Phage and Their Lysins as Biocontrol Agents for Food Safety Applications

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Abstract

Bacteriophage (phage) are bacterial viruses and are considered to be the most widely distributed and diverse natural biological entities. Soon after their discovery, bacteriophage were found to have antimicrobial properties that were exploited in many early anti-infection trials. However, the subsequent discovery of antibiotics led to a decline in the popularity of bacteriophage in much of the Western world, although work continued in the former Soviet Union and Eastern Europe. As a result of the emergence of antibiotic resistance in a number of bacterial pathogens, focus has been redirected back to bacteriophage and bacteriophage lysins as a means of pathogen control. Although bacteriophage have certain limitations, significant progress has been made toward their applications in food and has resulted in the U.S. Food and Drug Administration approving the use of a bacteriophage-based additive for the control of *Listeria monocytogenes* contamination. Furthermore, a number of animal studies have revealed the potential of bacteriophage for the control of various foodborne pathogens within the animal gastrointestinal tract and to subsequently decrease the likelihood of foodborne outbreaks. From a biopreservative perspective, phage have a number of key properties, including relative stability during storage, an ability to self-replicate, and a nontoxic nature. The purpose of this review is to highlight the recent developments in the use of phages and their lysins for biocontrol and to address their potential future applications.

INTRODUCTION

Bacteriophage (phage or Φ) are one of the most abundant replicating entities on earth and can be found in virtually all places where their bacterial hosts exist (Hendrix 2003). Phage are self-replicating agents that are able to multiply by hijacking their host's DNA replication and protein synthesis machinery. They can possess two life cycles, lytic and lysogenic. Virulent phage function by invading specific bacterial cells, resulting in cell death and the subsequent release of progeny phage that in turn infect and destroy neighboring bacterial cells (Sulakvelidze et al. 2001). In contrast, temperate phage can multiply via the lytic cycle or they can enter the lysogenic cycle by integrating their genome as a prophage into the host chromosome. This can facilitate the horizontal transfer of bacterial genes between different host bacteria (Sulakvelidze et al. 2001). From a safety perspective, virulent phage (phage that are exclusively lytic) offer many advantages as biocontrol or therapeutic agents, including their ability to target their host bacterium with high specificity without impacting on the other microbiota present (Sulakvelidze et al. 2001). This property favors the use of phage over antibiotics and other chemical agents for the control of specific pathogens in complex microbial niches such as food and the gastrointestinal tract because collateral damage inflicted on beneficial flora is mostly avoided.

Because of their importance, concentrated research efforts have been devoted to phage research, particularly over the past two decades. At present, more than 400 phage genomes have been completely sequenced, and progression in analytical techniques has improved our overall understanding of the evolution of phage genomes and the various functions of their genes (Hendrix 2003, Hendrix et al. 1999). The tailed phage are by far the most numerous and belong to the order of *Caudovirales*, which contains three families: *Myoviridae*, *Siphoviridae*, and *Podoviridae*. Phage in these families possess both contractile and noncontractile tails of varying length (Ackermann & Eisenstark 1974, Ackermann 1987). This is attached to the head of the phage containing the phage genome, which is packaged in an energy-dependent process (Rossmann et al. 2004) (**Figure 1**). Phage were first characterized in the pre-antibiotic era by Felix d'Herelle who published his findings in 1917 (Dublanchet & Fruciano 2008), although their antibacterial behavior was previously described by Twort in 1915 (Pennazio 2006). The use of phage for the treatment of infectious disease came about in 1919 when an epidemic of fowl typhoid in France led d'Herelle to study phage as potential antibacterials in greater detail (Dublanchet & Fruciano 2008). The first successful human administration of phage was accomplished in 1921 when d'Herelle applied a phage suspension to relieve dysentery in a twelve-year-old boy. It was reported that the patient's symptoms ceased after a single dose of the bacteriophage (Sulakvelidze et al. 2001). Later, various companies around the world produced therapeutic phage including l'Oréal, Paris and Eli Lilly, USA (Carlton et al. 2005, Garcia et al. 2008). Commercial production of phage was discontinued in the West with the advent of antibiotics (Garcia et al. 2008). Nevertheless, research into the potential of phage as antibacterial agents continued in the former Soviet Union (Sulakvelidze et al. 2001).

Remarkably, the therapeutic and prophylactic application of bacteriophages has encountered a revival of interest over the past two decades (Fortuna et al. 2008). As work in this area expands, a number of different research projects in industry have begun investing in phage technology, leading to innovations in the use of phage and their components as antibacterial agents (Hanlon 2007). One of the major breakthroughs occurred in 2006, when the United States Food and Drug Administration (FDA) approved the use of the LMP-102 phage preparation from Intralytix as an additive for the control of *Listeria monocytogenes* on ready to eat (RTE) foods such as meat and poultry products (Bren 2007). Listex P100, a phage that targets *L. monocytogenes*, was also approved by the FDA and the United States Department of Agriculture (USDA) for the control of the pathogen in meat and cheese products (Carlton et al. 2005) (**Figure 2**). In addition, OmniLytics,

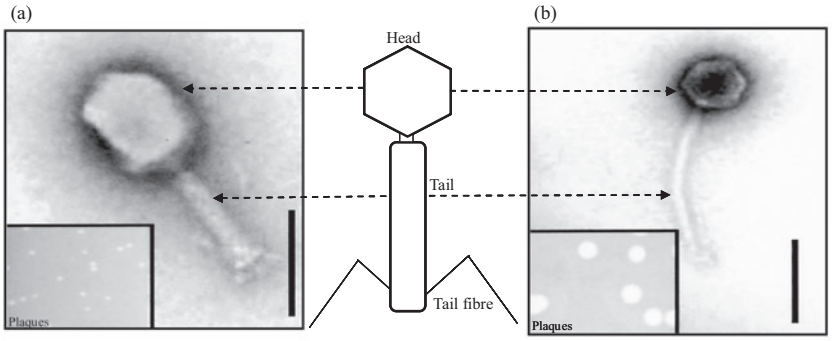

Figure 1

Electron micrograph (EM) images of *Escherichia coli* O157:H7 phage negatively stained with 1% (w/v) uranyl acetate and phage plaque morphology in insert (Reproduced from O'Flynn et al. 2006). EM identifications: (*a*) Phage e4/1c and associated plaques, (*b*) Phage e11/2 and associated plaques. Scale bar represents 100 nm.

Inc., Salt Lake City, USA, which also develops phage solutions for pathogen control, received U.S. Environmental Protection Agency (EPA) approval for the use of its product, Agriphage, to combat phytopathogenic bacteria (Garcia et al. 2008, OmniLytics). Such developments represent a clear and formal recognition of phage as realistic antibacterial agents in food production.

Foodborne disease is a costly issue in any country and figures for the United States alone have been estimated between 5 and 6 billion dollars per annum in direct medical expenses and lost productivity. Phage biocontrol can offer many advantages for the control of such disease from both therapeutic and biopreservative perspectives. This review therefore focuses on the application of phage to limit infection by four of the main foodborne pathogens, namely *L. monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7, and *Campylobacter* (**Table 1** shows the principal phage discussed in the review).

