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Minireview

Bacteriophage biocontrol in animals and meat products

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Summary

Since their discovery almost a century ago, bacterial viruses (bacteriophages or 'phages') have been used to prevent and treat a multitude of bacterial infections (phage therapy: PT). In addition, they have been the basis for many advances in genetics and biochemistry. Phage therapy was performed on human subjects in the United States, Europe and Asia in the few decades following their discovery. However, Western countries largely abandoned PT in favour of antibiotics in the 1940s. The relatively recent renaissance of PT in the West can be attributed partly to the increasing prevalence of antibiotic resistance in human and animal pathogens. However, the stringent controls on human trials now required in the United States and Europe have led to a greater number of domestic animal and agricultural applications as an alternative to PT in man. This trend is set to continue, at least in the short term, with recent approval from the Food and Drug Administration allowing commercial phage treatments to be used in human food in the USA. Nevertheless, despite these significant milestones and the growing number of successful PT trials, significant obstacles remain to their widespread use in animals, food and ultimately medicine in many parts of the world. This review will provide a brief overview of the history of PT in the West and will summarize some of the key findings of phage biocontrol studies in animals and meat products.

Discovery and early applications

Bacteriophages (or 'phages') were independently discovered by Twort (1915) and D' Herelle (1917) although initial observations of these viruses date back to Hankin in 1896 (Kutter, 2005). Although d' Herelle was quickly convinced that phages were viruses, many scientists believed their bactericidal effect was the result of an enzyme (Sulakvelidze *et al.*, 2001; Kutter, 2005). Indeed during this early period, leading microbiologists such as Bordet and Gratia argued strongly against d' Herelle's virus hypothesis (Stent and Adelberg, 1960). Definitive proof that phages were able to propagate at the expense of their host came when Ellis and Delbruck (1939) performed their classic one-step growth curve experiment. If a single phage infects a bacterium, it replicates inside the host until a point when the viral progeny lyse the cell and diffuse into the environment (lysis from within or 'lytic infection'). Delbruck (1940) also demonstrated that if many phages infect one bacterium simultaneously, they can kill their host without replication (lysis from without). Delbruck's evidence was supported by the first electron micrographs of phages by Ruska in 1940 (Pennazio, 2006).

D' Herelle was the first to realize the potential of phages to treat bacterial infections. In 1919 he performed the first successful phage therapy (PT) trial on a 12-year-old boy suffering from bacterial dysentery (Sulakvelidze *et al.*, 2001). d' Herelle repeated this success with three more dysentery patients, who all improved markedly within 24 h (Summers, 2001). Around this time, Bruynoghe and Maisin (1921) used phages to treat staphylococcal skin infections in what was to become the first published account of PT (Sulakvelidze *et al.*, 2001; Kutter, 2005). D' Herelle quickly expanded the range of bacterial diseases which could be treated with phages. He went on to successfully treat four bubonic plague patients in Egypt in 1925 and dramatically reduced the mortality rates of cholera patients in India from around 30% to 0% in hospital trials (Sulakvelidze and Kutter, 2005). However, not all early PT trials were successful. Pyle (1926) failed to repeat the success of an early d' Herelle trial (treating fowl typhoid) despite promising results *in vitro*. Around the same time, another trial failed to treat experimental *in vivo* streptococcal infections (Clark and Clark, 1927).

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Decline of phage therapy in the west

Despite showing early promise, PT in the West was in decline by the 1930s, even before the widespread use of antibiotics. A paucity of rigorously controlled studies and standardized methods frustrated early attempts to objectively evaluate PT. In the years following d' Herelle's success, enthusiastic entrepreneurs and medics were keen to exploit the potential of PT and a range of phage products were manufactured and marketed (Summers, 2001; Sulakvelidze and Kutter, 2005). These products were often marketed using false claims, for example that they were effective against viral diseases. Moreover, as d' Herelle himself reported, some of these products contained no active phages at all, rendering them ineffective against even bacterial diseases (Kutter, 2005). This was not altogether surprising as crude lysates often contained cell components which inactivate phages during storage (Randall-Hazelbauer and Schwartz, 1973). The common use of organomercury compounds as preservatives in these preparations may also have contributed to reduced phage viability (Merril *et al.*, 2006). Additionally, crude phage lysates often contained high levels of lipopolysaccharide. In sufficient quantities, lipopolysaccharide may lead to endotoxic shock and death in debilitated individuals (Diefenbach, 1949). The generally poor efficacy of commercial phage preparations led to widespread criticism and disagreement about the effectiveness of phages in treating disease.

