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Bacteriophages of *Clostridium perfringens*

Bruce S. Seal et al.*
Poultry Microbiological Safety Research Unit, Agricultural Research Service, USDA, USA

1. Introduction

Bacterial viruses were first reported in 1915 by Fredrick William Twort when he described a transmissible "glassy transformation" of micrococcus cultures that resulted in dissolution of the bacteria (Twort, 1915). Subsequently, Felix Hubert d'Hérelle reported a microscopic organism that was capable of lysing *Shigella* cultures on plates that resulted in clear spaces in the bacterial lawn that he termed "plaques" (d'Hérelle, 1917). The term "bacteriophage" was introduced by d'Hérelle (1917) as he attributed the replicate nature of this phenomenon to bacterial viruses. During 1919 d'Hérelle utilized phages isolated from poultry feces as a therapy to treat chicken typhus and further utilized this approach to successfully treat dysentery among humans (Summers, 2001). Prior to the discovery and widespread use of antibiotics, bacterial infections were treated by administering bacteriophages and were marketed by L'Oreal in France (Bruynoghe & Maisin, 1921). Although Eli Lilly Co. sold phage products for human use up until the 1940's, early clinical studies with bacteriophages were not extensively undertaken in the United States and Western Europe after the 1930's and '40's. Bacteriophages were and continue to be sold in the Russian Federation and Eastern Europe as treatments for bacterial infections (Sulakvelidze *et al.*, 2001).

Bacteriophages have been identified in a variety of forms and may contain RNA or DNA genomes of varying sizes that can be single or double-stranded nucleic acid (Ackermann, 1974; 2003; 2006; 2007). Of all the bacteriophages examined by the electron microscope, 95% of those reported are tailed with only 3.7% being filamentous, polyhedral or pleomorphic (Ackermann, 2007). The tailed bacteriophages contain a linear, double-stranded DNA genome that can vary from 11 to 500 kb in the order *Caudovirales* which is further divided into three families based on tail morphology (Ackermann, 2003; 2006). These bacterial viruses have icosohedral heads while those phages with contractile tails are placed in the *Myoviridae*, those phages with a long non-contractile tail are placed in the *Siphoviridae* and phages with short tail structures are members of the *Podoviridae*. Although bacteriophages of the *Caudovirales* (tailed-phages) may be physically similar it has been difficult to classify them by use of DNA or protein sequences due to the tremendous diversity because of

* Nikolay V. Volozhantsev[2], Brian B. Oakley[1], Cesar A. Morales[1], Johnna K. Garrish[1], Mustafa Simmons[1], Edward A. Svetoch[2] and Gregory R. Siragusa[3]
[1]*Poultry Microbiological Safety Research Unit, Agricultural Research Service, USDA, USA*
[2]*State Research Center for Applied Microbiology & Biotechnology, Russian Federation*
[3]*Danisco USA Inc., USA*

horizontal gene transfer (Casjens, 2005). Also, Unlike the case for *Bacteria* and *Archaea*, both of which can be classified using the 16S rRNA gene (Woese and Fox, 1977), due to the mosaic nature of bacteriophage genomes (Hendrix *et al.*, 1999), there appears not to be one candidate conserved gene that can be utilized to categorize all phages for a suitable classification scheme (Nelson, 2004). One approach has been to construct a "phage proteomic tree" based on predicted protein sequences of a bacterial virus (Rohwer and Edwards, 2002) while another approach is to divide bacteriophages based on genome type (ssRNA or DNA) with a further demarcation by physical characteristics such as tailed or filamentous types (Lawrence *et al.*, 2002). Proux *et al*. (2002) proposed a phage taxonomy scheme based on comparative genomics of a single structural gene module (head or tail genes). This partially phylogeny-based taxonomical system purportedly parallels many aspects of the current *International Committee on Taxonomy in Virology* (ICTV) classification system.

