

UNDER THE MICROSCOPE

## Bacteriophage applications: where are we now?

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### Abstract

Bacteriophages are bacterial viruses and have been used for almost a century as antimicrobial agents. In the West, their use diminished when chemical antibiotics were introduced, but they remain a common therapeutic approach in parts of eastern Europe. Increasing antibiotic resistance in bacteria has driven the demand for novel therapies to control infections and led to the replacement of antibiotics in animal husbandry. Alongside this, increased pressure to improve food safety has created a need for faster detection of pathogenic bacteria. Hence, there has been a resurgence of interest in bacteriophage applications, and this has encouraged the emergence of a large number of biotech companies hoping to commercialize their use. Research in Europe and the United States has increased steadily, leading to the development of a range of applications for bacteriophage agents for the healthcare, veterinary and agricultural sectors. This article will attempt to answer the question of whether bacteriophages are now delivering on their potential.

### The nature of bacteriophages and bacteriophage infections

Bacteriophages are obligate parasites of bacteria, using the resources of the bacterial cell to replicate. They are typically highly specific, often being restricted to particular strains within a single bacterial species. However, some bacteriophages have a relatively broad host range, infecting multiple species within a genus and can even infect members of other genera closely related to their normal host. In some cases, this reflects the uncertain nature of taxonomy, and in others, the presence of common receptors. Bacteriophages will multiply when (and only when) their specific bacterial host is present, allowing the use of extremely low input doses when treating infection.

More than 90% of bacteriophages have large, double-stranded DNA genomes located in heads with icosahedral symmetry, with tails of varying lengths. They belong to three major morphological groups of bacteriophages. These are the *Myoviridae* (with long, rigid, contractile tails), the *Siphoviridae* (with long, flexible, noncontractile tails) and the *Podoviridae* (with short, noncontractile

tails). The morphology and genome type of the remaining bacteriophage families is highly variable, and they may have DNA or RNA genomes. One notable group with single-stranded DNA genomes (*Inoviridae*) appear as long filaments (Maniloff 2006).

The outcome of infection of the bacterial cell may vary. Some bacteriophages undergo a lytic infectious cycle, with infection resulting in rapid lysis and death of the cell within a very short time. Typically, this will result in the release of hundreds of new, infectious virus particles within minutes or hours, a process that can be repeated as long as their bacterial host is present in sufficient numbers to support replication (Harper and Kutter, 2008).

Many bacteriophages are strictly lytic (sometimes referred to as virulent) and are unable to produce any other kind of infection. Others, known as temperate bacteriophages, may infect cells but then become dormant in the latent state known as lysogeny and are replicated along with the host cell chromosome and are subsequently transmitted to each daughter cell following cell division. However, these dormant prophages may be activated into lytic infection by specific stimuli, such as DNA

damage. For some bacteriophages, host chromosomal DNA may be packaged into bacteriophage particles during bacteriophage replication instead of the bacteriophage genome. This can result in high levels of horizontal gene transfer within the bacterial population, a process known as transduction. In contrast to the lytic and lysogenic cycles of bacteriophage infection, the filamentous bacteriophages typically cause persistent infection of bacterial cells that does not kill the host but results in continued excretion of viral particles (Harper and Kutter 2008).

The use of bacteriophages as highly specific antimicrobial agents is widely documented in the literature (Kutter and Sulakvelidze, 2005), and the results of the first modern, regulated clinical trial of bacteriophages as a therapeutic agent have now been published (Wright *et al.* 2009). In general, for therapeutic uses, obligately lytic bacteriophages are highly desirable, because they result in rapid killing of their target host cell, bacteriophage numbers increase rapidly and transduction is rare. DNA sequencing of bacteriophage genomes is now used to confirm both identity and the absence of undesirable elements such as functional lysogenic components or bacterial toxins. Such toxins are known to be associated with some bacteriophages, for instance the Shiga toxins of *Escherichia coli*. These would be of extreme concern if present in a bacteriophage intended for therapeutic use.