Interest in PT in the academic community was curtailed severely following the bacteriophage enquiry by the American Medical Association in the 1930s whose assessment was broadly negative (Eaton and Bayne-Jones, 1934). This position was reinforced in a subsequent report 7 years later by Krueger and Scribner (1941) who stated that 'the nature of bacteriophage is no longer in question. It is a protein of high molecular weight and appears to be formed from a precursor originating within the bacterium' (Sulakvelidze and Kutter, 2005). The re-examination of early PT trials suggests many of the failures could be attributed to one or more of the following factors (modified from Kutter, 2005):

(i) The heterogeneity and ecology of both phages and bacteria were not understood.
(ii) Failure to select highly virulent phages against target bacteria in the patient.
(iii) The use of single phage preparations to treat infections involving mixtures of different bacteria.
(iv) The emergence of bacteriophage-insensitive mutants (BIMs).
(v) Failure to appropriately characterize or titre phage preparations.
(vi) Failure to neutralize gastric pH prior to oral administration.
(vii) Inactivation of phages by host immune responses.
(viii) Presence of endotoxins (ET) in phage preparation leading to toxic shock in the patient.
(ix) The scarcity of reliable bacteriology laboratories to correctly identify the pathogens causing an infection.

These factors, along with the discovery of antibiotics in the 1940s and the ensuing Second World War, resulted in a significant decline in PT research in the West. However, research and practical applications of PT continued in various institutes of Eastern Europe and the former Soviet Union. The most famous of these are the Bacteriophage Institute in Tbilisi, Georgia and the Polish Academy of Sciences in Wroclaw, Poland. An overview of some of this work is provided by Alisky and colleagues (1998).

Reappraisal of phage therapy

Animal models of disease

An important step towards the recent resurgence of PT research in the West was taken with a series of veterinary experiments by Smith and Huggins in the 1980s. Their early work focused on *Escherichia coli* septicaemia experimentally induced in mice using a strain of *E. coli* (O18:K1:H7 ColV+) from a child with meningitis (Smith and Huggins, 1982). A single intramuscular (i.m.) injection of one phage was more effective than multiple i.m. injections of various antibiotics (tetracycline, ampicillin, chloramphenicol, or trimethoprim plus sulfafurazole) in protecting mice against a potentially lethal, i.m.- or intracerebral (i.c.)-induced infection. Phage therapy was found to be at least as effective as multiple i.m. injections of streptomycin. The phages persisted for 24 h in the bloodstream, and for several days in the spleen. Prophylactic administration of the phages 3–5 days before the *E. coli* challenge also protected mice against infection. The findings of a recent repeat of this work concur with those of the Smith and Huggins trial (Bull *et al.*, 2002).

Escherichia coli infections have been used in several PT models since then. Nishikawa and colleagues (2008) investigated the use of phages to treat murine bladder infections. The bladders of BALB/c mice were injected with 5×10^9 cfu of a uropathogenic *E. coli* isolate, ECU5, and then treated with different ratios of phages to host cells (multiplicity of infection: moi) of T4 or KEP10 phages. All of the mice in the untreated control group died after 3 days but all the mice treated with the highest phage moi (up to 60) were still alive after 7 days. Lower moi treatments were noticeably less effective, with survival falling to 40% with the lowest moi used (0.01). The importance of moi to the success of PT was also demonstrated by Wang and colleagues (2006). In this study, mice were given an intraperitoneal (i.p.) injection containing *E. coli* 9853, which produces extended-spectrum β-lactamases. After

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40 min the mice were given an i.p. injection containing phage ϕ9882 (isolated from hospital sewage). Mice treated with a moi of 10^{-4} or greater all survived after 30 days, compared with the untreated group which all died after 14 h. Survival was reduced appreciably for mice treated with a moi of 10^{-7}. Noteworthy, animals treated with heat-inactivated phages all died within a similar time scale to the untreated animals indicating that the biological activity of the phages was essential for the success of PT. Another study showed that repeated oral treatment of mice experimentally infected with *E. coli* O157 initially reduced pathogen numbers (Tanji *et al*., 2005). However, the numbers of *E. coli* recovered to similar levels as untreated animals within 9 days.

Other PT trials using rodent models of disease have shown that phages can be used to successfully reduce *Clostridium difficile* (Ramesh *et al*., 1999), *Acinetobacter baumanii* and *Pseudomonas aeruginosa* (Soothill, 1992). Work by Cerveny and colleagues (2002) showed that bacteriophages could protect mice which had been intravenously injected with over 10 times the lethal dose of *Vibrio vulnificus*. Biswas and colleagues (2002) developed an *Enterococcus faecium*-infected mouse model to determine the efficacy of PT in preventing a fatal septicaemia elicited by an i.p. injection of 10^9 cfu of a vancomycin-resistant strain of *Enterococcus faecium*. A single i.p. injection (administered 45 min after the bacterial challenge) of 10^9 or 10^8 plaque-forming units (PFU) of phages protected all of the infected mice. When treatment was delayed until the animals were moribund, 50% of them were rescued by a single injection of the phage preparation. As with the study by Wang and colleagues (2006) described above, mice injected with heat-inactivated phages had a similarly high mortality rate (~90%) to untreated animals.

Phage applications in food-producing animals

Bacteriophages are purportedly the most abundant forms of life on the planet with an estimated population of 10^{30}–10^{32} virions in the biome (Boyd and Brussow, 2002; Rohwer and Edwards, 2002). The presence of phages throughout the human food chain has been widely documented (Kennedy and Bitton, 1987). Some of these phages have the potential to be beneficial (for example as biocontrol agents) while others can be detrimental, e.g. by infecting starter cultures used for yoghurt manufacture (Sanders, 1987). Most PT trials in food producing animals have been directed against important zoonotic pathogens, principally *E. coli*, *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp. Antibiotic resistance among some of these bacteria is a major and growing concern (Threlfall *et al*., 2000a,b). With regulatory bodies in the European Union (EU) banning the routine use of antibiotics in livestock (Dibner and Richards, 2005) and restricting chemical treatments of carcasses during processing, alternative interventions are urgently required.