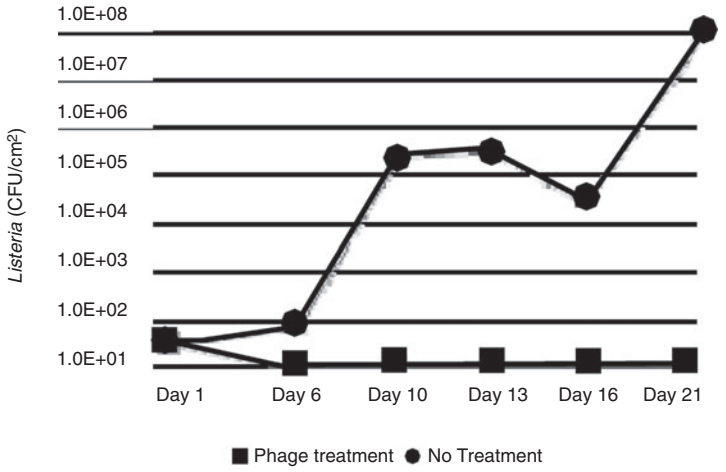

Figure 2

Graph demonstrating the effectiveness of Listex^{TM} P100 for controlling *Listeria monocytogenes* on the surface of smear ripened cheese. [Reproduced from EBI Food Safety (**http://www.ebifoodsafety.com**)].

Table 1 In vivo phage studies

Pathogen	Phage	Phage biocontrol	Reference
Listeria monocytogenes	LMP-102	First of its kind to be regulated as a food additive, a combined mixture of 6 *Listeria* specific phage	(Bren 2007)
	LM-103 and LMP-102	14 and 6 lytic phage cocktail, respectively combined with nisin and applied to reduce *Listeria* counts on Honeydew melons	(Leverentz et al. 2003, 2004)
	A511 and P100	Applied to a range of RTE foods in varying conditions	(Guenther et al. 2009)
Salmonella	SP6	Administered to chickens to combat effects of *Salmonella*	(Hurley et al. 2008)
	Felix O1	Microencapsulation technique to ensure protection of phage in the gastrointestinal tract	(Ma et al. 2008)
	P7	Applied to *Salmonella*-treated meat at low and high concentrations	(Bigwood et al. 2009)
E. coli O157:H7	KH1/SH1	Administered orally and rectally to sheep and cattle	(Sheng et al. 2004)
	e11/2, e4/1c and PPO1	Applied to meat surface	(O'Flynn et al. 2004)
	ECP-100	Applied to surface of hard surface foods	(Abuladze et al. 2008)
Campylobacter	29C	Applied to *Campylobacter*-infected chicken skin	(Goode et al. 2003)
	CP8 and CP34	Chickens inoculated with *C. jejuni* followed by phage	(Loc Carrillo et al. 2005)

LISTERIA MONOCYTOGENES

Listeria monocytogenes is an important foodborne pathogen, responsible for listeriosis, a potentially fatal illness that results primarily from the ingestion of contaminated RTE foods (Chan & Wiedmann 2009). Symptoms of listeriosis include diarrhea, abortion, and infection of the brain and central nervous system (Carlton et al. 2005). A further concern regarding this ubiquitous bacterium is its phycrotrophic nature, which permits it to survive and multiply to high levels at low temperatures, leading to proliferation in contaminated refrigerated foods during storage (Hendrix et al. 1999, Hendrix 2003). This hardy pathogen can also withstand varying environmental conditions, such as those encountered in food preparation, for instance, high salt levels, low pH (<pH6), lack of oxygen, and as mentioned lower temperatures (Guenther et al. 2009, Seeliger & Jones 1986). As a result of its high fatality rate (25–30%), listeriosis is categorized as one of the most severe foodborne illnesses (Carlton et al. 2005). Indeed, it has been estimated that 2000 hospitalizations and 500 deaths occur annually in the United States alone as a direct result of the consumption of *Listeria*-contaminated foods (Mead et al. 1999). Reported outbreaks of *L. monocytogenes* were usually associated with foods containing levels of 10^3 CFU/ml of the bacterium (Tompkin 2002). Nevertheless, because of high fatality rates associated with this pathogen, the FDA and USDA Food Safety Inspection Service have implemented a zero tolerance for this pathogen, which has resulted in a number of food recalls (2009). In acknowledgment of the importance of this pathogen, a recent study investigated the prevalence of *L. monocytogenes* in RTE foods and the potential threat of illness from these products. When a total of 711 different RTE food product samples were selected randomly from supermarkets and pastry shops and analyzed, 26.6% of the samples were found to be positive for *Listeria* (Mengesha et al. 2009).

Information on available *Listeria* phage is somewhat limited (Loessner et al. 2000), although at least twelve genomes are publicly available on the National Center for Biotechnology Information (NCBI) database. In this respect, it is important to emphasize that knowledge on the genetic make-up of phage destined for food applications is essential, especially in terms of the potential for genetic transfer of virulence factors such as toxins and antibiotic resistance between bacteria by phage (Kelly et al. 2009). Φ A511 was isolated from a sewage sample and was found to be extremely rare, as most *Listeria* phage are temperate. However, Φ A511 is a virulent phage which can infect up to 95% of common *L. monocytogenes* strains (Klumpp et al. 2008, Loessner et al. 2000). It contains a genome of 137,619 bp and has a nonflexible contractile tail. Following bioinformatic analysis, this phage was found to be closely related to another *Listeria* phage Φ P100. The broad host range *Listeria* Φ P100 was first isolated in 1997 from a sewage effluent sample taken from a dairy plant in south Germany. Φ P100 has a genome size of 131,384 bp, containing 174 open reading frames (ORFs). Importantly, none of the putative proteins bore any resemblance to toxins, pathogenic factors, or allergens (Carlton et al. 2005). Other *Listeria* phage have also been investigated including Φ A118, which has a genome size of 40,834 bp, encoding a total of 72 ORFs, 26 of which have been assigned functions, including DNA packaging proteins and lysis components (Loessner et al. 2000). Comparison of the genome structure of Φ A118 with other bacteriophage revealed extensive similarities where bioinformatic analysis showed specific portions of the Φ A118 genome resembled certain functional areas of other bacteriophage genomes, namely phage that infect *Lactobacillus*, *Streptococcus thermophilus*, and *Bacillus* hosts.