Early work on the therapeutic use of bacteriophages in animals or in humans was undertaken without understanding the nature of these agents. While their early proponent, d'Herelle, was consistent in claiming that these were live viral agents, many others disagreed. Even after the first electron microscopic images became available (Ruska 1940), authoritative sources dismissed the viral nature of bacteriophages, stating for example that 'Phage is a protein of high molecular weight' (Krueger and Scribner 1941). From the late 1940s onwards, bacteriophages, with their viral nature now clear, became an essential tool in the expanding science of molecular biology. As a consequence of this work, their nature and effects became much better understood, and this understanding has provided the basis for the more recent work in this field. There are also many articles relating to bacteriophages and their use in detection, therapy, agriculture or as food disinfectants. In the latter role, two products are approved for use against *Listeria monocytogenes* in the United States, while other products have been approved for agricultural use.

### Historical use of bacteriophages: from early promise to snake oil

The potential use of bacteriophages for therapy of bacterial infections was quickly recognized, and work in this

area dates back to 1919. Bacteriophages were independently discovered only shortly before this by Felix d'Herelle and Frederick Twort in 1917 and 1915, respectively (Harper and Kutter 2008). Further development work on their therapeutic use was performed between 1920 and 1940, including work performed by d'Herelle treating cases of cholera and other enteric diseases. Encouraged by the work of d'Herelle, large-scale work by Lieutenant-Colonel Morison in Assam, India targeted both cholera and dysentery and involved treating over a million patients.

From 1927 onwards, a range of commercial products were offered for sale by such groups as Laboratoire de Bactériophage/Robert et Carrière in France, Antipiol in Germany, Medico-Biological Laboratories in the United Kingdom, and Lilly, Swan-Myers, Squibb and Parke-Davis in the United States. In some cases, claims for therapeutic use included such unlikely conditions as herpes (a virus disease) or gallstones.

From his continued observations and experiences treating patients, d'Herelle quickly realized that not all bacteriophage preparations were effective and that care had to be taken both when preparing and when applying bacteriophages. Hence, it probably was of no surprise to him that after producing tens of thousands of doses of therapeutic bacteriophages in Brazil, da Costa Cruz declared that bacteriophage therapy did not achieve effective treatment. In his memoirs, D'Herelle noted that many of the commercial preparations being sold to the public were incapable of effective treatment of infectious disease. At the same time, the advent of effective chemical antibiotics in the 1930s and 1940s led to the therapeutic use of bacteriophages in the West being curtailed. However, clinical use continued in the countries of the former Soviet bloc.

Interest in bacteriophage therapy was reignited by increasing concern over antibiotic resistance and also by publication of successful results achieved by of Smith *et al.* (Smith and Huggins 1982, 1983; Smith *et al.* 1987), even demonstrating the apparently superior efficacy of bacteriophage therapy compared to antibiotics in a mouse model of *E. coli* infection (Smith and Huggins 1982). The advent of multi-drug resistant pathogens has forced the re-examination of bacteriophage therapy, with work being carried out to modern regulatory standards. This is supporting the continuing development of bacteriophage therapeutics and other applications (Table 1).

### Healthcare application of bacteriophages

A large amount of indicative data supporting the effectiveness of bacteriophage therapy is available from studies involving human patients in eastern Europe, with few reported adverse events. This evidence for safety, while not up to current regulatory standards, is further