Poultry. Poultry have been the most commonly used models for PT in food-producing animals. The large scale, high throughput and mechanization of poultry production lend itself to the benefits of PT in a way that large animal meat production does not. The high population density of chickens in conventional rearing systems, which can reach hundreds of thousands on a single farm, increases the risk of a rapid spread of disease and concomitant economic losses. However, the same high population density also favours the spread of phages through a flock, facilitating and reducing the cost of treating a large number of animals. One poultry disease which has been targeted by PT is colibacillosis, an *E. coli* infection which causes airsacculitis, pericarditis, perihepatitis in chickens and is a significant cause of morbidity and mortality in the industry. Over a number of studies, Huff and co-workers have used PT to treat chickens with experimental *E. coli* air sac infections. In their first study, they used an aerosol spray containing two different phages to halve chicken mortality when used on the same day of the *E. coli* challenge (Huff *et al*., 2002). A higher titre of phages in the aerosol spray was able to significantly reduce mortality when the birds were challenged with *E. coli* 3 days after PT. In a second study, a similar infection and treatment model was used and mortality in the phage-treated chickens was reduced to 7%, compared with approximately 48% of the untreated animals (Huff *et al*., 2006).

Salmonella spp. has been targeted in several PT trials involving poultry. This pathogen is a major cause of acute bacterial enteritis in man, with over 180 000 cases of salmonellosis reported in the EU during 2004 (EFSA, 2006). Contaminated poultry and eggs are widely accepted as a major source of *Salmonella* spp. In addition, some serovars of *Salmonella* can cause morbidity and mortality in chickens and survive in the farm environment for prolonged periods of time. Berchieri and colleagues (1991) used phages isolated from human sewage to reduce experimental *Salmonella* Typhimurium colonization of the chicken intestine by approximately 1 $\log_{10}$ cfu and significantly reduced mortality compared with untreated animals. However, the phages only persisted whilst *Salmonella* spp. could also be recovered. Phage-resistant *Salmonella* colonies recovered following phage treatment exhibited a rough morphology and were less virulent than the challenge strain. Fiorentin and colleagues (2005) used bacteriophages isolated from free-range chickens to reduce *Salmonella* Enteritidis PT4 colonization of broiler chicks. Day old broilers were challenged with *S.* Enteritidis and treated with 10^{11} PFU of a cocktail of three bacteriophages 7 days afterwards. A 3.5 $\log_{10}$ reduction in caecal carriage was recorded 5 days after phage

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treatment (compared with the control). An appreciable reduction in *Salmonella* colonization in the PT group remained for up to 25 days after phage treatment. Atterbury and colleagues (2007) demonstrated that the caecal colonization of both *S.* Enteritidis and *S.* Typhimurium in broiler chickens could be reduced by up to 4.2 $\log_{10}$ cycles through the oral administration of high titre (10^{11} PFU) phage suspensions. However, attempts to reduce a third serovar (Hadar) were unsuccessful. Phage-resistant sub-populations of salmonellas re-colonized the birds within 72 h of bacteriophage treatment. Interestingly, when phage-resistant mutants were used to challenge a new group of chickens, the salmonellas recovered from these birds appeared to revert to a phage-sensitive phenotype, suggesting that phage resistance in this case imparted a fitness penalty. Significant reductions in intestinal *S.* Enteritidis carriage in broiler chickens following PT were also recorded by Borie and colleagues (2008). As with Huff and co-workers, this study delivered phages via aerosols which resulted in a 1.63 $\log_{10}$ cfu ml^{-1} reduction in *Salmonella* numbers recovered from the intestinal contents.

Pathogenic *E. coli* can cause significant morbidity and mortality in poultry flocks. A study by Barrow and colleagues (1998) used phages to prevent *E. coli* septicaemia in chickens when the mortality rate in untreated birds was almost 100%. Delaying PT until the onset of clinical symptoms still greatly reduced the severity of infection. A study by Xie and colleagues (2005) investigated the effect of phages on neonatal diarrhoea in chickens. The effect of PT on the survival of chickens challenged with *E. coli* strain 3-1 was compared with antibiotic chemotherapy (chloromycetin, 1 mg per 10 g body weight). Phage therapy was found to reduce the incidence of diarrhoea in the chickens to 26%, compared with 51.6% for the control group. Survival in the PT group was approximately 10-fold higher than the control group and sixfold higher than the antibiotic-treated group. Noteworthy, the birds in the PT group had higher bodyweights than those in the antibiotic-treated group. Similar observations were made by Toro and colleagues (2005) who found that birds colonized with *S.* Typhimurium then treated with phages had higher mean bodyweights than untreated birds.

