REVIEW ARTICLE

Bacteriophage and their lysins for elimination of infectious bacteria

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Abstract

When phages were originally identified, the possibility of using them as antibacterial agents against pathogens was immediately recognized and put into practise based on the knowledge available at the time. However, with the advent of antibiotics a decline in the use of phage as therapeutics followed. Phages did, however, become more useful in the study of fundamental aspects of molecular biology and in the diagnostic laboratory for the identification of pathogenic bacteria. More recently, the original application of phage as therapeutics to treat human and animal infections has been rekindled, particularly in an era where antibiotic resistance has become so problematic/commonplace. Phage lysins have also been studied and utilized in their own right as potential therapeutics for the treatment of bacterial infections. Indeed the past decade has seen a considerable amount of research worldwide focused on the engineering of phages as antibacterial agents in a wide range of applications. Furthermore, the US Food and Drug Administration and/or the US Department of Agriculture have recently approved commercial phage preparations to prevent bacterial contamination of livestock, food crops, meat and other foods. Such developments have prompted this review into the status of phage research as it pertains to the control of infectious bacteria.

Introduction

Bacteriophages (phages) are bacterial parasites, and as such are genetically and structurally simple with life cycles as short as 20–60 min. They have either DNA or RNA as their genetic material encapsulated in a protein coat (Fig. 1). Phages are essentially ubiquitous and are known to infect >140 bacterial genera and can be regarded as the most abundant biological entities, with estimations of 10^{31} phage particles in the world (Bergh *et al.*, 1989; Whitman *et al.*, 1998). Following their discovery, it became evident that they possess two types of life cycle, lytic (used by both virulent and temperate phages) and lysogenic (used by temperate phages). Generally, phages bind to a receptor on the bacterial cell surface, insert their DNA and hijack the host cell machinery for subsequent replication of DNA and synthesis of phage proteins. Progeny phages then form intracellularly by a self-assembly process before being released following cell lysis (for review see Guttman *et al.*, 2005). In contrast, temperate phages can multiply via the lytic cycle or they can enter the lysogenic cycle by integrating their genome into the host chromosome. When the phage is residing in the chromosome, it is known as a prophage and is replicated along with the bacterial genome during cell replication. In some cases, prophage may encode virulence genes, which can be horizontally transferred from one bacterium to another by transduction (Boyd & Brussow, 2002).

Phages were first described and their viral nature appreciated by Felix d'Herelle in 1917, although their antibacterial activity had been independently recognized by Hankin in 1896, Gamaleya in 1898 and Twort in 1915 (Fig. 2) (Sulakvelidze *et al.*, 2001). D'Herelle subsequently published extensively on phage and helped to establish the International Bacteriophage Institute in Tbilisi, Georgia in 1923 (Summers, 1999; Sulakvelidze, 2001). The Bacteriophage Institute in Tbilisi (now the George Eliava Institute of Bacteriophage, Microbiology and Virology) is still researching phage therapy applications and supplies phage for the treatment of various bacterial infections. For reviews on the history of phage therapy and early human applications, see Summers (1999, 2001) and Sulakvelidze & Kutter (2005).

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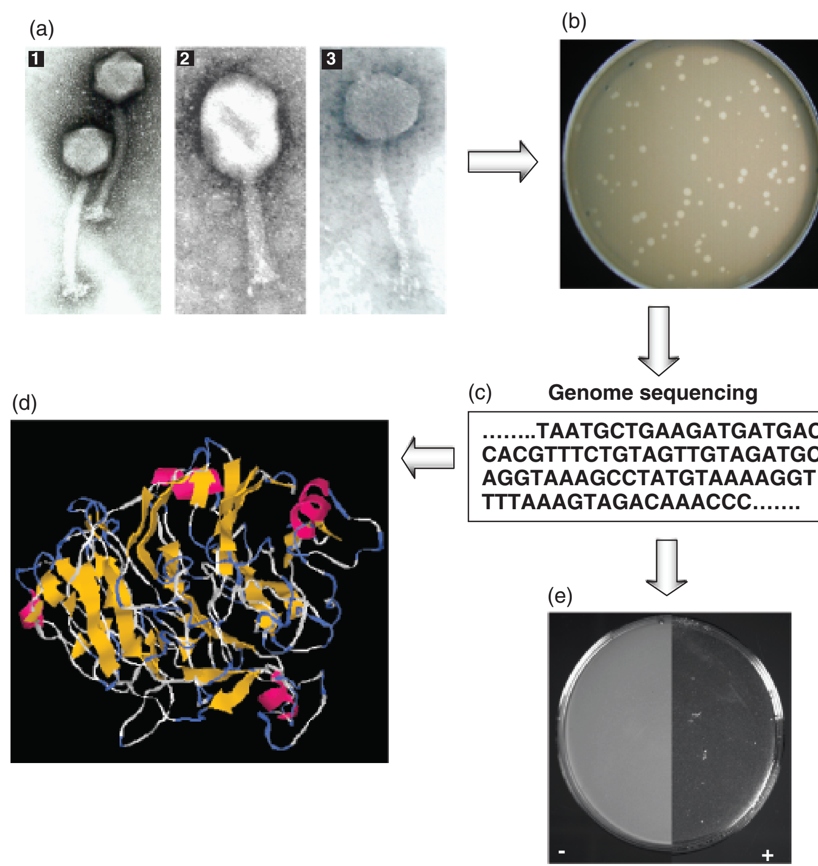

Fig. 1. Diagrammatic representation of the potential of phages and phage lysins as therapeutics. (a) Electron Micrographs of three phages infecting three relevant genera: 1, phage K (*Staphylococcus aureus*); 2, phage pp01 (*Escherichia coli*); 3, phage st104b (*Salmonella enterica*). (b) Representative Petri dish showing plaque formation by staphylococcal phage K. (c) Increase in high-throughput techniques and lower costs of sequencing has allowed a large number of phage genomes to be sequenced, which has resulted in the identification of additional valuable phage products such as phage lysin. (d) I-Tasser (Zhang, 2008) predication of the 3D structure of LysK, which has activity against a wide range of staphylococci. (e) *Staphylococcus aureus* incubated with (+) and without (−) LysK which demonstrates the anti-staphylococcal activity of LysK.

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Results of the early phage therapy experiments were variable, with reports of both success and failure. Nevertheless, D'Herelle's first phage therapy experiments against dysentery were extremely promising with elimination of infection being attributed to a phage preparation (Sulakvelidze *et al.*, 2001). Where failures in phage therapy occurred in later years, they could generally be attributed to a variety of factors including (1) a lack of understanding of phage biology, (2) poor experimental techniques, (3) poor quality of phage preparations and (4) a lack of understanding of the underlying causes of ailment being treated. The commercialization of antibiotics in the 1940s led to a concomitant decline in the use of phage as human therapeutics in Western civilizations; however, in the East, exploitation of phage either alone or in combination with antibiotics continued (Sulakvelidze *et al.*, 2001).

In tandem with the resurgence of interest in phages as therapeutics and the advent of high throughput sequencing, the number of phage genomes sequenced to completion has drastically increased in recent years from a number of 105 genomes by 2002 (Rohwer & Edwards, 2002) to the current total of *c.* 520. Analysis of these genomes has led to increased understanding of phage evolution (Hendrix *et al.*, 1999), phage–host interactions (Chibani-Chennoufi *et al.*, 2004a), bacterial pathogenicity (Boyd & Brussow, 2002), phage ecology (Weinbauer, 2004) and indeed the origin of phages themselves (Hendrix *et al.*, 2000). The past decade has seen a considerable amount of worldwide research focused on the exploitation of phages as antibacterial agents for a wide range of applications. One of the most relevant advances (discussed below) in the use of phages as biocontrol agents occurred in 2006 when the food and drug administration (FDA) approved the use of a six-phage cocktail designated LMP-102 TM for use on 'ready to eat' meat to control *Listeria monocytogenes* contamination. In this review, we concentrate on recent phage exploitation as antimicrobials both as intact phage or phage-based products.