Listeria Lysins

Lysins are highly evolved enzymes encoded by phage that play a role in the digestion of bacterial cell walls, permitting the release of newly formed phage from the bacterial cell (Fischetti 2008). Lysins are synthesized in the bacterial cytoplasm and can target various peptidoglycan bonds in the cell wall. They are thus generally dependent on phage-encoded holin enzyme, which disrupts the cytoplasmic membrane to allow them access to the cell wall substrate (Wang et al. 2000a). As lysins are capable of making direct contact with the cell wall peptidoglycan when added externally (lysis from without), they can be highly effective at killing Gram-positive bacteria but are generally ineffective against Gram-negative bacteria when added externally (Fischetti 2008). One of the many advantages of lysins is also the absence of bacterial resistance, given that generally a cell has to change the structure of its wall structure to become lysin resistant. Indeed, even after 40 cycles of exposing bacteria to a particular lysin, resistant strains did not develop (Fischetti 2008). Another important advantage of lysins is that within a bacterial genus, they do not exhibit limited host specificity, unlike their phages. Lysins have shown promise for the biocontrol of *Listeria*. Indeed, one study demonstrated that recombinant *Lactococcus lactis* cells, containing the genes encoding the listerial lysins Ply 118 and Ply 511, induced the lysis of *L. monocytogenes* cells in surrounding medium. Secretion of a functional lysin was also observed in *L. lactis* cells in the presence of lactose suggesting that the system could be used during fermentation of milk. These observations demonstrate the potential to impart starter cultures with an intrinsic antimicrobial system to selectively protect dairy products from *L. monocytogenes* contamination (Gaeng et al. 2000). However, such pathobiotechnology approaches do raise some safety concerns, as these lysin-producing starter cultures have been engineered with genetic material from a pathogen-associated source. Moreover, there are significant technical challenges to the use of *Listeria* lysins (from either a biopreservative or therapeutic perspective). (*a*) These relatively large proteins are subject to proteolysis in some foods or indeed in the gut, being the site of infection of *Listeria*;

(*b*) they are relatively expensive to produce; and (*c*) their safety in terms of food use needs to be proven. One can certainly envisage lysins of *Listeria* being used for surface applications to eliminate this ubiquitous problematic pathogen.

Phage for Biocontrol of *Listeria*

As previously mentioned, the FDA recently approved the use of a bacteriophage preparation for use in RTE foods in an attempt to combat *L. monocytogenes*, namely LMP-102 (Bren 2007). This preparation was the first of its kind to be regulated as a food additive and contains a mixture of six *Listeria*-specific phage that are known to be effective against 170 strains of *Listeria* (Bren 2007). The inclusion of six different phage served to reduce the occurrence of pathogen resistance to an individual phage. It is recommended that this phage preparation of 1ml per 500 cm^2 be used on the surface of RTE meat and poultry products prior to packaging (Bren 2007). Purification steps and strict protocols followed in the preparation of this phage cocktail ensure that no toxin factors are present in any of the six separate phage genomes and likewise that any other toxic elements from the host organism have been removed from the final product ensuring its safety for inclusion as a food additive (Bren 2007).

Other studies in this area have demonstrated synergistic activity between bacteriophage and other treatments. Combined phage and nisin treatments reduced bacterial populations of *L. monocytogenes* on honeydew melons more significantly than with nisin treatments alone (Leverentz et al. 2003). Phage preparations used in this study were LM-103 and LMP-102, which contained 14 and 6 lytic bacteriophages specific to *L. monocytogenes*, respectively, and were produced by Intralytix Inc. The results demonstrated that the application of both phage and nisin reduced *Listeria* numbers up to almost a millionfold. Although ready-made meals, dairy products, and meat are some of the foods at highest risk from contamination, fruit and vegetable contamination also occurs. In another study by the same group, melon pieces were sprayed with a cocktail of *Listeria*-specific phage at various time points before and after inoculation of *L. monocytogenes*. Interestingly, the phage treatment was most effective when it was applied at 1, 0.5, and 0 hours before application of *L. monocytogenes* with reduction levels of up to 6.8 log units after seven days of storage (Leverentz et al. 2004). One adaptation of this type of phage control would be to spray fruit immediately after cutting, thus optimizing the opportunity for reducing bacterial counts.

In another recent study, it was found that broad host range phage are very efficient in the biocontrol of *L. monocytogenes* in a range of foods, including meat, fish, dairy, and plant foods. To establish this, RTE food samples were deliberately infected with *L. monocytogenes* cells and *L. monocytogenes*-specific broad host range virulent phages, namely A511, and P100. Following storage at 6°C for six days, it was found that either phage could bring about a reduction in bacterial counts by up to five log units on most ranging foods. When the phage were applied to liquid foods such as chocolate milk and mozzarella cheese brine, a rapid decrease in bacterial counts (below the level of detection) was observed even over extended storage periods. This study showed that the amounts of phage needed for efficacy varied depending on the food matrix. This study also showed that higher phage titres were more effective than low doses at reducing bacterial counts. Specifically, it was found that in order to obtain optimum efficiency, it is necessary to apply concentrations of 10^8 PFU/g of food (Guenther et al. 2009). Subsequent work with Φ P100 was done in which various cheeses were contaminated with *L. monocytogenes* followed by application of Φ P100. It was demonstrated that Φ P100 reduced *L. monocytogenes* numbers by up to 3.5 logs and in some cases total elimination of the pathogen was achieved. In addition, no problems with phage resistance were encountered (Guenther et al. 2009, Carlton et al. 2005).

In conclusion, these results clearly demonstrate that broad host range phage, such as Φ A511 and Φ P100, have a strong potential to control *L. monocytogenes* levels in a variety of RTE food. Their application offers a highly attractive option to aid in the management of this pathogen.

SALMONELLA

Salmonella are rod-shaped, Gram-negative, facultatively anaerobic, pathogenic bacteria closely related to *E. coli*. When consumed by animals and humans, the organism can result in the development of a condition known as salmonellosis. Symptoms of salmonellosis are characterized by nausea, vomiting, abdominal cramps, diarrhea, fever, and headache between 6 hours and 48 hours after consumption (Barbara et al. 2000). In humans, salmonellosis occurs when the organism passes from the gastrointestinal tract into the epithelial tissue resulting in inflammation (Barbara et al. 2000). *Salmonella enterica* subsp. *enterica* serotype Enteritidis is recognized as one of the most important and common causes of *Salmonella* outbreaks in humans (Barbara et al. 2000). Indeed, foodborne salmonellosis has been reported to cause up to 600 deaths and 1.4 million illnesses in the United States annually (Mead et al. 1999). Sources of outbreaks range from leafy greens and tomatoes (Wright et al. 2009) to poultry products (Mishu et al. 1994), and consequently, an effective means of controlling this pathogen is urgently required.

Although there is an abundance of information relating to the genomics of *Salmonella* phage, Φ P22 is best characterized (**Figure 3**). This phage is a typical member of the family *Podoviridae* with characteristics including an icosahedral head and a short, noncontractile tail (Ackermann & Eisenstark 1974). It is a temperate phage and binds to its host via the interaction between the phage tail spike proteins and the lipopolysaccharide (LPS) O side chains of serovar typhimurium *rfb* cluster found on the bacterial surface (Steinbacher et al. 1997). Φ P22 is extremely similar to phage lambda in DNA sequence, genetic organization, and physiology (Susskind & Botstein 1978).

Another well-studied *Salmonella* phage is Φ ES18, a temperate phage that was isolated in 1953 (Callow 1959, Casjens et al. 2005). Φ ES18 is a member of the lambdoid phage group, and its 46 kb genome consists of 79 ORFs. Φ ES18 is particularly interesting because of the fact that it can infect both smooth (O antigen producing) and rough (O antigen defective) strains of *S. enterica*, as well as some *E. coli* strains, providing they display the *Salmonella* surface receptor. Some genes located on the Φ ES18 sequence show high similarity to Φ lambda including gene 29 which is similar to the *J* gene and is responsible for determining the host range for Φ lambda (Wang et al. 2000b). The Φ ES18 genome is typical of all lambdoid phages because of its similarities in transcription, predicted functions, and other characteristics to Φ lambda.