Another important zoonotic pathogen in poultry which has been targeted by PT is *Campylobacter* spp. Poultry are widely accepted as an important reservoir of *Campylobacter* spp. in the human food chain (Jacobs-Reitsma, 2000). Campylobacters readily colonize the chicken gut (Beery *et al*., 1988) and have been recovered from a large proportion of fresh poultry products (Kramer *et al*., 2000; Jorgensen *et al*., 2002). One risk analysis suggested that interventions resulting in a 2 $\log_{10}$ cfu reduction in *Campylobacter* contamination on carcasses may lead to a 30-fold reduction in human campylobacteriosis (Rosenquist *et al*., 2003).

Campylobacter numbers have been reduced in the caeca of broiler chickens by 1–2 $\log_{10}$ cfu following repeated doses of phages (Wagenaar *et al*., 2005). A study by Loc Carrillo and colleagues (2005) also evaluated the ability of phages to reduce the intestinal colonization of campylobacters. Significant reductions of up to 5 $\log_{10}$ cfu g^{-1} were recorded for some phage–host combinations in several parts of the intestinal tract. The authors suggested that such reductions could be highly beneficial as a pre-slaughter treatment to reduce *Campylobacter* contamination on the carcass. Recent studies have found that Mu-like prophages exist within the genome of some *Campylobacter* strains, and that phage resistance can be associated with a reduced colonization potential in the chicken intestine (Scott *et al*., 2007a,b). Reflecting the findings of other studies with *Salmonella* and *E. coli*, phage-resistant campylobacters appear to revert to a sensitive phenotype when re-colonizing the chicken intestine in the absence of bacteriophage predation, suggesting that there is a fitness cost to phage resistance (Scott *et al*., 2007a). Moreover, one study found that at least one group of well-characterized *Campylobacter* phages interact with phase-variable structures such as capsular polysaccharide and flagella as part of their infection cycle (Coward *et al*., 2006). The effects of prophage and phase variability of adsorption sites could have profound implications for PT and further emphasizes the importance of empirical data when evaluating the efficacy of PT *in vivo*.

The fact that many PT trials use phages isolated from the same environment as the host bacterium leads to questions about the coexistence of virus and host in the natural environment. Atterbury and colleagues (2005) found that the *Campylobacter* populations in the caeca of naturally colonized broiler chickens are generally lower when phages are also present. A total of 29/41 phage-positive chickens used in the study harboured *Campylobacter* below detectable limits. This suggests there is some natural predation of campylobacters by phages in commercial flocks. The wide range of bacteriophage titres recorded in the intestine (1.5–6.9 $\log_{10}$ PFU g^{-1} of caecal contents) may be a result of the continually changing composition of *Campylobacter* species/strains in the caeca and the availability of a susceptible sub-population of hosts at any given time.

Large food-producing animals. Following their early trials with mice, Smith and Huggins (1983) used cocktails of phages to treat enteritis in calves, piglets and lambs. The calves were either fed or deprived of colostrum and then challenged with enterotoxigenic *E. coli* strain O9:K30.99. None of the nine colostrum-fed calves treated with a high titre (10^{11} PFU) cocktail of two different phages became ill (compared with 93% in the control group). Two out of the

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13 colostrum-deprived calves treated with phages at the onset of diarrhoea died (compared with 100% in the control group). This study demonstrated that phages could greatly reduce morbidity and mortality even when used at the onset of clinical symptoms. Similar successes were recorded with enterotoxigenic *E. coli* strains used to challenge piglets and lambs. Callaway and colleagues (2008) used a cocktail of phages to significantly reduce numbers of *E. coli* O157:H7 in the intestinal tract of sheep. In contrast to some other PT studies, the highest phage moi used (100 and 10) were less efficacious than a moi of 1. In a study by Barrow and colleagues (1998), a lytic bacteriophage which specifically attached to the *E. coli* K1 capsular antigen was used to treat colostrum-deprived calves which had been challenged with enteropathogenic *E. coli*. Bacteraemia was delayed in phage-treated animals and their lifespan was extended. In a study by Sheng and colleagues (2006) PT was unable to reduce the numbers of *E. coli* O157 colonizing sheep. However, O157 numbers in the rectum of Holstein steers were significantly reduced (by up to 1.5 $\log_{10}$ cfu compared with controls) by applying a high titre (10^{10} PFU) of phages at the rectoanal junction and a low titre (10^6 PFU) in the drinking water. Approximately 5% of cattle are purported to carry high numbers of *E. coli* O157 in their intestinal tract and pose the greatest risk to human health (Matthews *et al.*, 2006). Although the study by Sheng and colleagues (2006) did not demonstrate the total elimination of *E. coli* O157 in the intestinal tract of the steers, using PT to reduce the carriage of this pathogen in a small targeted population of heavily colonized animals could yield considerable public health benefits.

Surface decontamination. Phages have not only been used to reduce the burden of zoonotic pathogens in the intestinal tracts of animals, but have also been applied directly to contact surfaces and carcasses. This has the advantage of selectively reducing pathogen numbers in an environment which (in many cases) will not permit the regrowth of BIMs. Phages have been applied in a variety of food matrices. However, most studies have concentrated on chicken and beef. Phages have been isolated from the gastrointestinal tracts of animals and are present in a wide variety of foods. As such, it is likely that phages are consumed regularly by humans without ill effects (Kennedy and Bitton, 1987).