Application of phages in biocontrol and therapeutic design

The increasing incidence of antibiotic resistance in bacterial pathogens has justified a reassessment of the value of phages as antibacterial agents for medical and veterinary applications. Antibiotic resistance is now widespread among pathogens such as *Staphylococcus aureus* (Lowy, 2003), *Salmonella* (Fluit, 2005), *Mycobacterium tuberculosis* (Di Perri & Bonora, 2004), *Acinetobacter* (Jain & Danziger, 2004),

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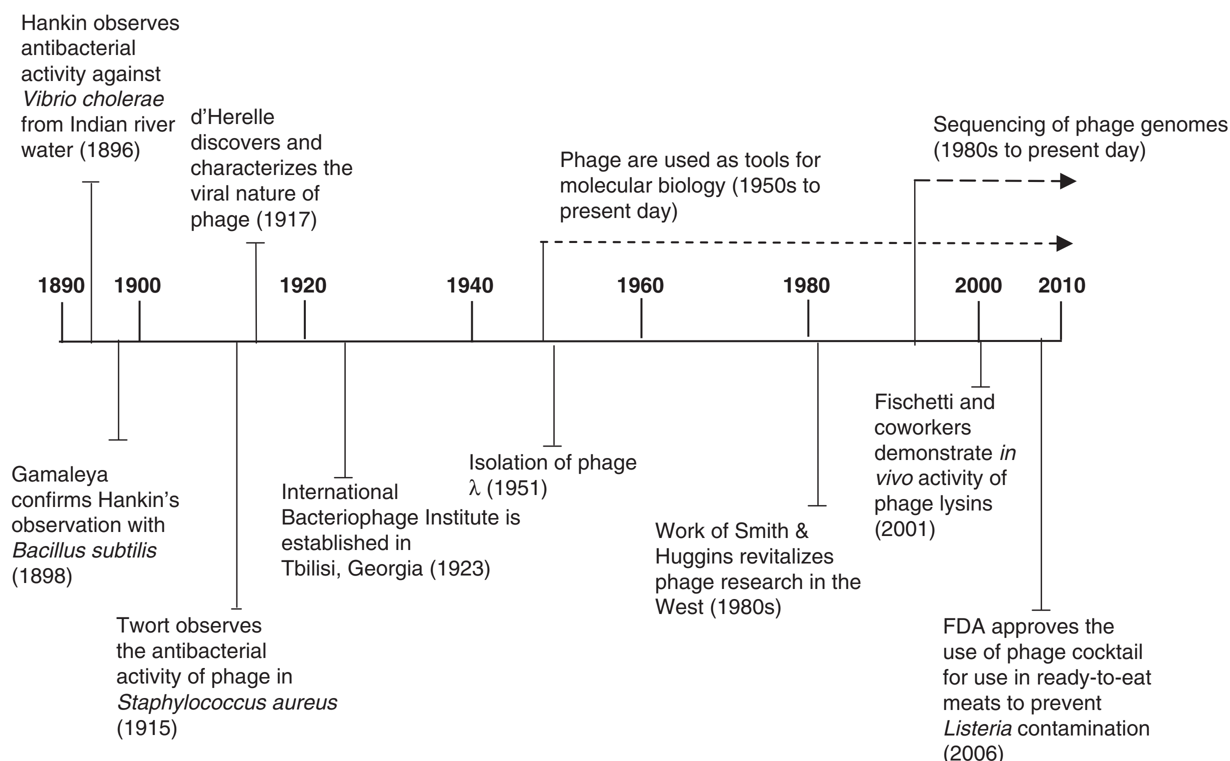

Fig. 2. Timeline of major milestones in phage history.

Escherichia coli (Saenz *et al*., 2004), *Streptococcus pneumoniae* (Jacobs, 2004), *Campylobacter jejuni* (Lindmark *et al*., 2004), *Helicobacter pylori* (Megraud, 2004), *Pseudomonas aeruginosa* (Ong *et al*., 2004), *Haemophilus influenzae* (Bozdogan & Appelbaum, 2004) and *Clostridium difficile* (Razavi *et al*., 2007). Indeed, pharmaceutical industries are investing less in the discovery of novel antibiotics, mainly due to poor returns on their investments (Projan & Shlaes, 2004; Norrby *et al*., 2005). This problem has intensified the need for the implementation of new effective measures to control infections by bacterial pathogens. These measures include (1) improvements in hospital hygiene and management, (2) stringent control over usage of existing antibiotics, (3) development of novel antibacterial drugs, including peptides and lipids as well as low molecular weight compounds and (4) the re-evaluation of phage therapy in the context of a far deeper understanding of phage biology. The merits of all these measures are considerable and this review will concentrate on the research carried out on the latter.

Use of phages as antimicrobial agents in humans

A number of reviews written over the past decade have focused on application of phage therapy in humans and certainly much of the work cited comes from the former Soviet Union states and Poland (Slopek *et al*., 1983, 1987; Carlton, 1999; Weber-Dabrowska *et al*., 2000; Chanishvili *et al*., 2001; Sulakvelidze, 2001; Summers, 2001). Some of these, particularly Sulakvelidze (2001), detail the wide range of infections and illnesses that have been treated successfully. In the recent past, two books on a wide range of phage topics including phage therapy have been published, namely Sulakvelidze & Kutter (2005) and Mc Grath & Van Sinderen (2007).

Looking at the area, it is evident that among the most prominent and comprehensive research in recent years was performed by the Polish group of Gorski and Weber-Dabrowska (Slopek *et al*., 1983, 1987; Weber-Dabrowska *et al*., 2000; Gorski *et al*., 2007) at Wroclaw, Poland, and by the bacteriophage group at the Bacteriophage Institute in Tbilisi, Georgia (Chanishvili *et al*., 2001).

In Poland, phage preparations were generally administered to patients whose infections were unresponsive to antibiotic therapy. Patients had a wide range of diseases caused by *Staphylococcus*, *Klebsiella*, *Escherichia*, *Proteus* and *Pseudomonas*. The patient ages ranged from 1 week to 86 years of age. Therapeutic phages were generally administered orally three times per day, locally by direct application on wounds or by dropping a phage suspension into the eye, ear or nose. In most cases, bacterial sensitivity to phage was monitored and different phages were applied in situations

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where bacteriophage resistance had occurred. In one study, phage therapy results from 550 cases were reported from 1981 to 1986 (Slopek *et al.*, 1987). These results demonstrated that 92.4% of patients were cured, 6.9% of patients showed an improvement in condition in contrast to 0.7% of patients where phage therapy was found to be ineffective (Slopek *et al.*, 1987). Furthermore, in a later study by the same group, similar results were reported. In this case, phage therapy in a group of 1307 patients ranging in age from 4 weeks to 86 years from 1987 to 1999 were investigated (Weber-Dabrowska *et al.*, 2000). Full recovery occurred in 85.9% of cases, an improvement in condition in 10.9% of cases, while no improvement was observed in 3.8% of cases. As with the earlier study, patients had a wide range of bacterial infections caused by the pathogens *Staphylococcus*, *Klebsiella*, *Escherichia*, *Enterobacter*, *Proteus* and *Pseudomonas* (Weber-Dabrowska *et al.*, 2000).

Additional published work by this group highlighted the use of phage therapy to treat chronic suppurative skin infections in 31 patients ranging in age from 12 to 86 years old, whose infections were caused by *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Proteus*, and *E. coli* (Cislo *et al.*, 1987). Of the 31 cases, 77% showed improvements in condition. However, in the remaining 23%, treatment was stopped either due to a lack of improvement or the development of side effects (Cislo *et al.*, 1987). In another study, bacterial infections in cancer patients were treated with phage therapy (Weber-Dabrowska *et al.*, 2001). In this case, 20 cancer patients ranging in age from 1 to 66 years old had concurrent bacterial infections caused by *S. aureus*, *P. aeruginosa*, *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *E. coli*. Importantly, before phage application, antibiotic treatment in all 20 patients had failed. Patients received phage orally three times a day and the infection was cured in all cases following phage treatment, which varied from 2 to 9 weeks in duration (Weber-Dabrowska *et al.*, 2001). More recently, antibiotic-resistant septicaemia has been treated with phage therapy in 94 patients (Weber-Dabrowska *et al.*, 2003). In 71 of these cases, antibiotic treatment was continued in conjunction with phage therapy and in the remaining 23 cases phage alone was administered. Of the 94 cases, complete recovery was achieved in 85.1% of cases, whereas in 14.9% of cases phage therapy was ineffective. Today phage therapy is generally considered an experimental treatment in Poland where it is administered to patients in whom generally antibiotic therapy has failed (for review see Gorski *et al.*, 2007). Patients and an institutional review board both have to give their consent. In general, the average success of phage therapy is 85%. Although these studies were not set as randomized clinical trials, they clearly indicate a high degree of efficacy of phage therapy to combat bacterial pathogens where antibiotic therapy was not effective. Additional studies are needed where a direct comparison can be made between phage-treated and -untreated control groups. In addition, considering these cases did not demonstrate a favourable outcome with antibiotic therapy, the option for phage therapy is more than desirable especially in view of the reported success rate.