Phage for Biocontrol of *Salmonella*

Many studies involving phage therapy for the bio-control of *Salmonella* have been undertaken with promising outcomes. Most of these have focused on the use of phage to reduce carriage of *Salmonella* in poultry rather than as a food additive. In addition, in vitro, in vivo, and in-silico phage-host modeling studies have also been undertaken, one using Φ SP6, a T7-like virulent *Salmonella* phage (Hurley 2008), and another using *Salmonella* Φ P7 (Bigwood et al. 2009). Such studies can assist in predicting optimal phage treatments for this pathogen.

In the context of biocontrol in live animals, Borie et al. (2008) isolated three lytic phage from the sewage system of commercial chicken flocks and administered them as a cocktail to ten-day-old chickens in their drinking water and by spray application, before challenge with *S. enteritidis*. Both phage applications were found to reduce the levels of *S. enteritidis* in the intestinal tract

(Borie et al. 2008). Based on these results, the authors concluded that phage could be a suitable replacement for antibiotic therapy in these circumstances. A number of other studies have had similar successful outcomes (Bigwood et al. 2008, Bren 2007, Mead et al. 1999). Nevertheless, it is important to understand that the efficacy of the approach always depends on the phage host range, burst size, the structural integrity of the phage particle in the potentially damaging gut environment, physical access of the phage to all target bacteria in the gastro-intestinal tract, and the physiological state of the target bacterium. Not all phage interventions automatically give rise to elimination of the pathogen, as shown by Hurley et al. (2008) with *Salmonella* Φ SP6. Specifically, on the point of protecting the phage from the adverse acidic conditions, digestive enzymes and other potentially damaging substances in the gut (Joerger 2003), Ma et al. (2008) addressed the problem by employing a phage microencapsulation method to ensure delivery of the phage unharmed to the main site of *Salmonella* colonization. This study utilized the well-known *Salmonella* Φ Felix O1 in a porcine gut environment. Assays carried out prior to encapsulation of the phage demonstrated that activity of Φ Felix O1 was significantly affected in extreme acidic conditions and following contact with bile salts. Fortunately, the encapsulation procedure did not reduce the ability of the Φ Felix O1 to function. This approach of phage protection during gut transit is likely to be of use with many phage candidates.

With regard to using *Salmonella* phage as a food additive, Bigwood et al. (2008) investigated whether phage Φ P7 retained its ability to lyse *Salmonella* when present on a meat surface. The meat was inoculated with the host strain and subsequently treated with Φ P7 at the two temperatures of 5°C and 24°C. These applications were carried out at low and high host concentrations, as well as low and high multiplicity of infection (MOI) values. Within 24 hours of incubation the *Salmonella* cell numbers were significantly reduced on both raw and cooked meat samples, and this phage was able to effectively reduce cell numbers when host cell density and MOI were both high (Bigwood et al. 2008). This study shows that *Salmonella* phage certainly has a strong potential to work as an effective food additive for the removal of this important pathogen.

ESCHERICHIA COLI O157:H7

The chief route of human infection by *E. coli* O157:H7 is via foods of bovine origin (Armstrong et al. 1996). Studies have shown that the occurrence of *E. coli* O157:H7 on the hide of the animal before slaughter can lead to the contamination of meat during the slaughtering process (Elder et al. 2000). In addition, *E. coli* O157:H7 has a very low infectious dose, with as few as 10 cells being capable of causing infection. Being a gut bacterium, it can survive well under intestinal conditions (Kaper 1998). Outbreaks of *E. coli* O157:H7 have been primarily associated with the ingestion of contaminated beef products (Armstrong et al. 1996). Once ingested, the organism travels in the gastrointestinal tract where it adheres closely to the mucosal cells of the bowel (Mead & Griffin 1998). This condition can progress into hemorrhagic colitis, with symptoms that include severe abdominal cramps and bloody diarrhea. Infections occur in all age groups but are more common in children and the elderly. In extreme cases hemolytic uremic syndrome (HUS) can develop as a result of the binding of shiga toxins to endothelial cells of the kidneys. This can lead to anemia as a result of destruction of red blood cells, leading to kidney failure. HUS is more likely to occur in children under five years and in some cases can be fatal (Mead & Griffin 1998, Karmali et al. 1983). Furthermore, complications can arise from HUS leading to a condition known as thrombocytopaenia (TTP), a syndrome that includes the main clinical features of HUS but with additional neurological involvement and is more common in the elderly (Karmali et al. 1983).

In light of the seriousness of the disease caused by *E. coli* O157:H7, much research has been directed at determining the primary sources of contamination. Although studies have shown that

contamination of the hide before slaughter leads to contamination of meat during the slaughtering process (Elder et al. 2000), possible contamination of carcass tissue during this process is the most significant threat to food safety (McEvoy et al. 2003). Regrettably, control of this is difficult because of the sporadic nature of *E. coli* O157:H7 shedding in positive animals. Indeed, verifying a pattern for the shedding of *E. coli* O157:H7 in an animal has been complex, as an animal which initially tests positive may not test positive again for days or even weeks (Mechie et al. 1997, Barrow et al. 1998). Other sources of *E. coli* O157:H7 contamination include the intake of contaminated drinking water or fruit juices, contaminated recreational water, contact with culture-positive animals, and consumption of food types that have come into contact with manure from infected animals (Barrow 1998, Bigwood et al. 2009, Danis et al. 2009, Dublanchet & Fruciano 2008).

E. coli O157:H7 causes more than 73,000 illnesses in the United States each year and approximately 60 deaths (Mead & Griffin 1998). A recent multi-state outbreak of *E. coli* O157:H7 involved ground beef purchased from a retail store where 49 cases had been confirmed. As a result of this outbreak twenty-seven patients were hospitalized, and one patient developed HUS (CDC 2007). Another recent case resulted in the recall of 21.7 million pounds of frozen ground beef patties. In this outbreak, 21 patients were hospitalized and two developed HUS. It was shown in this case that many of the patients had consumed the same brand of frozen ground beef patties (CDC 2008).