Poultry products have been widely used to study the efficacy of phage treatments of meat surfaces. Unsurprisingly, *Campylobacter* and *Salmonella* have been the most frequently targeted zoonotic pathogens on chicken meat using such treatments. Experiments by Atterbury and colleagues (2003) and Goode and colleagues (2003) showed that the application of phages onto the surface of chicken skin artificially contaminated with *Campylobacter jejuni* led to a reduction of 1–1.3 $\log_{10}$ cfu within 24 h. Combining phage treatment with freezing the skin sections at –20°C was more effective than either treatment used independently (Atterbury *et al.*, 2003). In a further trial, Goode and colleagues (2003) demonstrated that applying a high titre of phages could reduce *S.* Enteritidis numbers on contaminated chicken skin to below detectable levels within 48 h. In an effort to represent a more realistic distribution of pathogens on the surface of chicken carcasses, Atterbury and colleagues (2006) took skin sections from the carcasses of chickens which had been experimentally infected with *S.* Enteritidis or *S.* Typhimurium during rearing. The application of a high titre phage suspension (10^9 PFU) reduced *S.* Enteritidis numbers to below detectable levels in 73% of treated skin sections after 20 min incubation at room temperature. Whole carcasses of broiler chickens and turkeys were also used as models for phage biocontrol by Higgins and colleagues (2005). Carcasses artificially contaminated with low numbers ($< 10^2$ cfu) of *S.* Enteritidis were treated with different titres of phages (up to ~10^{10} PFU). The proportion of *Salmonella*-positive carcasses was significantly reduced following phage treatment. The higher phage titres were generally much more effective in reducing *Salmonella* recovery than the lowest titres. Chighladze and colleagues (2001) used chicken carcasses artificially contaminated with *Salmonella* as a model for surface disinfection using phages. They found that a cocktail of phages could reduce *Salmonella* recovery by > 1000-fold compared with untreated controls. A small number of studies have examined the efficacy of phages against *Salmonella* in chicken portions and processed products. For example, phages have been used to reduce numbers of *S.* Typhimurium DT104 inoculated onto chicken sausages (Whichard *et al.*, 2003).

In contrast to chicken products, a substantial number of phage biocontrol studies in beef have focussed on spoilage organisms. Phages which are able to replicate at low temperatures have been isolated from beef (Kennedy and Bitton, 1987). The preservative effect of *Pseudomonas* phages in raw chilled beef has been thoroughly examined in experimentally inoculated meat, showing a significant extension of the retail shelf life of phage-treated beef (Greer, 1982; 1986; 1988). However, in experiments with naturally spoiling beef, the efficacy of phage treatment was limited by the narrow range of specificity of the phages used, so the majority of natural contaminants resisted phage attack, and continued to proliferate and spoil the meat (Greer and Dilts, 1990).

As with poultry products, phage biocontrol of zoonotic pathogens has also been investigated for beef. O'Flynn and colleagues (2004) reduced the numbers of *E. coli* O157 on experimentally contaminated beef surfaces to below detectable limits in 78% of phage-treated samples.

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In ground beef samples experimentally contaminated with ~3.5 $\log_{10}$ cfu g^{-1} of *E. coli* O157, Abuladze and colleagues (2008) were able to reduce the pathogen load to less than 2.0 $\log_{10}$ cfu g^{-1} 24 h after phage treatment. The control of *Listeria monocytogenes* in meats raises additional difficulties due to the ability of this pathogen to grow at low temperatures. In a study by Dykes and Moorhead (2002), bacteriophages alone had no effect on the growth of *L. monocytogenes* in beef broth. However, an enhanced effect was seen when phages and nisin were combined, although this could not be replicated on a vacuum-packed beef model. Bigwood and colleagues (2008) investigated the use of phages against *S.* Typhimurium and *C. jejuni* in cooked and raw meat at different temperatures. The greatest reduction in pathogen numbers was obtained when both the population density of target bacteria and moi were high. Incubation temperature also appeared to be important, with greater reductions in pathogen numbers occurring at the higher temperatures used in the study (~24°C). The reduction in pathogen numbers following phage treatment could be maintained for up to 8 days when the meat samples were incubated at 5°C. This was despite no recorded increase in bacteriophage numbers after 24 h.

Relatively few studies have used pork as a model for phage biocontrol. Phages have been used under experimental conditions to significantly reduce the growth of *Brochothrix thermosphacta* on pork adipose tissue over 2 days (Greer and Dilts, 2002). However, phage-resistant bacteria grew in the days following phage treatment with total numbers of *Brochothrix* recovering to similar levels to the control after 10 days' storage. A recent study demonstrated that phages could reduce the numbers of *Listeria* in hot dogs by $\geq$ 4.2 $\log_{10}$ cfu (Guenther *et al.*, 2009). The greatest reductions in *Listeria* were recorded when the highest titres of phages were applied. The phages remained viable on the food surface for 6 days when stored at 6°C, with only a negligible (< 0.6 $\log_{10}$ PFU) reduction in titre during this period.