Other notable work was performed in the former Soviet Union at the Eliava Institute for Bacteriophage, Microbiology and Virology in Tbilisi, Georgia, and this has been reviewed by Chanishvili *et al.* (2001) and Sulakvelidze & Kutter (2005). Mass-produced phages generated at the Eliava Institute were used throughout the entire Soviet Union. These preparations have been successfully used for prophylactic treatment in addition to the treatment of gas gangrene and emergency wound infections in soldiers (Chanishvili *et al.*, 2001). More recently, screening for phages from the Institute's collection was performed using antibiotic-resistant bacteria, including methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) as hosts (Chanishvili *et al.*, 2001).

Until recently, relatively little work on the phage therapy topic has been demonstrated in Western Europe and commercial phage preparations have not been available since the early phage products of Eli Lilly and L'Oréal (France) fell into decline. Interestingly, a safety test on phage administration has been performed with human volunteers (Bruttin & Brussow, 2005). In this study, subjects received T4 coliphage orally in their drinking water at a concentration up to 10^5 PFU mL^{-1}. No adverse affects were identified in subjects receiving phage T4. Human clinical trials with phages have also been initiated by various phage companies to treat ear infections, leg ulcers and burn wounds (Fortuna *et al.*, 2008). For example, Biocontrol Ltd performed a double-blind placebo-controlled phase II clinical trial in the UK with 24 patients. This trial targeted ear infections caused by *P. aeruginosa*. Results demonstrated a 50% reduction of symptoms in the phage-treated group compared with a 20% in the untreated group. In addition, after 3 weeks of phage application the mean bacterial cell numbers in the patient's ears were reduced by 80% in the phage-treated group, whereas the bacterial cell numbers demonstrated a small increase in the untreated group (Fortuna *et al.*, 2008). However, despite the renewed interest in phages as therapeutic agents for human disease and the few initial small-scale human trials, there have been no reports, to date of large-scale human clinical trials. An important use of phage to kill the pathogen *S. aureus* was reported by Jikia *et al.* (2005). In this case the product PhagoBioDerm was used to treat two men from Georgia who were exposed to strontium-90 and subsequently developed *S. aureus* infections. These infections were not treated satisfactorily with typical mediations such as antibiotics and topical ointments. Doctors used PhagoBioDerm (a biodegradable polymer that contains both the antibiotic ciprofloxacin and phage)

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designed specifically for wound healing. Interestingly, the *S. aureus* strain treated was shown to be resistant to ciprofloxacin and other antibiotics, therefore the clinical improvements were attributed to the phage impregnated in the product (Jikia *et al*., 2005). The two men were treated with PhagoBioDerm a month after hospitalization and demonstrated elimination of the *S. aureus* strain 7 days after application. This is an important case as it demonstrates that when conventional medicine had failed, phage application can be successful. Therefore, although there have not been reports of large-case clinical trials, isolated reports of successful phage treatment, where conventional treatment has failed, is encouraging and an argument for the use of phages in these situations is warranted. The knowledge and technology is currently available to screen patients from such cases for specific strains and match with the phage in the laboratory first or apply phage cocktails for treatment as has been successfully carried out for patients in Poland and the former Soviet Union. Therefore at the present time while further research is being undertaken, one could argue that in specific cases phage should most definitely be considered as a treatment option.

Phages as antimicrobial agents in animal models of human infection

With any new drug or anti-infective, animal models of infection are generally used to evaluate their efficacy. This is also the case with phages, where numerous animal models of infection are used to study phages as potential therapeutics, particularly in the context of antibiotic-resistant infections (Table 1). Chibani-Chennoufi *et al.* (2004b) have quantified phage activities against *E. coli* both *in vitro* and in an *in vivo* model of mice infection. Phages were isolated from environmental water samples and from stool samples of paediatric patients and were subsequently administered to the drinking water of mice. Interestingly, the *in vitro* part of the study demonstrated that the murine intestinal *E. coli* strains were susceptible to phage elimination. However, in the *in vivo* element of the study, the overall titre of *E. coli* was only minimally affected. The authors suggested that the resident *E. coli* were physically or physiologically protected from phage infection. For example, phage infection could have been inhibited due to the presence of large amounts of nontarget bacteria (physical), which may have been in the stationary phase of growth (physiological) (Chibani-Chennoufi *et al*., 2004b). In addition, phage therapy has been investigated in a mouse model of infection against β-lactamase-producing *E. coli* strains (Wang *et al*., 2006b). One phage was isolated from hospital sewage and designated Φ9882, which exhibited a broad lytic spectrum against clinical isolates of these antibiotic resistant *E. coli*. Mice were injected with the minimal lethal dose of *E. coli* and mice that received no phage died within 24 h. In contrast, all mice that received phage Φ9882 [multiplicity of infection (MOI) 10^4] administered 40 min after the bacterial inoculation survived. However, when phage treatment was delayed by 20 or 60 min, only 60% of the mice survived. This was further reduced to 20% when phages were administered 3 h after the bacteria (Wang *et al*., 2006b). These researchers used the same strategy to study the efficacy of phage against imipenem-resistant *P. aeruginosa* (Wang *et al*., 2006a). In this case, treatment with the appropriate phage (ΦA392) within 60 min of bacterial inoculation resulted in a 100% survival rate in the mice. All mice that were not treated with phage died within 24 h (Wang *et al*., 2006a). In a mouse burn wound model, phages were used to control *P. aeruginosa* infection (McVay *et al*., 2007). A cocktail of three phages was administered intramuscularly, subcutaneously or intraperitoneally to groups containing 18 mice. Overall survival in the control group was 6% compared with survival rates of 22–87% depending on the route of injection with intraperitoneal injections of phages resulting in the most significant protection (87%) (McVay *et al*., 2007).

Uropathogenic *E. coli* (UPEC) is another emerging multidrug-resistant pathogen that has recently been targeted with phage (Nishikawa *et al*., 2008). Phage T4 and a newly isolated phage (KEP10) were examined in mice that were administrated with a UPEC strain. Following 7 days, 100% and 90% of mice treated with T4 and KEP10, respectively, at an MOI of 60, had survived (Nishikawa *et al*., 2008). In the control group, where no phage was administered, all the mice died within 3 days (Nishikawa *et al*., 2008).

The potential of phage therapy to control *S. aureus* infection in a rabbit model of wound infection has also been reported (Wills *et al*., 2005). The bacterial strain used in this study had previously caused infection on a rabbit farm. Two groups of eight rabbits were used in a prophylaxis study where both groups received 8×10^7 CFU mL^{-1} of *S. aureus* and one group received 2×10^9 PFU mL^{-1} of phage LS2a. One of the eight phage-treated rabbits developed an abscess of 64 mm^2, compared with the eight in the case of the group of untreated rabbits. These had abscesses ranging from 32 to 144 mm^2. In addition, in a dose–response study, all but one of 12 rabbits that had received 8×10^7 CFU mL^{-1} of *S. aureus* formed an abscess. The one rabbit that had no abscess had received the highest dose of phage (6×10^7 PFU mL^{-1}). An experiment in which treatment with phage was delayed (6, 12 or 24 h after bacterial injection) was also performed. In this case, all rabbits presented abscesses with no difference in severity between these and the negative control group (Wills *et al*., 2005).

Biswas *et al.* (2002) used a mouse model to investigate bacteraemia caused by a clinical isolate of VRE. In this case, mice infected with 10^9 CFU mL^{-1} of enterococci were protected after injection of phage (3×10^8 PFU mL^{-1}). These

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Table 1. *In vivo* and *in situ* phage studies