Much work has focused on phage therapy for the biocontrol of *E. coli* O157:H7 (**Table 2**), and this in turn has helped in the understanding of the genomic structure of O157-specific phages. One such bacteriophage, Φ PPO1, isolated from a swine stool sample, was found to infect strains of *E. coli* O157 serotype but not strains of other serotypes (Hendrix 2003). This phage was shown to be a member of the T-even phage group. Research into the use of this phage indicated that contact with the host cell for extended periods of time led to the development of Φ PPO1 resistant *E. coli*

Table 2 Collection of O157:H7-specific bacteriophages

Bacteriophage	Isolation/Source	Host bacteria	Reference
e11/2	Bovine slurry	*E. coli* O157:H7	(O'Flynn et al. 2004)
e4/1c	Bovine slurry	*E. coli* O157:H7	(O'Flynn et al. 2004)
PPO1	Swine stool sample	*E. coli* O157:H7	(O'Flynn et al. 2004)
rV5	Public Health Agency Canada, Laboratory for Foodborne Zoonoses	*E. coli* O157:H7	(Rozema et al. 2009)
wV7	Public Health Agency Canada, Laboratory for Foodborne Zoonoses	*E. coli* O157:H7	(Rozema et al. 2009)
wV8	Public Health Agency Canada, Laboratory for Foodborne Zoonoses	*E. coli* O157:H7	(Rozema et al. 2009)
wV11	Public Health Agency Canada, Laboratory for Foodborne Zoonoses	*E. coli* O157:H7	(Rozema et al. 2009)
SP15	Stock animals and sewage effluent	*E. coli* O157:H7	(Tanji et al. 2005)
SP21	Stock animals and sewage effluent	*E. coli* O157:H7	(Tanji et al. 2005)
SP22	Stock animals and sewage effluent	*E. coli* O157:H7	(Tanji et al. 2005)
Cev1	Sheep feces	*E. coli* O157:H7	(Raya et al. 2006)
KH1	Bovine and ovine samples	*E. coli* O157:H7	(Kudva et al. 1999)
KH4	Bovine and ovine samples	*E. coli* O157:H7	(Kudva et al. 1999)
KH5	Bovine and ovine samples	*E. coli* O157:H7	(Kudva et al. 1999)
ECP-100 (3 phage cocktail of ECML-4, ECML-117, and ECML-134)	Fresh- and saltwater environments	*E. coli* O157:H7	(Abuladze et al. 2008)

O157:H7 mutants. This initially proved to be a limiting factor for this phage but also prompted further work into determining how the development of resistance could be overcome. Phage specificity is determined by receptors on the surface of the host cell being compatible with phage binding (Hendrix 2003). The receptor on *E. coli* O157:H7 host strains for Φ PPO1 is the OmpC membrane protein. Further work demonstrated that it is possible to broaden the host range of Φ PPO1 by altering its genome sequence. Phage T2 genes responsible for host specificity were successfully incorporated into the Φ PPO1 genome, which resulted in Φ PPO1 having a greater host range and also reduced the occurrence of phage resistant mutants (Hendrix 2003). Specificity of infection for various phage is an independent factor and varies from phage to phage. However, if scientific adaptation of phage genomes is introduced and accepted, this strategy may assist the progress of molecular breeding of superior phage for biocontrol and therapeutic use (Yoichi et al. 2005).

Several studies have shown that genetic exchange can occur between bacteria and phage (Byl & Kropinski 2000), and this is a major concern when selecting phages for use as food additives and for therapeutic applications. One example cited in genomic studies (Hendrix et al. 1999) was the T4-like phage JS98, whose genome harbored a variety of bacterial virulence genes that were thought to have transferred by genetic exchange from host to phage (Chibani-Chennoufi et al. 2004). It is now recognized that phage to be used as antibacterials are sequenced and examined for the presence of virulence factors as well as determinants enabling integration into the host genome.

Phage Biocontrol of *E. coli* O157

In recent years, there has been an increased interest in the occurrence of preharvest intervention strategies to reduce various pathogenic bacteria in food animals prior to slaughter (Sargeant et al. 2007). In this context, in vitro studies have demonstrated the efficacy of phage in killing *E. coli* O157:H7 (LeJeune & Wetzel 2007) (**Figure 4**). Recently, the potential of phage to combat *E. coli* O157:H7 infection in cattle was supported by in vivo studies using murine models (Tanji et al. 2005), although subsequent studies in ruminants proved less successful (Sheng et al. 2006). Indeed, the failure of phage to remove intestinal *E. coli* O157:H7 in sheep and calves was proposed to be due to differences in numbers of the pathogen at various sites of the gastrointestinal tract

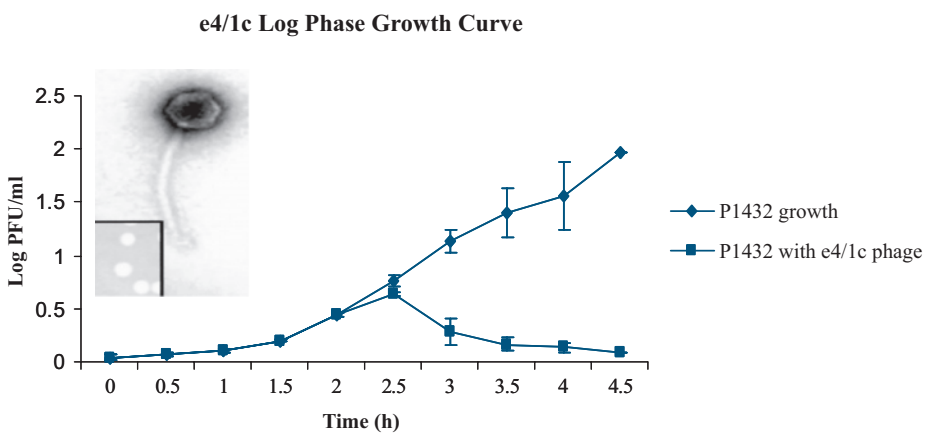

Figure 4

Demonstration of the lytic ability of phage e4/1c to lyse *E. coli* O157:H7 (P1432) in vitro, (♦) P1432 control grown in the absence of phage, and (■) P1432 infected with phage e4/1c.

(Sheng et al. 2006). In this ruminant study, Sheng et al. (2006) examined the effects of site application of phage to control intestinal *E. coli* O157:H7 in ruminants whereby two phage, namely Φ KH1 and Φ SH1, were administered orally and rectally to both sheep and cattle. However, the results demonstrated no reduction in the carriage of *E. coli* O157:H7 when applied orally, although a combination of the two phage administered rectally did reduce numbers of *E. coli* O157:H7, but were unable to clear the pathogen from the animals (Sheng et al. 2006).

Indeed, it has been shown that the primary site of *E. coli* O157:H7 colonization in cattle is in the rectoanal junction (Grauke et al. 2002), and this study went on to demonstrate that application of phage directly into the rectoanal junction can aid the reduction of *E. coli* numbers. Indeed, in vivo studies by Callaway et al. (2004) using a cocktail of phage, isolated from cattle feces, showed a reduction in *E. coli* O157:H7 populations in the feces of sheep. Moreover, it was noticed upon necropsy that *E. coli* O157:H7 populations were reduced by the phage treatments in the cecum and also the rectum (Callaway et al. 2004).

In terms of meat decontamination, a recent study demonstrated the potential of a mixture of three *E. coli* O157:H7–specific phage for the elimination of *E. coli* O157:H7 from the surfaces of seven of nine specimens of experimentally contaminated sliced beef (O'Flynn et al. 2004). It was also noted that using the cocktail of lytic phage specific for the same bacteria prevented the occurrence of bacterial resistance. One other study carried out by Abuladze et al. (2008), revealed how a cocktail of *Myoviridae* type phages, ECML-4, ECML-117, and ECML-134, together designated as ECP_100, were used to treat four different *E. coli* O157:H7–contaminated foods, tomato, spinach, broccoli, and ground beef. The phage preparation significantly reduced contamination of the foods, with the reduction ranging from 94% to 100% compared with controls. Results obtained from this study support the idea that *E. coli* O157:H7–specific lytic phage are capable of significantly reducing contamination of inanimate hard surfaces with *E. coli* even though the pathogen may be surrounded by dried organic matter (Abuladze et al. 2008).