There are few examples of phage treatments in foods of animal origin other than those described above. A recent study demonstrated the potential of phages for the reduction of *L. monocytogenes* in sliced turkey meat and mixed seafood (Guenther *et al.*, 2009). Samples of each food were inoculated with *L. monocytogenes* (~3.0 $\log_{10}$ cfu g^{-1}) then treated with ~8.0 $\log_{10}$ PFU of bacteriophages and incubated at 6°C over 6 days. The two strains of *L. monocytogenes* used in the study were reduced by 0.4–5.0 $\log_{10}$ compared with the controls during the course of the trial. A small (0.8 $\log_{10}$ cfu) reduction in *L. monocytogenes* was achieved on the surface of smoked salmon following phage treatment. However, the numbers of one *L. monocytogenes* strain in the phage-treated samples recovered to similar levels to the untreated controls after 6 days. Generally speaking, higher phage numbers applied to the food surface resulted in greater reductions in pathogen numbers. Another study which used contaminated salmon as a model recorded a 3.0 $\log_{10}$ cfu reduction in *Listeria* when a high titre of phages was applied (Hagens and Loessner, 2007). The use of a lower phage titre did not result in a significant reduction in *Listeria* numbers.

A limited number of studies have investigated the use of phages to control pathogen numbers in processing plants or metallic surfaces. This could be particularly important in high-throughput meat processing plants which receive animals from a wide geographical area (e.g. large broiler chicken processors) and have limited periods of cleaning and disinfection. Due to its propensity for growth at low temperatures and incorporation into biofilms, *L. monocytogenes* has been the focus of phage treatment of biofilms in food-processing plant surfaces. Hibma and colleagues (1997) showed that the formation of *Listeria* biofilms on metal discs was reduced in the presence of bacteriophages. Moreover, *Listeria* in mature biofilms could be reduced by ~3 $\log_{10}$ cfu following phage application. This was equivalent to treatment with 130 p.p.m. of lactic acid. A study by Roy and colleagues (1993) found that *Listeria* biofilms on stainless steel discs could be reduced by 3 $\log_{10}$ cfu following phage treatment. This reduction could be increased to 5 $\log_{10}$ cfu with the addition of a quaternary ammonium compound.

Phage behaviour *in vivo*

The presence of viable phages in a preparation seems to be the critical requirement for efficacy during the treatment of established bacterial infections (Dubos *et al.*, 1943; Biswas *et al.*, 2002; Wang *et al.*, 2006). Many studies describing the efficacy of phages in treating experimentally infected animals have been published, and various mathematical models have been employed to analyse the complex kinetics of these 'self-replicating pharmaceuticals' (Payne and Jansen, 2000; Payne and Jansen, 2001; Bull *et al.*, 2002).

One potential obstacle to using phages for the treatment of systemic disease is that phages can be rapidly removed from the circulatory system when injected intravenously into healthy animals. For example, Inchley (1969) demonstrated that the mouse liver phagocytized > 99% of $_{51}$Cr-labelled T4 phages within 30 min of intravenous (i.v.) injection. However, when injected into the body of an animal already suffering from infection, phages are known to persist and penetrate tissues. In a seminal study, Dubos and colleagues (1943) gave groups of mice an i.c. injection containing a lethal dose of *Shigella dysenteriae*. Some mice were then given an i.p. injection of phages. Phages were not only detected in the blood stream but their titres

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rapidly increased in the brain. This was in contrast to a group of uninfected animals in which phage numbers fell in the bloodstream within hours of administration and few were recorded in the brain. Such findings may be of great importance in the treatment of bacterial meningitis for example (Sulakvelidze and Kutter, 2005).

Both specific and non-specific immunity may hamper PT in certain circumstances. Repeated i.v. injections of phages may result in the production of neutralizing antibodies. The effect of such antibodies on the efficacy of PT depends largely on the target antigen and location of the bacterial host in the body. For example, the attachment of antibodies to the base plate of Myoviridae phages can prevent attachment to the bacterium and reduce therapeutic efficacy. However, the outcome of PT at sites unlikely to produce large quantities of antibodies (e.g. in the gastrointestinal tract) is unlikely to be affected. Generally speaking, serologically related phages (as determined by neutralizing antibodies) are morphologically identical (Ackermann and DuBow, 1987). However, there are some exceptions to this rule, such as coliphages P1, P2 and P4 which are serologically related by morphologically different (Ackermann and DuBow, 1987). Antigenicity varies considerably between phage families. For example, T4 is a good immunogen but T1 and T5 much less so. Antigenic cross reactivity has not been recorded among Podoviridae, Myoviridae, Siphoviridae and even closely related immunogenic T4-like phages show little or no neutralization cross reactivity (Sulakvelidze and Kutter, 2005).