Hosts for bacteria	Bacteria	Phages	Main outcome	References
Animal models for human infection				
Mice	*Escherichia coli* O157:H7	SP15, SP21 and SP22	Successive daily phage administration was required to reduce cell numbers from the gastrointestinal tract	Tanji *et al*. (2005)
Mice	β-Lactamase-producing *E. coli*	Φ9882	100% survival at 24–168 h after phage administration (40 min after bacterial administration)	Wang *et al*. (2006b)
Mice	*E. coli*	Anti-K1 phage	Better mice survival rates with phage administration. Bacterial mutants were shown to be of lesser virulence	Smith & Huggins (1982)
Mice	*E. coli*	ΦLW and ΦLH	Mortality rates in mice varied depending on the phage used	Bull *et al*. (2002)
Mice	UPEC	T4 and KEP10	100% survival rate with T4. 90% survival rate with KEP10	Nishikawa *et al*. (2008)
Mice	*E. coli*, *Salmonella enterica* serovar Typhimurium	λ and P22	Identification, isolation and subsequent use of long circulating phage	Merril *et al*. (1996)
Mice	*Enterococcus faecium* (VRE)	ENB6	100% survival 45 min after phage administration	Biswas *et al*. (2002)
Mice	*Enterococcus faecalis*	EF24C	100% survival rate with a phage MOI of 0.1	Uchiyama *et al*. (2008)
Mice	*Pseudomonas aeruginosa*	ΦA392	100% survival rate 60 min after phage administration. Reduced survival rates when phages were administrated at 180 and 360 min	Wang *et al*. (2006a)
Mice	*P. aeruginosa*	Pa1, Pa2 and Pa11	87% protection against bacterial infection in mouse burn model compared with 6% in the untreated group after intraperitoneal injection	McVay *et al*. (2007)
Mice	*P. aeruginosa*	CSV-31	100% protection observed when phages were administrated 45 min after bacterial challenge	Vinodkumar *et al*. (2008)
Mice	*Staphylococcus aureus* (MRSA)	ΦMR11	Better mice survival rates with phage administration (MOI > 0.1) straight after bacteria administration	Matsuzaki *et al*. (2003)
Guinea pigs	*P. aeruginosa*	BS24	Skin graft protection from bacteria by phage	Soothill (1994)
Hamster	*Clostridium difficile*	CD140	5/6 hamster survived in the phage-treated group compared with none in the control	Ramesh *et al*. (1999)
Rabbit (wound infection)	*S. aureus*	LS2a	Reduction in abscess size in phage-treated animals, no difference when phage administration was delayed	Wills *et al*. (2005)
Mice	*Vibrio vulnificus*	CK-2, 153A-5 and 153A-7	Different results of mice protection depending on the phage used. CK-2 and 153A-5 protected mice, whereas 153A-7 did not	Cerveny *et al*. (2002)
Mice	*Klebsiella pneumoniae*	SS	Immediate administration of phage resulted in 100% protection, this was decreased after 3 h and no protection at 6 h was observed postbacterial challenge	Chhibber *et al*. (2008)
Other phage trials with animal models				
Calves, piglets and lambs	*E. coli*	B44/1 and B44/2	Prevention of *E. coli* induced diarrhea	Smith & Huggins (1983)
Calves	*E. coli*	Phage cocktail (7 phage)	Prevention of *E. coli* induced diarrhoea	Smith *et al*. (1987b)
Mice, sheep and cattle	*E. coli* O157:H7	SH1 and KH1	KH1 did not reduce intestinal levels of bacteria in sheep. Reduction but not elimination of *E. coli* O157:H7 in steers	Sheng *et al*. (2006)
Sheep	*E. coli* O157:H7	CEV1	Reduction (2 logs within 2 days) but not elimination of *E. coli* O157:H7	Raya *et al*. (2006)
Chickens	*E. coli*	R	Protection of septicaemia and meningitis-like infections even when phage administration was delayed. Phage multiplied in the blood	Barrow *et al*. (1998)
Chickens	*S. enterica* serovar Enteritidis	CNPSA1, CNPSA3 and CNPSA4	Reduction of *Salmonella* in caecal contents	Fiorentin *et al*. (2005)
Chickens	*S. enterica* serovar Typhimurium	S2a, S9 and S11	Beneficial effect on weight gain and reduction in *Salmonella* numbers in the caecum with phage cocktail	Toro *et al*. (2005)

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Table 1. Continued.

Hosts for bacteria	Bacteria	Phages	Main outcome	References
Chickens	*S. enterica* serotypes Enteritidis, Hadar and Typhimurium	Φ151 Φ25 and Φ10	Reduction of caecal cell numbers with Φ151 and Φ10 but not Φ25	Atterbury *et al*. (2007)
Chickens	*Campylobacter jejuni*	69 and 71	Reduction of *C. jejuni* in caecal contents	Wagenaar *et al*. (2005)
Chickens	*C. jejuni*	CP8 and CP34	Reduction of *C. jejuni* in caecal contents. Resistant bacteria were less virulent and reverted to phage sensitivity	Loc Carrillo *et al*. (2005)
Chickens	*E. coli*		Various results (see text)	Huff *et al*. (2002a, b, 2003a, b, 2004, 2005, 2006)
Yellowtail fish	*Lactobacillus garvieae*	PlgY-16	Protection against infection after phage administration	Nakai *et al*. (1999)
Ayu fish	*Pseudomonas plecoglossicida*	PPpW-3 and PPpW-4	Reduced mortality rates in fish that had received phage	Park *et al*. (2000)
Holstein cows	*S. aureus*	K	The cure rate (16.7%) in the phage-treated group was not significantly improved compared with the untreated group	Gill *et al*. (2006a)
Phage trials with food				
Melon and apple	*S. enterica* serovar Enteritidis	Phage cocktail	Reduction of cell numbers on melon slices. No significant difference between phage-treated and -untreated samples on apples	Leverentz *et al*. (2001a)
Cheese	*Listeria monocytogenes*	P100	Reduction and or elimination of *L. monocytogenes* cell numbers on smear ripened cheese surfaces	Carlton *et al*. (2005)
Melon and apple	*L. monocytogenes*	Phage cocktail	Reduction of *L. monocytogenes* cell numbers more effective on melon slices than apple slices	Leverentz *et al*. (2003)
Tomato, spinach, broccoli, ground beef and hard surfaces	*E. coli* O157:H7	Three phage cocktail; ECP-100	Successful reduction of bacterial numbers at a concentration of 10^9 PFU mL^{-1}	Abuladze *et al*. (2008)
Chicken skin	*C. jejuni*	Φ2	Reduction of *C. jejuni* numbers	Atterbury *et al*. (2003)
Chicken skin	*C. jejuni* and *S. enterica* serovar Enteritidis	Phages 12673, 12, HT*int*, 29C	Reduction of cell numbers on treated chicken skins	Goode *et al*. (2003)
Frankfurters	*S. enterica* serovar Typhimurium DT104	Felix 01 variant	Reduction of cell numbers on treated frankfurters	Whichard *et al*. (2003)
Beef steaks	*Pseudomonas* spp.		Increased retail shelf life of beef	Greer (1986)
Beef steaks	*Pseudomonas* spp.	Phage cocktail	Retail case life of steaks treated with the phage cocktail was not significantly different than control steaks	Greer & Dilts (1990)
Pork adipose tissue	*Brochothrix thermosphacta*	A3	Increase in shelf life from 4 to 8 days	Greer & Dilts (2002)
Phage trials with bioflims				
Polysaccharide capsule	*E. coli*	K29	This phage demonstrated the ability to penetrate the polysaccharide capsule of *E. coli*.	Bayer *et al*. (1979)
Biofilm	*E. coli*	T4	*E. coli* in biofilms was successfully lysed with phage T4	Doolittle *et al*. (1995)
Biofilm	*E. coli*	Modified T7	Engineered phage reduced biofilm cell counts by over 99%	Lu & Collins (2007)
Stainless steel and polypropylene surfaces	*L. monocytogenes*	H387, H387-A and 2671	Synergistic effect observed with a quaternary ammonium compound and phage	Roy *et al*. (1993)
Biofilm	*Pseudomonas fluorescens*	ΦS1	85% reduction in biofilm mass	Sillankorva *et al*. (2004)
Biofilm	*S. aureus*	K	Reduction in biofilm mass varied depending on strain tested	Cerca *et al*. (2007)

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researchers also demonstrated that the survival rate was reduced to 50% when phage treatment was deliberately delayed (18 and 24 h) in mice that were moribund (Biswas *et al*., 2002). Furthermore, it was demonstrated that the recovery of the bacteraemic mice was not due to a nonspecific immune effect but instead was clearly associated with the antibacterial activity of the phage (Biswas *et al*., 2002). More recently, treatment with phage EF24C has been associated with prevention of *E. faecalis* sepsis in a mouse model of infection (Uchiyama *et al*., 2008). This phage whose genome has also been sequenced, saved 100% of mice from sepsis at an MOI of 0.1 (Uchiyama *et al*., 2008).

Cerveny *et al*. (2002) examined the potential use of phages as therapeutic agents against *Vibrio vulnificus* infection in a mouse model. *Vibrio vulnificus* is a Gram-negative opportunistic pathogen of humans, which contaminates filter-feeding shellfish such as oysters (Linkous & Oliver, 1999). In one study, two groups of four mice were injected intravenously with 10^6 CFU mL^{-1} of *V. vulnificus* and immediately injected with 10^8 PFU mL^{-1} of phage CK-2 (test group) or phosphate-buffered saline containing 0.01% (w/v) gelatin (control group). The survival rates of control mice were 0% compared with complete survival in the phage-treated mice (Cerveny *et al*., 2002).