CAMPYLOBACTER

Campylobacter jejuni is a Gram-negative, spiral-shaped, motile bacterium and is one of the most important human pathogens worldwide, being a major cause of acute human diarrheal illness in the developed world (Lindqvist et al. 2001). Consumption of the organism even at low doses (400–500 cells) can result in the onset of campylobacterosis. This disease is characterized by watery diarrhea, which may contain blood, and can be accompanied by abdominal pain, nausea, headache, and muscle pain, which can occur between seven to ten days after consumption (Brouwer et al. 1979). Infection due to *Campylobacter* is one of the most prevalent causes of bacterial illnesses in England and Wales, where estimates of incidents reached more than 359,000 in the year 2000 alone (Adak et al. 2002). It has been proposed that infection occurs because of inadequate hygiene, contaminated drinking water, or external factors such as transfer on boots or farm equipment (Evans & Sayers 2000). Also, the ingestion of undercooked poultry or cross contamination of other food types poses a further risk of infection (Humphrey et al. 2001). *C. jejuni* is found in the poultry environment and in warm-blooded animals including pets, rodents, and wild birds, adding to the difficult task of controlling this pathogen (Newell & Fearnley 2003). A further source of infection is within cattle, as they regularly accommodate *Campylobacter* in their gastrointestinal tract, thus raw milk can be contaminated by *Campylobacter* through the feces during the milking process (Oliver et al. 2005). In a recent case study carried out within the Republic of Ireland and Northern Ireland, the most vital risk factors for *Campylobacter* infection were found to include consumption of chicken, lettuce, eating from take-away restaurants, and contact with sheep, and resulted in problems with stomach ulcers or lower bowel difficulties (Danis et al. 2009).

Table 3 *Campylobacter* **phages and corresponding propagating strains**

Bacteriophages Reference Number NCTC	ATCT	Species	Propagating Strains NCTC No.
12669		*C. jejuni*	12658
12670		*C. jejuni*	12659
12671		*C. jejuni*	12660
12672		*C. jejuni*	12660
12673	35,925-B2	*C. jejuni*	12661
12674	35,922-B2	*C. jejuni*	12661
12675		*C. jejuni*	12622
12676	35,920-B1	*C. jejuni*	12663
12677	35,920-B2	*C. jejuni*	12663
12678		*C. jejuni*	12664
12679	35,922-B3	*C. jejuni*	12664
12680	35,924-B1	*C. jejuni*	12665
12681		*C. coli*	12666
12682		*C. coli*	12667
12683		*C. coli*	12668
12684		*C. coli*	12668

NCTC, National Collection of Type Cultures; ATCC, American Type Culture Collection.
Reproduced from Sails et al. (1998).

It has been stated that *Campylobacter* phage can be isolated from poultry excreta and hence pose as very suitable therapeutic agents for campylobacter control, thus ruling out the introduction of new biological entities into the food chain (Loc Carrillo et al. 2005).

However, there is a deficit of taxonomic classification work reported on *Campylobacter* phage (Sails et al. 1998). One study characterized 16 virulent *Campylobacter* phage (**Table 3**) using electron microscopy, pulse-field gel electrophoresis, and restriction endonuclease analysis. This study found all phage to be members of the *Myoviridae* family because of the possession of icosahedral heads and contractile tails, and the phage were further divided into three groups according to their genome and head size, which is unique to other phage classification strategies (Sails et al. 1998).

In relation to *Campylobacter* control by phage, the issue of bacteria developing phage resistance has been investigated (Scott et al. 2007b). Interestingly, it has been found that among *C. jejuni* that survive phage attack in broiler chickens, phage-resistant types that display evidence of genomic rearrangements also exist. These rearrangements were identified as intragenomic inversions between Mu-like prophage DNA sequences to invert genomic segments up to 590 kb in size. The resulting strains exhibited three phenotypes: resistance to infection by virulent phage, inefficient colonization of the broiler chicken intestine, and the production of infectious phage CampMu. These genotypes were recovered from chickens in the presence of virulent phage but not in vitro. Reintroduction of these strains into chickens in the absence of phage resulted in further genomic rearrangements at the same locations, leading to reversion to phage sensitivity and colonization proficiency. The findings from a further study (Scott et al. 2007a) showed that horizontal genomic transfer of up to 112 kb from one *C. jejuni* strain that was sensitive to phage attack to another strain that was insensitive to phage attack led to the development of a new *C. jejuni* strain that was associated with the acquisition of phage sensitivity to 6 of the 25 lytic phage isolated from

the flock (Scott et al. 2007a). The consequence of this horizontal transfer resulted in extensive genomic diversity within *C. jejuni* strains thus increasing the dilemma of phage resistance (Scott et al. 2007a).

Changes on the surface of host bacteria can lead to the inability of phage to recognize attachment sites, thus in order to combat this, additional approaches such as the use of phage cocktails or genome manipulations to broaden host range may be necessary to overcome such problems (Bren 2007).

Phage Biocontrol of *Campylobacter*

The need for intervention strategies for the reduction of *Campylobacter* is immense. More than 80% of birds in the UK alone are said to harbor this organism, and it is reported that *Campylobacter* has a very low infectious dose of between 500 and 800 cells (Black et al. 1988). Consequently, this pathogen is recognized as one of the major causes of human gastroenteritis worldwide (Blaser 1997). Measures to improve farm bio-security or measures to ensure carcass quality post slaughter need to be addressed (Loc Carrillo et al. 2005). However, it would be more advantageous to introduce such control measures within the food chain or before chickens go to slaughter (Herman et al. 2003).

To date, there are more than 170 phage of *Campylobacter* species reported (Sails et al. 1998) that have been isolated from various sources such as abattoir effluents, broiler chickens, sewage, and also from retail poultry (Connerton et al. 2004), and work with these phage has produced some promising results for future control of *Campylobacter*. It is believed that *Campylobacter* phage are most plentiful in areas where their host cells are located, particularly in the gut of poultry. This in turn leads to the occurrence of phage being excreted in the feces of chickens, resulting in high numbers occurring in sewage or housing environments (Connerton et al. 2004). An investigation by Goode et al. (2003) reported that the administration of Φ 29C significantly reduced *Campylobacter* numbers on chicken skin inoculated with the pathogen (Goode et al. 2003). In 2004, a study investigated *Campylobacter* colonization and phage presence in organic and free-range chickens (El-Shibiny et al. 2005). Organic-produced birds are slaughtered at 73 days and must adhere to strict regulations in relation to the use of substances administered; birds are fed an organic feed and are allowed access to the outside. Free-range birds, however, are slaughtered at 56 days and are allowed access to the outside in low stocking numbers. Isolation of *Campylobacter* was undertaken with 68.5% isolated from organic birds and 90% from free-range birds. Moreover, *Campylobacter* was detected much earlier in free-range birds at 8-days-old in comparison to 31-day-old chicks in the organic birds. Results from this study revealed that 51% of *Campylobacter*-positive organic birds carried phage in their cecal contents, and the phage were present at levels of 5.5 PFU/g cecal content with the first appearance of phage occurring at day 34, three days after initial colonization of *Campylobacter*. However, phage were not prevalent in the free-range birds with only one being isolated (El-Shibiny et al. 2005). The fact that the phage appeared so quickly within the organic birds after *Campylobacter* colonization suggests that there will be an abundance of host-specific phage where the host cell is residing. To date, there is still little information regarding the potential of phage therapy to reduce *Campylobacter* in animals (El-Shibiny et al. 2005). The application of Φ CP8 and Φ CP34 to control experimentally inoculated chickens has been reported by Loc Carrillo et al. (2005) (**Figure 5**). These phage were isolated from broiler chicken excreta and free-range chicken excreta (Loc Carrillo et al. 2005). In this study, birds were inoculated with *C. jejuni* at approximately 20 days old, followed by phage five days later. At 24 hour intervals, birds were slaughtered, and *Campylobacter* and phage numbers were evaluated. Evaluation assays between these two phage revealed Φ CP8 to be the most effective phage during in vitro assays.