Innate immunity may pose a different set of problems. When phages are administered to the subject, especially intravenously, the reticuloendothelial system may sequester the virions before they are able to achieve maximal effect on the target bacterial population (Bergh *et al.*, 1989). A study by Merril and colleagues (1996) showed that a virulent lambda phage (λ_{vir}) injected into healthy mice was rapidly sequestered by the reticuloendothelial system. However, the serial passage of phages which remained viable in the circulatory system of mice for the longest periods eventually resulted in a 'long-circulating' derivative. In subsequent therapeutic trials, this phage was found to be more effective in rescuing bacteraemic mice. Capparelli and colleagues (2006) used similar techniques to obtain a bacteriophage (ϕD) which was capable of persisting in mice for 38 days. This phage was effective in clearing an experimental *E. coli* O157 infection within 48 h. However, this approach is laborious and may be too time-consuming for clinical applications (Barrow and Soothill, 1997).

Modelling phage therapy

Both classic and recent *in vivo* studies of PT have found that phages do not always eliminate their prey (Alexander, 1981; Smith and Huggins, 1983; Loc Carrillo *et al.*, 2005; Atterbury *et al.*, 2007). Often in these cases, the population densities increase and decrease with characteristic 'predator–prey' oscillations (Van Den Ende, 1973). This phenomenon has also been described in predatory protozoa (Van Den Ende, 1973), *Bdellovibrio* (Alexander, 1981) and appears to be the case for *Campylobacter* and their phages (Wagenaar *et al.*, 2005). Ultimately, bacterial populations may adapt to coexist with a predatory phage (Fischer *et al.*, 2004).

Some mathematical models of phage–host interactions suggest that a minimum density of host cells is required in order to support phage replication and significantly reduce the target population of bacteria (Payne and Jansen, 2000; Payne and Jansen, 2001). One study concluded that phages do not affect the number or activity of bacteria in liquid environments where the density of the host species is below the host cell threshold of about 10^4 cfu per ml (Wiggins and Alexander, 1985). However, the necessity of a minimum bacterial density as a prerequisite for successful phage biocontrol is not universally accepted (Kasman *et al.*, 2002) and studies on the control of spoilage bacteria on meat surfaces suggest that phages can be effective biocontrol agents when the population of host cells is as low as 46 cfu per cm^2 (Greer, 1988). These conflicting findings may be a result of factors such as different phage/host systems, the animal or matrix used, the presence of non-host decoys or the assumptions made when modelling. As such, the efficacy of phage biocontrol should be determined empirically on a case-by-case basis as the ability of current models to predict the efficacy of bacteriophage treatments is limited.

Critical appraisal of phage biocontrol

Bacteriophages are imbued with several traits which give them advantages over antibiotics (Sulakvelidze and Kutter, 2005). Phages are both self-replicating and self-limiting, they will only actively replicate as long as susceptible hosts are available. They can be targeted towards specific pathogens which prevents the disruption of commensal microflora (dysbiosis) often seen following treatment with broad-spectrum antibiotics. The long-term use of phage to treat human infections in Eastern Europe with few ill effects implies that phages can be used without the frequent development of allergic reactions. Animal trials in the West have also shown that the administration of phages both orally and parenterally does not produce any abnormal histological changes, morbidity or mortality (Merril *et al.*, 1996; Biswas *et al.*, 2002; Carlton *et al.*, 2005). Moreover, human volunteers who ingested up to 10^5 PFU of phage T4 did not suffer any detectable ill effects (Bruttin and Brussow, 2005). Purified phages (e.g. ΦX174) have also been injected intravenously into

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individuals with HIV (Fogelman *et al.*, 2000); other immunodeficiency diseases (Ochs *et al.*, 1971) and healthy volunteers (Ochs *et al.*, 1993) without any apparent side effects.

Phages may be used prophylactically, therapeutically and in the sanitizing of surfaces. Their use in the human food chain has been calculated to be more cost-effective than other treatments such as irradiation, freezing or improving consumer hygiene in the kitchen (Mangen *et al.*, 2007). Bacteriophages are able to circulate in the bloodstream following both oral and parenteral administration and can pass through the blood–brain barrier (Barrow and Soothill, 1997). Unlike antibiotics, phages are often able to circumvent bacterial resistance, resulting in a greater versatility of the choice of phage cocktails/treatments to use.

The liberation of ET which accompanies bacterial cell lysis is known to occur following some forms of chemotherapy (e.g. β-lactam antibiotics). If the body is overwhelmed by a large and rapid release of ET following the death of bacterial cells, the patient may experience fever-like symptoms and a worsening of the presenting disease (Pound and May, 2005). This potentially fatal response (Diefenbach, 1949), otherwise known as the Herxheimer reaction, could also occur with phage-mediated lysis. While antibiotics diffuse from the site of administration and gradually lose potency, bacteriophages may further penetrate host tissues leading to uncontrolled liberation of ET. In a recent study investigating this phenomenon, a holin-deficient T4 phage was used to treat bacteraemic mice that had been challenged with *E. coli* BL21 (Matsuda *et al.*, 2005). The phage was capable of killing the *E. coli* host by digesting its genome, but the phage progeny would not be released from the cell thus limiting the release of ET. The survival of mice 48 h after treatment with the holin-deficient phages was significantly higher (81%) when compared with animals in the untreated (0%), wild type T4-treated (52%) and 25 mg kg^{-1} antibiotic (LMOX, 33%) treated groups. Significantly lower levels of free ET were recorded for the mice treated with the holin-deficient T4 mutant.