The studies discussed in this section and Table 1 demonstrate the importance of mouse models and other animal models of infection. These studies were performed under different conditions and demonstrate that there is no universal experimental condition set for phage therapy. Furthermore these studies illustrate the importance of timing, MOI and route of phage administration. Phage efficiency was reduced when administration was delayed in some cases for less than an hour, where in other cases phages were efficient when treatment was delayed over a number of days. These results indicate the importance of work performed by Merril *et al*. (1996) (discussed below) for the selection of long circulating phages. In addition, some studies demonstrated varied results depending on the route of administration. Therefore before phages are administered to humans and animals for treatment (especially for new phages) animal models are an important initial step to help determine potential experimental conditions down the line such as route and timing of administration, MOI, etc. In addition, phage sequencing should be performed to ensure that there is no presence of toxic genes and that virulent phages are used for therapy. The latter is an important criterion for the selection of phages for therapeutic purposes. The magnitude of this criterion is reflected in a significant recent study by Chen & Novick (2009), which demonstrated intergeneric transfer of toxin genes between *S. aureus* and *L. monocytogenes* by transducing phage.

Veterinary applications of phages

In addition to pathogens affecting humans, the use of phage as therapeutics to treat infections in animals themselves and to prevent the carriage of pathogens that might subsequently get into the food system is another vital area of phage research (Table 1). The important and much-cited research by Smith and Huggins in the 1980s at the Institute for Animal Disease Research in Houghton, Cambridgeshire, was the first substantial phage therapy study in Western Europe after the antibiotic era (Smith & Huggins, 1982, 1983; Smith *et al*., 1987b, c). In one of these experiments, single intramuscular injection of 3×10^8 PFU mL^{-1} of phage gave complete protection to mice, which had been injected with a potentially lethal dose of 3×10^8 CFU mL^{-1} of *E. coli* K1 (Smith & Huggins, 1982). During these experiments, some resistant cells were found, for example in mice inoculated with *E. coli* K1 and phage, 15 out of 360 *E. coli* isolates were K1 negative; however, these mutants were less virulent (Smith & Huggins, 1982). Furthermore, Smith & Huggins (1982) demonstrated that a single dose of phage was more effective than multiple doses of antibiotics such as ampicillin, tetracycline and chloramphenicol. This group also successfully used phage to prevent *E. coli*-induced diarrhoea in calves, piglets and lambs (Smith & Huggins, 1983). Administered as a prophylactic, a mixture of two phages protected calves against a potentially lethal dose of *E. coli* O9:K30,99 (Smith & Huggins, 1983). Furthermore, calves in pens previously occupied by calves that had been treated for diarrhoea by phage were also protected from developing diarrhoea (Smith *et al*., 1987b). Interestingly, Bull *et al*. (2002) have repeated the experiments of Smith and Huggins achieving similar results, with different phages and *E. coli* strains. This group found that mortality rates in mice varied depending on the phage used. This work by Smith and Huggins was very important as it reawakened the possibility of using phage to successfully cure bacterial infections, and many researchers in the West subsequently started to investigate this field.

More recently, reports of the use of phages to control *E. coli* O157:H7 numbers in sheep and cattle have been reported (Sheng *et al*., 2006). Although sheep and cattle do not suffer from *E. coli* O157:H7 infection, they are considered as important reservoirs of the pathogen to the human population and hence a decrease in *E. coli* O157:H7 carriage by these animals is warranted. In the first study, Sheng *et al*. (2006) used two phages, SH1 and KH1 to limit bacteria numbers in mice, sheep and cattle. While SH1 (which was more effective than KH1) did not eliminate all bacteria, the numbers of *E. coli* O157:H7 was reduced in steers treated with SH1 alone or a combination of SH1 and KH1, compared with control animals.

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Attempts to treat bovine mastitis infection caused by *S. aureus* with phage have also been reported (Gill *et al*., 2006a). However, Gill *et al*. (2006a) found that the cure rate (16.7%) in the phage-treated group was not significantly improved compared with the untreated group. Phage K, which was evaluated against mastitis-causing staphylococci was shown to have a surprisingly broad host range within the entire genus *Staphylococcus* (O'Flaherty *et al*., 2005d). Indeed, genome analysis by O'Flaherty *et al*. (2004) demonstrated that phage K was exclusively lytic and its entire 127-kb genome possessed no GATC (Sau3A) restriction sites. Where phage resistance, due to restriction modification activity, was encountered in staphylococci, it could be circumvented by generating modified phage K (O'Flaherty *et al*., 2005d). Interestingly, studies with this phage showed that phage K activity was inhibited in raw bovine milk and whey (O'Flaherty *et al*., 2005b; Gill *et al*., 2006b). The poor lytic activity of phage in the raw milk environment may be due to immunoglobin activity against the target bacteria resulting in clumping (O'Flaherty *et al*., 2005b). This phenomenon may explain the poor efficacy reported to treat some infections such as mastitis with phage (Gill *et al*., 2006a). Given the drug-resistant nature of this pathogen further work, including isolating additional anti-staphylococcal phage (O'Flaherty *et al*., 2005c) and/or using anti-staphylococcal phage lysins (Fig. 1, Obeso *et al*., 2008), is warranted.

Treatment of respiratory infections caused by *E. coli* in chickens, with phage therapy has also been investigated. These researchers found phage therapy to be ineffective in successive experiments where the phage was administered in drinking water (Huff *et al*., 2002b). In addition, the efficacy of either aerosol or intramuscular injection of phage to treat an *E. coli* infection in broiler chickens was studied (Huff *et al*., 2003b). In this case, an intramuscular injection of phage reduced mortality from 53% to 17%, 46% to 10%, and 44% to 20% when given immediately (0), 24, or 48 h after challenge with 10^4 CFU mL^{-1} of *E. coli*, respectively. *Salmonella* infection and carriage in chickens and broilers has also been treated with phage (Fiorentin *et al*., 2005; Toro *et al*., 2005). In one study, after oral administration of phage, *Salmonella enterica* serovar Enteritidis CFU levels were reduced by only 3.5-fold per gram in the caecal contents of broilers after 5 days (Fiorentin *et al*., 2005). In another study, Toro *et al*. (2005) used a *Salmonella*-specific phage cocktail to reduce *Salmonella enterica* serovar Typhimurium colonization in chickens. A reduction in *Salmonella* numbers in the caecum and ileum was observed in phage-treated birds. In addition, the phage cocktail caused a beneficial effect on weight gain performance (Toro *et al*., 2005). Like *E. coli* and *Salmonella*, *C. jejuni* is a member of the normal commensal microbial community of broiler chickens. This zoonotic bacterium is pathogenic to humans and can be passed along the food chain in meat products. In a biocontrol study by Wagenaar *et al*. (2005), phages were administered for 6 days starting 5 days after bacterial colonization. Initially, results showed a 3-log decrease in *Campylobacter* numbers in the caeca, which stabilized to a 1-log reduction after 5 days. Another study by Loc Carrillo *et al*. (2005) described a reduction in numbers of *Campylobacter* in caecal contents in the phage-treated group over 5 days. Resistant bacteria were isolated, but following analysis, these later reverted to a phage-sensitive phenotype.

The use of phage therapy to control fish pathogens has also been reported. Drug-resistant bacteria can be a particular problem in aquaculture; hence, there is potential for phage application in this area (Nakai & Park, 2002). The protective effects of phage against infection by *Lactobacillus garvieae* in yellowtail fish were demonstrated after intraperitoneal or oral administration of phages. In this study, 100% of fish inoculated with *L. garvieae* survived following phage administration, compared with only 10% survival in the control group, where no phage was administered (Nakai *et al*., 1999). Protection was also reported against *Pseudomonas plecoglossicida* infection in Ayu fish (*Plecoglossus altivelis*) with phage administration (Park *et al*., 2000). In one trial, fish were first orally challenged with *P. plecoglossicida* in pellets (10^7 CFU g^{-1}). Fifteen minutes later the fish were then administered with phage-impregnated (10^7 PFU g^{-1}) or phage-free feed. Following 2 weeks, the mortality rate in fish that received the phage-free feed was 65% ($n=40$). However, the mortality rate was 22.5% ($n=40$) in fish that received the phage-impregnated feed. Interestingly, no neutralizing antibodies were detected in either study (Nakai & Park, 2002). Importantly, these researchers extended their studies to a field trial where phage-impregnated feed was administered to Ayu fish in a pond. In this case, the disease was not artificially induced but occurred naturally in the pond. Mortality rates (*c*. 18 kg or 900 fish per day) had decreased by a third when compared with the control group (Park & Nakai, 2003).