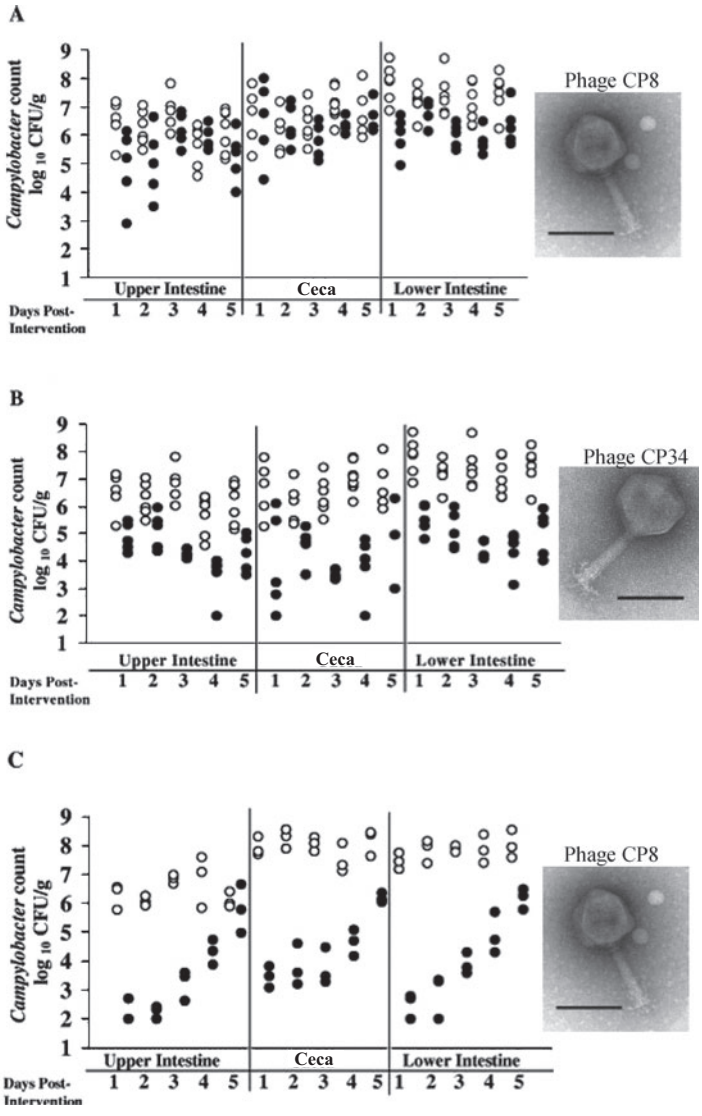

Figure 5

Comparison of the efficacy of phages CP8 and CP34 against *Campylobacter jejuni* HPC5 and GIIC8. Chickens colonized by *C. jejuni* HPC5 or GIIC8 were treated with a 10^7 PFU dose of either CP8 or CP34. (○) Control *Campylobacter* counts of intestinal contents; (●) bacteriophage-treated *Campylobacter* counts (log10 CFU/g). (*a*) Phage CP8-treated chickens colonized with *C. jejuni* HPC5; (*b*) phage CP34-treated chickens colonized with *C. jejuni* HPC5; (*c*) phage CP8-treated chickens colonized with *C. jejuni* GIIC8 (reproduced from Loc Carrillo et al. 2005).

However, it was shown that Φ CP34 was able to replicate on the host cells and reduce cell numbers in the ceca with more success than Φ CP8. Φ CP8 still has many merits for use in controlling *Campylobacter* levels, as it was able to survive ingestion and was still able to replicate on host cells, even though it failed to significantly reduce numbers (Loc Carrillo et al. 2005). Wagenaar et al. (2005) reported the benefits of phage therapy for both therapeutic and preventive use for *Campylobacter* in chickens. Bacteriophage 71, a *Myoviridae* phage, was used in this study because

of its broad host range of *C. jejuni* strains. The research involved 10-day-old chicks inoculated with a *C. jejuni* strain before (therapeutic group) and after (preventive group) treatment with Φ 71. Findings revealed that phage treatment administered before *C. jejuni* did not prevent colonization of the bacteria but was able to delay it. In addition, phage numbers remained established in the ceca, and *C. jejuni* numbers were significantly lower compared with those in the control group. Indeed, in the therapeutic group, a thousand-fold reduction in *C. jejuni* numbers was observed.

SAFETY AND REGULATORY ASPECTS OF BACTERIOPHAGES

Bacteriophage therapy has numerous advantages that have been addressed in this review; however, some uncertainties may still arise, particularly because of their rapid evolving nature. One such problem is the transfer of genetic material from host to phage. Recently, Chen & Novick (2009) discussed the possibility of phage being involved in genetic transfer of toxin-encoding genes. It was originally thought that phage-mediated genetic transfer occurred within a genus. However, as a result of the occurrence of broad host range phage, genetic transfer may not only be genus specific. The significant factor in the midst of this is the threat of intergenus transfer of virulence factors (Cheetham & Katz 1995). Chen & Novick (2009) investigated the likelihood of virulence genes being transferred in a cow's milk medium in which both *Staphylococcus aureus* and *L. monocytogenes* were present. It was evident that spontaneous prophage induction arose where toxin-carrying pathogenicity islands of *S. aureus* were transferred to *L. monocytogenes* (Chen & Novick 2009). Furthermore, another study in this area demonstrated that genetic material transfer by phage could be a frequent occurrence (Canchaya et al. 2004). In this case, it was reported that many bacterial species have a number of prophage within their genomes that encode for a variety of genes. One such example is *E. coli* O157:H7, where strain O157 Sakai has been reported to possess 18 prophage, which contain numerous genes with virulence features (Canchaya et al. 2004). It had been revealed that some of these prophage contain deletions and disruptions, thus as such they were disregarded. However, bioinformatic analysis of these imperfect prophage has indicated that they are not just flawed genetic elements but possess considerable capability to act as mobile elements that may be used for the transfer of virulence genes from *E. coli* O157:H7 to other bacterial species (Asadulghani et al. 2009). It is therefore essential to sequence phage genomes to obtain information on the potential presence of virulence factors, verifying the individual phage suitability for food application.