**Table 1** Commercial phage-based products

Product	Description	Company	Website
AgriPhage™	Targets bacterial spot or bacterial speck on crops, with specific formulations for strains of <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> or <i>Pseudomonas syringae</i> pv. <i>Tomato</i>	Omnilytics	<a href="http://www.phage.com">http://www.phage.com</a>
BioTector	Animal feed for control of <i>Salmonella</i> in poultry	Cheiljedang Corporation	<a href="http://www.cj.co.kr/">http://www.cj.co.kr/</a>
EcoShield™	Targets <i>Escherichia coli</i> O157:H7 contamination in foods and food processing facilities	Intralytix	<a href="http://www.intralytix.com">http://www.intralytix.com</a>
FASTPlaque-Response™	Rapid detection of rifampicin resistance in smear-positive sputum specimens containing <i>M. tuberculosis</i>	Biotech Laboratories/Lab21	<a href="http://www.biotech.com">http://www.biotech.com</a>
FASTPlaqueTB™	Rapid detection of <i>Mycobacterium tuberculosis</i> in human sputum samples	Biotech Laboratories/Lab21	<a href="http://www.biotech.com">http://www.biotech.com</a>
ListShield™	Targets <i>Listeria monocytogenes</i> contamination in foods and food processing facilities	Intralytix	<a href="http://www.intralytix.com">http://www.intralytix.com</a>
LISTEX™ P100	A food processing aid that targets <i>L. monocytogenes</i> strains on food products	EBI Food Safety	<a href="http://www.ebifoodsafety.com">http://www.ebifoodsafety.com</a>
MRSA/MSSA Blood culture test	Determining of <i>Staphylococcus aureus</i> methicillin resistance or susceptibility directly from blood cultures	Microphage	<a href="http://microphage.com">http://microphage.com</a>
MRSA Screening test	Identifies methicillin-resistant <i>Staph. aureus</i> (MRSA) for use in Infection Control programs	Microphage	<a href="http://microphage.com">http://microphage.com</a>
MicroPhage MRSA/MSSA test	Differentiation of methicillin-resistant (MRSA) and methicillin-susceptible (MSSA) <i>Staph. aureus</i>	Microphage	<a href="http://microphage.com">http://microphage.com</a>

supported by the exposure of humans to high levels of bacteriophages via everyday activities because of the ubiquitous nature of bacteriophages in the environment.

Human safety trials have also been performed with increasing frequency including extensive safety trials undertaken on Staphage Lysate by Delmont Laboratories (USA) (Sulakvelidze and Barrow 2005). This product, which contains high concentrations of antistaphylococcal bacteriophages, was administered to humans intranasally, topically, orally, subcutaneously and intravenously. In over 12 years of use in humans, only minor side effects were observed (Sulakvelidze and Barrow 2005). In a formal safety study, Harold Brussow based at the Nestle Research Centre, Switzerland, demonstrated no safety concerns when bacteriophages targeting *E. coli* were administered to human volunteers (Bruttin and Brüssow 2005).

An FDA-approved phase I physician-led trial has been completed at a wound care centre in Lubbock, Texas (Rhoads *et al.* 2009) using a mixture of bacteriophages targeting *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *E. coli* and also showed no safety concerns.

Moving on from trials that provide supportive safety data, the first fully regulated, placebo-controlled,

double-blind, randomized phase II clinical trial of the efficacy of a bacteriophage therapeutic was completed in 2007 and reported a successful outcome against long-term infections with *Ps. aeruginosa*, despite using only a single dose of input bacteriophages in the nanogram range (Wright *et al.* 2009). This supports the prediction that successful bacteriophage infection will lead to therapeutically useful replication of the therapeutic agent if susceptible host bacteria are present at the site of application.

A large (450 patient) trial evaluating the efficacy of multiple bacteriophage products against diarrhoea caused by *E. coli* is under way in Bangladesh, regulated by local authorities and supported by the multinational food company, Nestlé.

Powerful supporting data on the potential for bacteriophage therapy is also provided from animal models. The protective and therapeutic effects of bacteriophages against a wide range of bacterial pathogens has been demonstrated. Bacteriophages targeting bacterial pathogens including *Klebsiella pneumoniae* (Chhibber *et al.* 2008), *Ps. aeruginosa* (Soothill 1992; McVay *et al.* 2007; Chhibber *et al.* 2008), *Staph. aureus* (Soothill 1992; Wills *et al.* 2005), vancomycin-resistant *Enterococcus faecium*

(Biswas *et al.* 2002) and *Acinetobacter baumannii* (Soothill 1992) have all demonstrated efficacy in treating infections in animal models. Use of bacteriophages for treatment of respiratory pathogens in an animal model has also been reported (Carmody *et al.* 2010). Extending this work, the first clinical trial of bacteriophages as an animal therapeutic to treat ear infections in companion dogs reported no safety concerns and demonstrated short-term efficacy (Soothill *et al.* 2004).