The work of Smith and Huggins (Smith & Huggins, 1983; Smith *et al*., 1987a, b) interestingly described phage-resistant mutants of *E. coli*, which emerged after phage therapy in calves. These mutants were greatly reduced in virulence when compared with the parent *E. coli* strain. Apart from the emergence of mutants another potential problem with the administration of phage in animal and human systems is the elimination of phage by the host immune system. A discovery, which circumvented this problem, was reported by Merril *et al*. (1996). Specific strains of phage were identified, which survived longer than normal in the murine circulatory system. These so-called 'long-circulating' phage variants were selected for *E. coli* phage λ and *Salmonella* phage P22 in mice. Furthermore, these mutants were subsequently shown to be more effective in protecting mice from

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bacteraemia than the parental phage strains (Merril *et al.*, 1996).

An advantage to the use of phages to treat animal models of infection may mean less stringent regulations, which in turn would be advantageous to the study of phage therapy as a whole. However, it is evident from these studies that careful phage selection is required in addition to careful selection of experimental conditions. In addition, phage sequencing, the use of cocktails to prevent resistance, studies on the route and timing of phage administration and environmental monitoring of phage for use in large animal trials are needed.

The use of phages as biocontrol agents against undesirable biofilms

Biofilms are the accumulation of microbial cells and their excreted products attached to living or inert surfaces. These excreted products include exopolysaccharide and various proteins. Food, pharmaceutical and environmental processing equipment, medical catheters, implants, shunts and prostheses can all become coated in biofilms, with concomitant contamination problems. Thus the removal of these films represents a major challenge. Moreover, bacteria embedded within the biofilm matrix are less accessible to antimicrobial agents, including phages. Interestingly, some phages, have been shown to possess enzymes that can degrade bacterial polysaccharide. An example is coliphage K29, which is capable of penetrating the polysaccharide capsule of *E. coli* and successfully causing lytic infection (Bayer *et al.*, 1979). In another study, Doolittle *et al.* (1995) lysed *E. coli* in biofilms using coliphage T4. These researchers also traced the interaction of the coliphage and biofilms with fluorescent and chromogenic probes (Doolittle *et al.*, 1996). Phages have also been used in combination with other antibiofilm treatments. For example, the application of *Listeria* phage with a quaternary ammonium compound displayed a synergistic effect. In this study, a significant reduction in *L. monocytogenes* numbers was observed on stainless steel and polypropylene surfaces (Roy *et al.*, 1993). An 85% reduction in *Pseudomonas fluorescens* biofilm mass was observed after treatment with phage ΦS1 (Sillankorva *et al.*, 2004). Recently, two staphylococcal phage lysins were evaluated for activity against biofilms of *S. aureus* (Sass & Bierbaum, 2007). In this case, the recombinant phage lysin from Φ11 successfully hydrolyzed staphylococcal biofilms. In addition, Cerca *et al.* (2007) showed that phage K was successful in reducing *S. epidermidis* biofilm biomass after a 24-h challenge. Phages have also been successfully engineered to express an enzyme, DspB, which hydrolyzes an adhesion crucial for biofilm formation by *Staphylococcus* and *E. coli* (Lu & Collins, 2007). The engineered T7 phage reduced *E. coli* bacterial biofilm cell counts by over 99%. The above reports provide evidence that phage do have potential in controlling biofilms and emphasizes the need for continued development of phage and phage-encoded enzymes for this application.

The use of phages as biocontrol agents for food protection

Control of bacterial pathogens, which may be present on fresh fruit and vegetables and ready to eat foods, is a major concern because these foods do not generally undergo any further processing or cooking that would kill pathogens before consumption. The existing literature would suggest that phages also have potential applications in the control of pathogens in these circumstances (Goodridge, 2004; Sulakvelidze & Barrow, 2005). Hence, because phages are applied in a nonmedical setting, the regulations governing their application might not be as stringent as for medical applications. Specific studies describing the application of phages to control food pathogens on fruits and vegetables are outlined below. Leverentz and colleagues at the US Department of Agriculture (USDA) in Maryland studied the use of phages to control a variety of bacterial pathogens on freshly cut produce focusing their attention on honey dew melons and apples (Leverentz *et al.*, 2001, 2003, 2004). These researchers demonstrated a reduction of 3.5 log in *S. enterica* serovar Enteritidis on melon slices stored between 5 and 10 °C, which was a greater reduction when compared with chemical sanitizers (Leverentz *et al.*, 2001). However, no significant decrease on apple slices was observed (Leverentz *et al.*, 2001). These researchers also combined phage with the bacteriocin nisin for the control of *L. monocytogenes* on fresh cut apples and melons. Phage alone reduced *L. monocytogenes* counts by between 2 and 2.6 logs on melon slices following two applications of phage cocktails. The inclusion of nisin resulted in a decrease of 5.7 logs (Leverentz *et al.*, 2003). These researchers also recently optimized the phage concentration and timing of phage application via aerosol on freshly cut honeydew melons (Leverentz *et al.*, 2004). In a different study, *L. monocytogenes* contamination was also controlled on surface-ripened red smear soft cheese by at least 3.5 logs with *Listeria* phage P100 (Carlton *et al.*, 2005).

Phages have also been evaluated to reduce *C. jejuni* numbers on chicken skin at 4 and −20 °C (Atterbury *et al.*, 2003). In this study, the highest titre of phage (10^7 PFU mL^{-1}) gave the most promising results. A 10-fold reduction in samples stored at 4 °C and a 2.5 log reduction in samples stored at −20 °C was obtained. In a separate study, inoculation of chicken skins with 10^4 CFU mL^{-1} of *C. jejuni* and subsequent administration of phage (10^6 PFU cm^{-2}) resulted in a 95% reduction of *C. jejuni* numbers (Goode *et al.*, 2003). This group also studied the reduction of *S. enterica* serovar Enteritidis on chicken skin.

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Following phage administration, a 99% reduction in bacterial numbers was observed when compared with the controls where no phages were applied (Goode *et al.*, 2003). In another study, the inhibition of *S. enterica* serovar Typhimurium DT104 on frankfurters has been assessed (Whichard *et al.*, 2003). In this case two phages were used, Felix 01 and a mutant of Felix 01, which had increased lytic activity against *S. enterica* serovar Typhimurium DT104 *in vitro*. Phage Felix 01 and the mutant phage reduced cell numbers by 1.8 and 2.1 logs, respectively, when compared with nontreated frankfurters. In addition, phage Felix 01 has demonstrated the ability to survive acidic conditions and to lyse a broad range of *S. enterica* species (O'Flynn *et al.*, 2006). Furthermore, phage-resistant derivatives of the *S. enterica* strain tested exhibited an irregular colony morphology indicating that the unusual morphology is due to reversion to phage sensitivity and consequent cell death within the colony as it forms (O'Flynn et al., 2007).

The shelf life of food is an important factor in the food-processing industry. Accordingly, phages have also been assessed in this context. For example, the shelf life of beef steaks was almost doubled (from 1.6 to 2.9 days) by phage-mediated control of *Pseudomonas* (Greer, 1986). However, in a different study, the utilization of a cocktail of seven phages, which was active against 57.2% of *Pseudomonas* isolates *in vitro* proved unsuccessful in the same application (Greer & Dilts, 1990). Nevertheless, these researchers did demonstrate the control of the pork spoilage organism *Brochothrix thermosphacta* with phage (Greer & Dilts, 2002). In this case, the storage life of pork adipose tissue treated with phage was increased from 4 to 8 days when compared with the non-phage-treated control (Greer & Dilts, 2002). In another meat study, *E. coli* O157:H7 was successfully reduced or eliminated on meat surfaces by O'Flynn *et al.* (2004) with a coliphage cocktail.

As a result of the resurgence and studies into the possibility of using phage as antibacterials, a natural transition is the establishment of companies to market and get regulatory approval for phage-based products. In fact, numerous companies have developed or are in the process of developing phage-based products and in some cases, discussed here, already have received regulatory approval. This is an important step for the study and promotion of phages and their lysins as novel antibacterials.

Omnilytics, a phage company based in the United States, which sells phage cocktails for use in agriculture, has been using phages in greenhouses and fields to prevent disease on crops for over 10 years (http://www.phage.com/home5.html). Their AgriPhage™ product is marketed to farmers as a natural, safe and effective treatment especially for bacterial spot infections. AgriPhage™ is currently approved by the EPA and has OMRI (Organic Material Review Institute) listing for the control of bacterial spot (caused by *Xanthomonas campestris* pv. *vesicatoria*) and bacterial speck (caused by *Pseudomonas syringae* pv. *tomato*). In addition, this company is in the process of seeking approval from the EPA and OMRI for the control of bacterial canker (caused by *Clavibacter michiganensis* ssp. *michiganensis*) and received no objections from the USDA's Food Safety and Inspection Service for the use of anti-*E. coli* 0157:H7 and *Salmonella* phage on live animals before slaughter and for the use of *Salmonella* phage on poultry. In addition, Intralytix (http://www.intralytix.com), received FDA approval in 2006 and EPA approval in 2008 for the use of a six-phage cocktail designated LMP-102™ for use on ready to eat meat, poultry products and in food-processing plants to control *L. monocytogenes* contamination (Lang, 2006). This company is also seeking approval from the FDA for phage-based product against *E. coli* 0157:H7.