Even with the many apparent advantages outlined for the use of phage as therapeutic agents for the control of foodborne pathogens, there is still a journey ahead before bacteriophage are widely accepted as antibacterial agents in human and animal applications. In Eastern Europe and the former Soviet Union, phage therapy/biocontrol has been implemented clinically for the treatment of bacterial infections for many years (Hanlon 2007, Housby & Mann 2009). However, in the West the advent of antibiotics caused phage therapy to fall into demise. Regardless, general recognition of bacteriophage is beginning to make advancements with some phage-based products being approved by the FDA. For example, in 2006 the approval of the LMP-102 phage preparation was announced (Bren 2007).

Another issue regarding the use of phage as biocontrol agents is the occurrence of phage-resistant strains (Mattey & Spencer 2008). The constant evolution of phage together with genetic rearrangement ensures that the interaction between phage and bacteria favors the phage at all times. Finally, the idea of applying a virus or virulent bacteriophage to food products may take considerable time to get acceptance from a terminology point of view. Numerous clinical trials need to be performed to guarantee the safety of applying phage to food, as well as full-scale toxicological studies to determine the length of time phage remain in the animal or human after application. Also, studies such as those from Bruttin & Brussow (2005) in which volunteers

ingested a sample of T4 phage without any consequences, are extremely important in the area of acceptance. Furthermore, it needs to be reiterated that the use of a virus to combat a pathogen is not unheard of, especially when we see that many vaccines consist of live viruses (Inal 2003). If such obstacles are wholly addressed, the use of phage-based therapies together with other antibacterial agents is a highly appealing answer for the successful treatment of bacterial infections (Petty et al. 2007). We are, however, on the road to consumer acceptance and belief in the efficacy of phage therapies, with a number of companies now investing in the production of phage-based products (**http://www.ebifoodsafety.com, 2007**).

POTENTIAL OF BACTERIOPHAGES/FUTURE ASPECTS

The many studies and trials involving human and animal applications of phage demonstrates the obvious benefit of their use as a means of bacterial pathogen control. However, there are numerous advantages to be reaped from the development of products incorporating phage and their lysins, including bacterial detection strategies and the generation of novel phage-based nanotechnologies (Petty et al. 2007).

In relation to phage with a narrow host range, the prospect of genetically engineering phage genomes can lead to the broadening of host specificity. This has obvious advantages, with the resulting phage being capable of infecting a broader number of pathogenic strains and overcoming phage resistance encountered in some strains (Carlton 1999).

As many studies have shown, the use of phage for pathogen control within the animal has shown highly promising results, and there is potential to develop this work further. Within the area of *E. coli* O157:H7 control, many studies have shown that certain O157-specific phage reduced bacterial numbers at the primary colonization site in cattle but did not eradicate *E. coli* O157:H7 completely (Sheng et al. 2004). To reduce pathogen numbers further, it has been suggested to use higher numbers of phage in the animal, thus ensuring more contact with the pathogen, as well as applying the phage orally and via the rectum together with topping up phage supplies in the drinking water (Sheng et al. 2004, 2006). Other work in which phage has been applied directly to food has shown a very high degree of success. Obviously, this approach can certainly be exploited more widely in the future.

CONCLUSION

Many of the studies mentioned in this review clearly demonstrate the efficacy of phage for the control of foodborne pathogens and suggest that the development of phage-based approaches is a highly promising strategy. Even though phage and their lysins have the added advantage of being natural entities, the concern with oral administration and applications in foods is still an obstacle that needs to be overcome in order to fully achieve public confidence. Also, there is the possibility that phage may not be successful in the biocontrol of pathogens in all food systems, as their ability to function largely depends on their environment. O'Flaherty et al. (2009) reported that phage K activity was inhibited in raw bovine milk and whey, and suggested that the poor lytic activity was as a result of immunoglobin activity against the target bacteria resulting in clumping and reducing physical contact with the phage (O'Flaherty et al. 2009). In all, there is no argument that phage and their lysins have the potential to become the main method of treatment for bacterial infections, however we believe that through their strategic development and intelligent use, such therapies have an important role to play in combating foodborne pathogens and many infectious diseases in the future.

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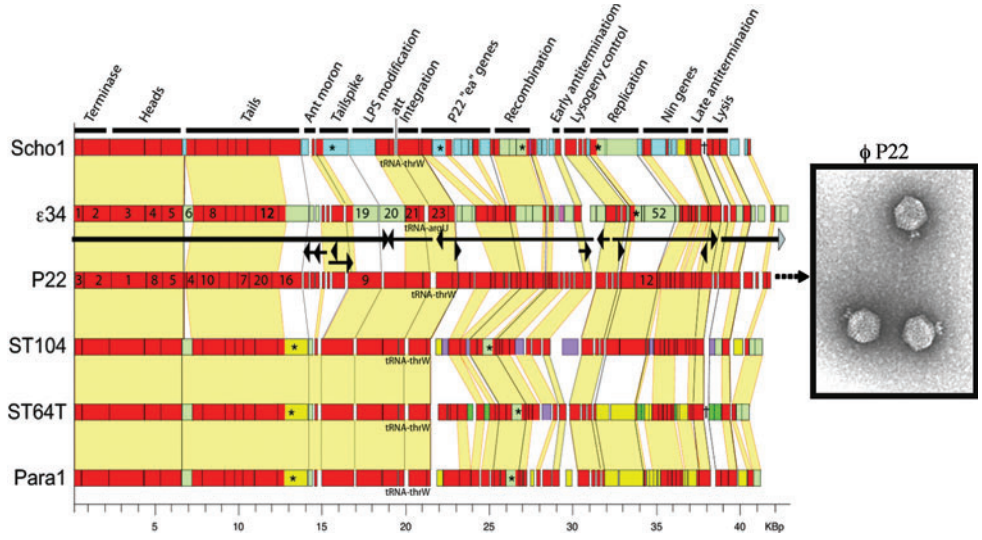

Figure 3

Genomes of phage P22 and the P22-like phages of *Salmonella*. The genomes of four *Salmonella* temperate phages (P22, ε34, ST104, and ST64T) and two apparently intact *Salmonella* prophages (Scho1 and Para1) are shown with the open reading frames indicated as colored rectangles. Similar rectangle colors indicate homology and these homologies are connected by yellow trapezoids between adjacent genomes; different open reading frame colors indicate apparent nonhomologies. The circular genome sequences are arbitrarily opened at the start of the small terminase gene. Above, the constant (among this type of phage) order of gene functions are indicated and thick black lines between the genomes denote the apparent boundaries between these regions. Asterisks (*) mark genes in which homology breaks clearly occur within genes and daggers (†) indicate the presences of tRNA genes (which read Asn GTT and Thr TGT codons in Scho1 and Asn GTT in Para1). The site of integration into the host genome is indicated at the attachment site (*att*) of each genome. Finally, the experimentally determined transcription pattern of phage P22 is indicated above the P22 genome (the *gray arrowhead* on the rightmost mRNA indicates that this transcript extends across the artificial break in the genome and continues at the other end). (Reproduced from Villafane et al. 2008.) Electron micrograph of phage P22 (**http://www.asm.org/division/m/foto/P22Mic.html**).

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Errata

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