### Use of bacteriophages in food and agriculture

There is a considerable literature from the early days of the 20th century relating to the treatment of bacterial infections of animals (rather than the use of animals as models of human infection as described earlier) but much of this has generated very variable results. Early studies by d'Herelle suggest that avian typhoid (*Salmonella enterica* serovar Gallinarum) was susceptible to bacteriophage treatment although this was not repeatable by Pyle with a bacteriophage that was effective *in vitro*. Topley *et al.* were not successful with treatment of murine typhoid caused by *Salm. enterica* serovar Typhimurium. Variable results were also obtained with attempts to treat staphylococcal and *Yersinia pestis* infections of rabbits and mice. The situation from a review of early work was confusing and potentially disappointing (Sulakvelidze and Barrow 2005).

The topic was taken up again in the early 1980s by Smith who was able to protect against *E. coli* infections. This was performed for septicaemia in mice and also for gastroenteritis in neonatal calves, pigs and lambs and involved strictly controlled experiments (Smith and Huggins 1982, 1983; Smith *et al.* 1987). The results showed therapeutic benefit and also that prophylaxis was possible even by treating animal bedding alone. These experiments were extended by his group with success when applied against *E. coli* septicaemia in poultry and calves (Barrow *et al.* 1998).

Use of bacteriophages against food-borne bacterial pathogens has been reviewed by Garcia *et al.* (2008), but showed variable success. Treatment of systemic salmonellosis in poultry was unsuccessful unless bacteriophages were administered immediately following the bacteria. Reduction in intestinal colonization, aimed to reduce entry of this organism into the human food chain, was also not successful (Berchieri *et al.* 1991). However, in 2010, the Korean CheilJedang Corporation introduced BioTector, a bacteriophage product intended to reduce *Salmonella* levels in poultry.

Connerton *et al.* were successful in reducing numbers of *Campylobacter jejuni* in the chicken gut by a factor of between 1 and 4 logs by bacteriophage administration immediately prior to slaughter (Loc Carrillo *et al.* 2005).

To avoid the development of bacteriophage resistance in production facilities, it is important that animals are treated after being removed from the production sites shortly before slaughter so that recycling is impossible. However, it has been found that bacteriophage-resistant strains of *Campylobacter* recovered after bacteriophage infection are less able to colonize new birds (Loc Carrillo *et al.* 2005).

Bacteriophages have also been found to be effective in experimental vancomycin-resistant *Ent. faecium* infection of mice and against *Lactococcus garviae* infection of yellowtail fish (Nakai and Park 2002). In *Pseudomonas plecoglossicida* infection of Ayu fish, feeding bacteriophage-treated feed pellets has been found to be effective, providing a practical approach for the treatment of large numbers of animals (Park and Nakai 2003).

In agriculture, bacteriophages have also been applied against plant infections including *Erwinia amylovora* infection of apple blossom (Schnabel and Jones 2001), and *Ralstonia solanacearum* and *Xanthomonas campestris* infection causing bacterial wilt and spot, respectively, in tomatoes (Fox 2000). Bacteriophages can also be applied in combination with other crop protection technologies (Obradovic *et al.* 2004). The successes in this research area lead to the development of a commercial biocontrol product (Agriphage) produced by the US company Omnilytics. This comprises tailored bacteriophage preparations and has been used primarily to treat tomato and pepper spot, but has shown to be successful for treatment of a wide range of diseases affecting other crops. The product is marketed throughout North and South America and has more recently been licensed for use in Asia. In 2006, AgriPhage was recognized by the Organic Materials Review Institute (OMRI) as being compatible with organic food production.

Bacteriophages have also been developed as a food surface decontaminant. The acceptance of bacteriophages as a natural food additive is based on the presence of high levels of bacteriophages in the digestive tract of humans and throughout the natural environment. However, before a new *Listeria*-specific bacteriophage (Listex P-100) was launched by the Dutch company EBI, an oral toxicity study was performed in rats, and no side effects were recorded (Carlton *et al.* 2005). This product was accepted by the US FDA in 2006 with approval under 'generally recognized as safe' (GRAS) regulations based on experience in Europe. It is designed for the treatment of foods in which *L. monocytogenes* may grow during refrigerated storage and has also been recently recognized as an allowed additive to organic food products in Europe. A competing product (ListShield) is produced by the US company Intralytix, who also list a product targeting *E. coli* O157 on their website.