In Europe, a company based in the Netherlands, EBI Food Safety, has been granted generally recognized as safe (GRAS) status from the FDA and USDA for the phage product; LISTEX™, for food products against *L. monocytogenes* (Fortuna *et al.*, 2008). Therefore, this product is exempt from formal premarket safety review. In addition, LISTEX™ is considered organic under EU law as it has been recognized by the Dutch regulatory body (http://www.ebifoodsafety.com). To date, phage-approved products discussed above are to treat food-related products. Many of these companies are also investigating phage-based products for animal and human use. For example Novolytics, a UK-based company is actively researching the use of phage against drug-resistant bacteria such as MRSA. This company is currently producing phage under GMP (Good Manufacturing Practices) conditions. The UK Medicines and Healthcare products Regulatory Agency has given outline approval and the company plans to start toxicity testing and phase I clinical trials, with the subsequent commencement of phase IIA clinical trials (N. Mann, pers. commun.; http://www.novolytics.co.uk/).These developments will provide a chance to observe consumer response and attitude to the use of phage on food. It will also be interesting to see if this development will pave the way for consumer acceptance and the approval by regulatory bodies for other phage preparations or products.

Phage lysins as therapeutics

The vast amount of genetic information accumulated from phage genomic sequencing may be seen as a blueprint from which to design novel antimicrobial agents. In this respect, our current mechanistic knowledge of how phage exploit host biosynthetic machinery and eventually lyse the cell is very important. Possibly the best example of this is phage lytic enzymes and indeed, increased research into the utilization of phage lysins as therapeutics is evident

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Table 2. Phage lysins that have been tested in animal models

Bacteria	Phages	Lysins	Activity	References
Streptococcus pneumoniae	Cp1	Cpl-1	Muramidase	Loeffler *et al*. (2001, 2003); Jado *et al*. (2003); Loeffler & Fischetti (2003); Entenza *et al*. (2005); McCullers *et al*. (2007); Grandgirard *et al*. (2008)
Streptococcus pneumoniae	Dp-1	Pal	Amidase	Jado *et al*. (2003); Loeffler & Fischetti (2003)
Streptococcus pyogenes	C1	C1	Amidase	Nelson *et al*. (2001)
Bacillus anthracis	γ	PlyG	Amidase	Schuch *et al*. (2002)
Bacillus anthracis	*	PlyPH	Amidase	Yoong *et al*. (2006)
Enterococcus faecalis and *Enterococcus faecium*	Phi1	PlyV12	Amidase	Yoong *et al*. (2004)
Staphylococcus aureus	MR11	MV-L	Endopeptidase and amidase	Rashel *et al*. (2007)
GBS	Phage NCTC 11361	PlyGBS	Muramidase and endopeptidase	Cheng *et al*. (2005)

*This lysin was identified and amplified from the *Bacillus anthracis* Ames strain (Schuch *et al*., 2002).

Table 3. Advantages and disadvantages of phage and lysin to treat infectious bacteria

Phage	Lysin
Phage advantages	Lysin advantages
Easy to isolate and propagate	Not self-replicating, more targeted defined control
Can overcome resistance	Protein therapeutic
Self-replicating	Resistance not yet reported
Act synergistically in a cocktail or in combination with other antibiotics	Possibility to genetically engineer lysins
Inhibits Gram-positive and Gram-negative organisms	Specific bacterial targets
Some phage products have regulatory approval or GRAS status	Could be used as a prophylactic and for treatment
Potential for use in numerous environments (human, animal, food, biofilm, etc.)	Potential for use in numerous environments (human, animal, food, biofilm, etc.)
Historically have been in use for nearly a century	Can be identified and used from temperate and virulent phages
Possibility to genetically engineer phage	
Specific bacterial targets	
Could be used as a prophylactic and for treatment	
Phage disadvantages	Lysin disadvantages
Need to select for virulent phage to prevent genetic transfer	Not self-replicating
Bacterial strains can develop resistance	Protein; therefore, susceptible to inactivation
Many phages can have a limited host range	
Regulatory and consumer acceptance still required	To date not yet successfully applied against Gram-negative bacteria

(Table 2). As these enzymes break down the cell wall, they have the potential to be used as therapeutic agents in their own right. Table 3 outlines the advantages and disadvantages of using phages and phage lysins as therapeutics for bacterial control. The continuing emergence of phage genome sequences enables the putative identification of many lysins. Indeed, the potential of many phage lysins as therapeutics or biocontrol agents has already been demonstrated and will be described here.

Fischetti and colleagues have exploited these enzymes, which they have termed 'enzybiotics', to kill a variety of Gram-positive pathogens (Nelson *et al.*, 2001) reported the prophylactic use of a phage lysin in an *in vivo* model. In this study, phage lysin encoded by the C1 phage was utilized, which is specific for groups A, C and E streptococci. The addition of 1000 U of purified lysin *in vitro* within 5 s resulted in 100% inhibition of 10^7 CFU mL^{-1} of group A streptococci. Furthermore, in a mouse model of infection, protection of mice from group A streptococci colonization was evident. In this case, a single dose of lysin (250 U) was added to the oral cavity of mice before the addition of 10^7 CFU mL^{-1} of group A streptococci. Indeed, in an additional experiment following administration of lysin (500 U) to mice that were heavily colonized with group A streptococci, no detectable streptococci were detected 2 h post-treatment (Nelson *et al.*, 2001). The use of phage lysins to control *S. pneumoniae* was also studied by Fischetti's group (Loeffler *et al.*, 2001; Loeffler & Fischetti, 2003). These

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included the use of the purified lysin, Pal, which is active against 15 common serotypes of pneumococci (Fig. 3). Indeed, in a mouse model of infection, mice colonized with *S. pneumoniae* and treated with Pal lysin had undetectable bacterial numbers 5 h post-treatment (Loeffler *et al.*, 2001). Interestingly, in this case, the authors found that the capsule did not inhibit access of the amidase Pal to the cell wall. In the second study, lysin Cpl-1 was also shown to be effective against *S. pneumoniae* in a mouse model of infection as a topical application and when injected into the bloodstream (Loeffler *et al.*, 2003). A combination of Pal and Cpl-1 lysins resulted in an increased killing effect *in vitro* against *S. pneumoniae* (Loeffler & Fischetti, 2003). In addition, Jado *et al.* (2003) used a murine sepsis model to study the ability of pneumococcal phage lysins Pal and Cpl-1 to cure bacteraemia caused by *S. pneumoniae* strain 6B (a multi-drug-resistant serotype and the most common serotype isolated from children with bacteraemia). The group found that by injecting lysin Cpl-1 or Pal 1 h after bacterial challenge with strain 6B, the mice survived, whereas untreated mice challenged with 5×10^7 CFU mL^{-1} of strain 6B died within 72 h. Moreover, a synergistic effect was observed *in vivo* when both Pal and Cpl-1 were used in combination, as survival rates in animals that received both lysins were higher than in animals that received each alone. More recently, Cpl-1 was successfully used in treating *S. pneumoniae* in a model of endocarditis and bacterial meningitis in rats (Entenza *et al.*, 2005; Grandgirard *et al.*, 2008). In additional studies in mice with Cpl-1 lysin, acute otitis media, which is an infection in children commonly caused by *S. pneumoniae*, was prevented (McCullers *et al.*, 2007).

Phage lysin has also been utilized for the detection and elimination of *Bacillus anthracis* (Schuch *et al.*, 2002). In this case, the lysin was identified from γ phage of *B. anthracis* and was found to kill vegetative cells in addition to germinating spores. Challenge with *B. cereus* strain RSVF1 in a mouse model of infection resulted in death in all cases. However, 13 out of 19 mice recovered fully after injection with PlyG and the remaining six mice survived (Schuch *et al.*, 2002). Interestingly, no resistance was observed to Pal, C1 or PlyG in these studies (Loeffler *et al.*, 2001; Nelson *et al.*, 2001; Schuch *et al.*, 2002). A second lysin active against *B. anthracis* has been reported by Yoong *et al.* (2006). This lysin was designated PlyPH, due to its activity across a large pH range, from 4 to 10.5. In a mice model of infection, *c.* 40% of mice survived in the PlyPH group compared with 100% death of the control group within 38 h (Yoong *et al.*, 2006).