Temperate phages are extremely common in the environment, human gut, human oral cavity, foods sold at retail, etc., and they have been found in almost all bacterial genera (Ackermann and DuBow, 1987; Schicklmaier and Schmieger, 1995; Eggers *et al.*, 2001; Langley *et al.*, 2003). Several temperate phages have been shown to carry bacterial toxin genes (Waldor, 1998; Boyd *et al.*, 2001; Merril *et al.*, 2003). Temperate phages should therefore be avoided as biocontrol agents in favour of exclusively virulent phages. This is not without its problems as the division between temperate and virulent phages is somewhat artificial and may change according to the phage–host combination. Advances in bioinformatics and high-throughput genomic sequencing allow the *ad hoc* screening of phages for genes associated with transduction or virulence factors. This should minimize the risk of selecting phages which could confer pathogenic traits onto previously innocuous bacteria.

Selective pressure for resistant mutants is a problem akin to both antibiotic and phage therapy. The frequency of such resistance can vary considerably depending on the phage–host combination. For example, the frequency of resistance in *E. coli* O157 populations following phage treatment has been reported to vary from 3.3×10^{-4} 1.9×10^{-6} cfu (O'Flynn *et al.*, 2006). Based on this data, the frequency BIMs is not dissimilar to the incidence of spontaneous antibiotic-resistant mutants (Neu, 1988; Kohler *et al.*, 1997; Medders *et al.*, 1998; Carlton, 1999). However, several studies have indicated that phage resistance often incurs a fitness penalty, such as impaired colonization (Loc Carrillo *et al.*, 2005; Atterbury *et al.*, 2007) or reduced virulence (Smith and Huggins, 1983).

Summary and conclusions

Bacteriophages have been used to control pathogenic bacteria in man and animals with varying degrees of success for over 80 years. They have also been a cornerstone of modern molecular biology and genetics. Many of the reasons why some early PT trials were unsuccessful are now understood. However, it seems clear that the subtleties of different phage–host interactions alongside issues such as immunity, viral replication dynamics, the presence of decoys and the physiology of the host bacterium may all need to be considered when developing new phage treatments. Recent PT trials in the West have been predominantly in the areas of agriculture and veterinary medicine. This has been partly a consequence of the prohibitive costs of developing human treatments. In food production, the widespread use of antibiotics analogous to those used in human medicine has purportedly been partly to blame for the increase in multidrug-resistant (MDR) bacterial pathogens (Linton, 1986; Threlfall *et al.*, 2000a,b).

Concerns regarding the use of chemical additives in food production have led the EU to ban many antibiotics and growth promoters used in the rearing of livestock. These include spiramycin and tylosin phosphate which were banned in 1999 (Dibner and Richards, 2005). The US Food and Drug Administration (FDA) now requires manufacturers of livestock pharmaceuticals to determine if newly proposed antibiotics could be associated with resistance developing against drugs currently used to treat human infections (Merril *et al.*, 2006). Indeed, the FDA has withdrawn the use of some antibiotics such as enrofloxacin for use in primary production (http://www.fda.gov/oc/antimicrobial/baytril.html). Banning

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or significantly reducing agricultural antibiotic usage may reduce produce quality, yields and microbiological safety (Sulakvelidze and Barrow, 2005) and so alternatives are urgently needed. Interestingly, several studies have highlighted the lack of genetic diversity among MDR bacterial pathogens which could be a consequence of clonal selection (Knight, 2002). For example, an estimated 75% of drug-resistant childhood pneumonia cases are caused by 10 strains of pneumococcus (Knight, 2002); with almost half of these cases caused by one strain (Spain 23-F). This lack of variability may mean that antibiotic-resistant bacteria are ideal targets for PT (Merril *et al.*, 2006).

Bacteriophages are ubiquitous in the environment (Rohwer and Edwards, 2002) and numerous studies suggest they permeate the human food chain and are regularly consumed without apparent ill effects (Kennedy and Bitton, 1987). As such, their application as biocontrol agents in foods does not add new biological entities into the food chain. Indeed, recent approval of phage treatments in foods by the US FDA has paved the way for a plethora of new products aimed at reducing or eliminating zoonotic pathogens and spoilage bacteria. However, approval has yet to be formally granted by the EU and there is a debate as to their legal status as either 'food additives' or 'processing aids' (von Jagow and Teufer, 2007).

One of the main issues which still surrounds PT is the possibility of toxin/virulence gene transfer via transduction. The careful screening of phage genomes for virulence genes should help to minimize the risk of this event, as would the selection of exclusively virulent phages. However, the risk of accidental transfer of bacterial DNA through generalized transduction is unlikely to be eliminated completely, especially considering the difficulties in preventing large numbers of phages from being released into the environment.

The spread of MDR pathogens in the past few decades has led to an intensification of research into alternative antibacterial agents. The potential of bacteriophage biocontrol is only just beginning to be realized in the West. While phages are never likely to offer a panacea, they could offer a valuable addition to the tools available to modern medicine, agriculture and veterinary science.

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