In this application, high numbers of bacteriophages are applied to the surface of the food, and this has led to some confusion concerning the mode of action. Bacteriophages can be highly effective at killing bacteria through the process of 'lysis from without', where a large number of bacteriophages infect each bacterial cell. If the number binding to the bacterial cell is sufficient, it can be destroyed by the resulting changes in membrane integrity without the processes of bacteriophage infection being completed and, in experimental systems, this mode of killing does not require bacteriophage replication (Atterbury *et al.* 2003; Goode *et al.* 2003). The use of bacteriophages as a food treatment is being reviewed by the European Union to assess its efficacy and safety for use with food producing animals and food products (Anon, 2009). When applied to food, however, where cell numbers are low and the surface area (relative to the size of the bacteriophages) is very large, it is questionable whether the killing achieved is because of lysis from without, as the spatial distribution of host cells and bacteriophages on a surface means that locally high ratios of bacteriophages to host cells are harder to achieve. Irrespective of this uncertainty concerning the exact kinetics of the infection process, these bacteriophage products have been shown to be effective at controlling pathogens in a range of food products (Guenther *et al.* 2009; Holck and Berg 2009; Soni *et al.* 2010).

### Rapid detection methods

The specificity of some bacteriophages for their target host cells and the speed of bacteriophage replication compared to the replication of the host makes bacteriophages suitable for rapid detection. Early work used genetically engineered marker genes to produce a detectable signal on infection of the host cell (Rees and Loessner 2005). However, this was costly, and the use of live GMOs in the assays proved unpopular. Instead, commercial tests have been developed based on the growth of native bacteriophages, and the diversity of both format and application of these assays continues to grow.

The first to be commercially developed was the FAST-PlaqueTB™ assay for the detection of *Mycobacterium tuberculosis* in human sputum samples by a UK-based company, Biotec Laboratories (Ipswich, UK). The assay simply detects the growth of the bacteriophage in a lawn of bacteria by the formation of plaques. To date, the assay has been primarily taken up in the developing world. However, the test has recently been further developed as a combination assay, with PCR being used to confirm the identity of the cells detected, allowing the assay to be applied to a wider range of Mycobacterial species and to

a wider range of samples, including raw milk (Stanley *et al.* 2007; Botsaris *et al.* 2010).

A rapid assay has also been developed to identify methicillin-resistant *Staph. aureus* (MRSA). The MicroPhage MRSA screening test is again designed for applications where rapid molecular diagnostics is not practicable as a faster alternative to conventional culture methods. In this instance, the assay detects growth of a specific bacteriophage following infection of the target cell using antibody capture, and results are available within 5 h. The test is formatted so that the cells present on a swab sample can be challenged with antibiotics, and bacteriophage will only grow if the cells are resistant to the antibiotics (because host cells must be viable for bacteriophage replication to occur). Antibiotic-resistant isolates are rapidly identified, and the assay is designed to be used as a practical, low cost screening procedure for patients arriving at care facilities for treatment.

### Discussion

Bacteriophage products are already in use in agricultural, food safety and diagnostic applications, demonstrating the utility and viability of such approaches. Bacteriophage products for use against human disease are now finally being taken through fully regulated trials, and with the pressing need for alternatives to conventional antibiotics the potential for bacteriophage-based therapeutics is very real.

Despite the progress being made with these applications, the focus of such developments remains in small companies. As yet, interest in these technologies by large pharmaceutical and biotechnology companies remains limited, although the therapeutic trial being conducted by Nestlé in Bangladesh provides a positive indication. Uptake by major commercial partners will be an important step in the development of this technology but as yet remains to be delivered.

### Conclusions

The effective use of bacteriophages in all applications must be supported by detailed understanding of the bacteriophages themselves and then by high quality work and effective trials of this technology to current regulatory standards. A great deal of anecdotal evidence already exists, but this is insufficient to permit approvals by organizations such as the European Medicines Agency or the US Food and Drugs Administration. It is clear that earlier work was not supported in this way, but recent developments do seem now to be delivering on the promise of this approach.



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