some cases, we don't know this fact yet, but the phage treatment is effective.
4. Ethical Studies: Are you aware of any ethical studies done on phage therapy? Which types of experiments would you like to see completed to provide greater insight into phage therapy? Should we do more studies on how phage are transported throughout the body?

### **Example Questions for Legal Experts**

- 1. Phage Therapy Laws:
  - a. What laws, if any, currently regulate phage therapy in the U.S.? Is this under FDA jurisdiction?
  - b. What changes do you think should be implemented?
  - c. Should we require the use of standardized protocols to minimize the harmful side effects from too high or too low a dose, or harm caused by delivering phage to the wrong area of the body?
  - d. Should we allow the use of “compassionate use” protocols to help acquire data when large-scale clinical trials are not feasible?

### **Interview Preamble**

We are a group of students from the Worcester Polytechnic Institute in Massachusetts, and for our research project we are conducting a series of interviews to investigate problems associated with phage therapy (and its tailocin and lysin alternatives) for treating antibiotic-resistant bacteria.

Your participation in this interview is completely voluntary, and you may withdraw at any time. During this interview, we would like to record our conversation for later analysis. We will also be taking notes during the interview on key points. Is this okay with you?

Can we also have your permission to quote any comments or perspectives expressed during the interview? This information will be used for research purposes only, and we will give you an opportunity to review any materials we use prior to the completion of our final report, which will be published on-line in WPI's archive of projects.

If the subject does not agree to be quoted, we will respond as follows: “Since you would not like to be quoted during this interview, we will make sure your responses are anonymous. No names or identifying information will appear in any of the project reports or publications.”

Your participation and assistance is greatly appreciated, and we thank you for taking the time to meet with us. If you are interested, we would be happy to provide you with a copy of our results at the conclusion of our study.