Recently, lysin PlyV12 was found to not only have activity against its host *E. faecalis* but also other Gram-positive pathogens such as staphylococci and streptococci. In this case, the authors suggested that this may be due to a common surface structure between these pathogens (Yoong

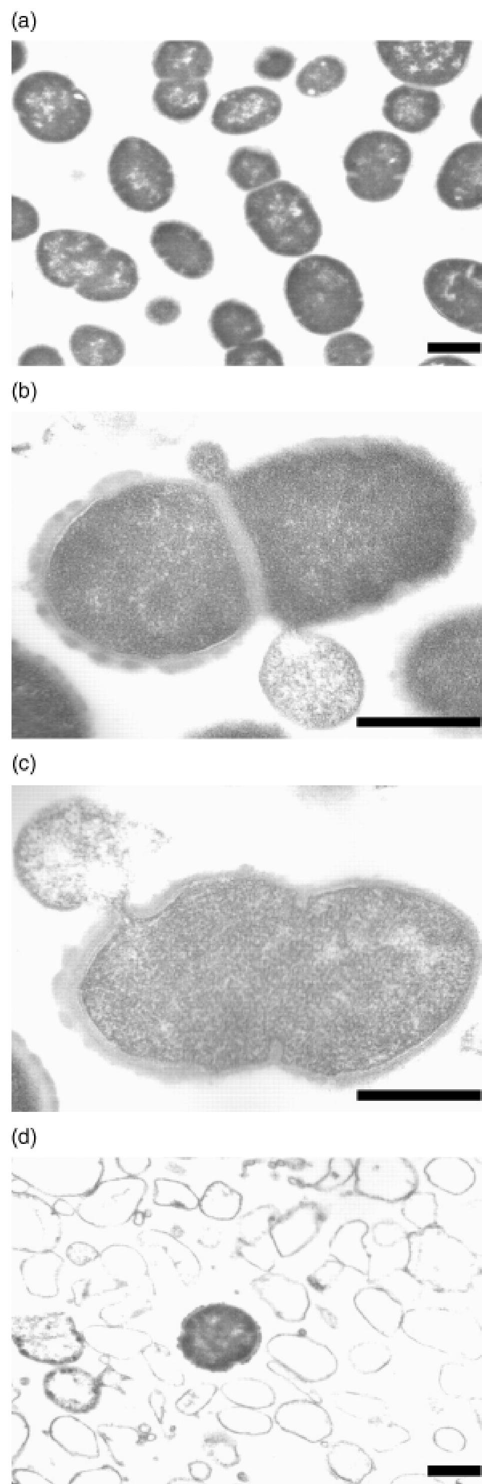

Fig. 3. Electron micrographs of *Streptococcus pneumoniae* exposed to Pal. (a) Unexposed control cells. Higher magnification of enzyme-treated cells after a 1-min exposure shows cell membrane protrusion (b) or cytoplasmic leaks from membrane rupture (c) through isolated breaks in the cell wall. After 5 min (d), killing is virtually complete and only empty cell walls are left. Scale bars = 0.5 μm. Reproduced from Loeffler *et al.* (2001) with permission.

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et al., 2004). Fischetti and colleagues have also studied a lysin active against group B streptococci (GBS), designated PlyGBS (Cheng *et al.*, 2005). PlyGBS was shown to have a broad spectrum of inhibition inhibiting all tested GBS serotypes *in vitro*. In a mouse model of infection, mice were vaginally challenged with 10^6 CFU mL^{-1} of GBS. Twenty-four hours later mice were treated vaginally with either buffer or PlyGBS lysin and swabs were taken 2 and 4 h later; when compared with the control, a 3 log reduction in GBS cell numbers was demonstrated in the mice that had received the lysin PlyGBS (Cheng *et al.*, 2005).

Phage lysin active against the pathogen *S. aureus* has also been described recently (Rashel *et al.*, 2007). Phage ΦMR11 has been described above where it protected mice from staphylococcal infection. This phage was subsequently sequenced and the lysin gene identified (Matsuzaki *et al.*, 2003; Rashel *et al.*, 2007). MV-L lysin was used to treat MRSA in the nasal cavities of mice and complete elimination of bacteria was observed in one of nine mice treated with MV-L lysin. The remaining mice had much lower CFU/nasal cavity numbers than the untreated mice. In an additional experiment with a model of systemic MRSA disease after 60 days, all mice treated with MV-L lysin directly or 30 min after bacterial administration survived compared with 60% mice survival 60 min postbacterial administration (Rashel *et al.*, 2007).

In addition, further *in vitro* work has been performed with lysins. For example, Pritchard *et al.* (2004) also cloned and expressed a lysin from a GBS phage. As GBS are a major cause of neonatal infections, this group studied the host range of the purified recombinant GBS phage lysin and found that it inhibited β-haemolytic streptococcal groups A, B, C, E and G. Zimmer *et al.* (2002) have also reported a phage lysin (ply3626) specifically active against *C. perfringens in vitro*. This organism is a causative pathogen of foodborne illnesses and also results in major economic losses in the poultry industry. Lysins such as LysK (O'Flaherty *et al.*, 2005a), LysH5 (Obeso *et al.*, 2008) and the endolysin from phage phi11 (Donovan *et al.*, 2006b) demonstrated *in vitro* activity against *S. aureus*, including drug-resistant strains in the case of LysK (Fig. 1).

Listeria monocytogenes is an important food-poisoning pathogen (McLauchlin *et al.*, 2004) and as such, is a prime target for new antimicrobials such as phage lysins. Loessner *et al.* (1995b) have described phage lysins Ply118, Ply511 and Ply500 encoded by *Listeria* phage A118, A511 and A500, respectively. Lysin Ply118 has been utilized for the disruption of the *Listeria* cell wall for DNA, RNA and protein extraction (Loessner *et al.*, 1995a). Moreover, Ply511 and Ply118 have also been cloned and expressed in *Lactococcus lactis* with the intention of producing lactococcal starter strains with anti-*L. monocytogenes* activity (Gaeng *et al.*, 2000).

Importantly, lytic enzymes originating from phages have huge potential from a therapeutic perspective as these enzymes show no adverse reactions during *in vivo* trials (Jado *et al.*, 2003) and no resistance to them has been discovered (Loeffler *et al.*, 2001; Nelson *et al.*, 2001; Schuch *et al.*, 2002). Another important feature of phage lysins is the capability to produce engineered lysins (Table 3). Lysins Pal and Cpl-1 from pneumococcal phages are among the most extensively studied phage lytic proteins that have demonstrated therapeutic potential. Work by López *et al.* (2004) demonstrated the two-domain structure of pneumococcal lysins; a catalytic and cell wall-binding domain. Further work by this group, which exploited the two-domain architecture of these lysins has included, (1) demonstration that both the binding and catalytic domains of lytic enzymes can be exchanged (Diaz *et al.*, 1990, 1991), (2) construction of a chimeric protein with two lytic activities (Sanz *et al.*, 1996) and (3) the exchange of enzyme specificity between bacterial species by construction of chimeric enzymes (Croux *et al.*, 1993; Lopez & Garcia, 2004). This work has demonstrated the possibility of engineering phage lysins by domain swapping to obtain lytic enzymes with multiple lytic activities and/or multiple binding domains, which have the potential to increase the therapeutic potential of phage lytic enzymes. Advances in structural engineering and proteomics will no doubt advance this field. Already there are studies where groups have truncated some of these lysins to their active 'core' (Donovan *et al.*, 2006a; Horgan *et al.*, 2009) where activity is still maintained in the smaller functional unit. In addition, the lysins discussed here are active against Gram-positive bacteria, but study into the possibility of lysing Gram-negative bacteria is needed. For example, Schuch *et al.* (2009) have described a genetic screen to identify lysins, which may also be applicable to Gram-negative bacteria. Although they lack the ability to self-replicate, as with antibiotics, studies into lysin dosage will be required in future studies. Furthermore, there is no reason why phage lysins cannot be used, where phages have been found to be applicable.

Concluding remarks

Phages have increasingly become the subject of renewed interest as agents to treat infections in recent years. The studies outlined above clearly show the efficacy of phage in killing human pathogenic bacteria in a number of microbial niches. While the results are very promising, there is still a critical need for well-designed double-blinded placebo-based human clinical trials to examine their efficacy in reducing carriage and treating infection. In an era where antibiotic resistance is causing many problems particularly in nosocomial situations, phage and phage-based technologies may prove to be valuable antimicrobial alternatives for widespread applications in the future.

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