2. Antibiotics, antibiotic resistance and the future role of bacteriophages

There is worldwide concern over the present state of antimicrobial resistance (AMR) issues with zoonotic bacteria potentially circulating among food-producing animals, including poultry (McDermott *et al*., 2002; Gyles, 2008). This has resulted in the general public's perception that antibiotic use by humans and in food animals selects for the development of AMR among food-borne bacteria that could complicate public health therapies (DuPont, 2007). A major issue is that antibiotic resistance may not only occur among disease-causing organisms but also become an issue for other resident organisms in the host which may accumulate in the environment (Yan & Gilbert, 2004). Sub-therapeutic use of antibiotics as growth promoters has been discontinued in the European Union (Regulation EC No. 1831/2003 of the European parliament and the council of 22 September 2003 on additives for use in animal nutrition; Castanon, 2007). This concern is justified due to the increase in antibiotic resistance among bacterial pathogens (NAS, 2006; Gyles, 2008), including bacteria from healthy broiler chickens (Persoons *et al.,* 2010). Consequently, there is a need for developing novel intervention methods including narrow-spectrum antimicrobials and probiotics that selectively target pathogenic organisms while avoiding killing of beneficial organisms (NAS, 2006).

There has been a resurgent interest in bacteriophage biology and their use or use of phage gene products as antibacterial agents (Merril *et al.,* 1996; Wagner and Walder, 2002; Liu *et al.,* 2004; Fischetti, 2010). The potential use of lytic bacteriophage and/or their lytic enzymes is of considerable interest for medicine, veterinary and bioindustry worldwide due to antibiotic resistance issues. Recently, the U.S. Food and Drug Administration approved a mixture of anti-*Listeria* viruses as a food additive to be used in processing plants for spraying onto ready-to-eat meat and poultry products to protect consumers from *Listeria monocytogenes* (Bren, 2007). In veterinary practice, experimental alimentary *E. coli* infections in mice and cattle were controlled by bacteriophage therapy (Smith & Huggins, 1982; 1987). Similarly Barrow *et al*. (1998) reported the use of lytic bacteriophages to protect against *E. coli* septicemia and meningitis in chickens and young cattle. Huff *et al*. (2002a,b; 2003) reported the use of a lytic bacteriophage to reduce effects of *E. coli* respiratory illness in chickens and bacteriophages have been proposed as a strategy for control of food-borne pathogens (Hudson *et al.,* 2005). Joerger (2002) reviewed the literature for application of lytic bacteriophage to control specific bacteria in poultry and concluded that evidence from several trials indicated that phage therapy may be effective under certain circumstances. However, obstacles for the use of phage as antimicrobials remain due to reasons such as limited host-range for many bacteriophages (Labrie et al., 2010).

In the European Union (EU) antimicrobial growth promoters have been banned from animal feeds because of concerns over the spread of antibiotic resistances among bacteria (Bedford, 2000; Moore *et al.*,2006) and the EU-wide ban on the use of antibiotics as growth promoters in animal feed entered into effect on January 1, 2006 (Regulation 1831/2003/EC). Removal of these antimicrobials will induce changes within the chicken intestinal microbial flora, dictating the need to further understand the microbial ecology of this system (Knarreborg *et al.,* 2002; Wise and Siragusa, 2007), so that appropriate antibiotic alternatives may be developed based on this knowledge (Cotter *et al.,* 2005; Ricke *et al.,* 2005). There has been a limited number of new antibiotic drugs marketed recently with only two, linezolid which targets bacterial protein synthesis and daptomycin wherein the mechanism of action is unknown, appearing since 2000. This is disconcerting considering that this is happening at a time when there is an increasing emergence of antibiotic resistant bacteria with a meager number of new drugs being developed active against such agents (Projan *et al.,* 2004). The view that there is no compelling reason to pursue development of novel therapeutic agents is unwise (Projan & Youngman, 2002), especially considering emergence of “pan-resistant” or multiple-antibiotic resistant strains of Gram-positive bacteria (French, 2010). Consequently, bacteriophage or perhaps more importantly their gene products may provide us with new antimicrobials to combat antibiotic resistant bacteria or that could be used synergistically with traditional antibiotics.

3. Biology of *Clostridium perfringens*, human and veterinary medical issues

Clostridium perfringens is a Gram-positive, spore forming, anaerobic bacterium that is commonly present in the intestines of people and animals. *C. perfringens* is classified into one of five types (A, B, C, D, or E) based on toxin production (Smedley *et al*., 2004; Sawires & Songer, 2006). Spores of the pathogen can persist in soil, feces or the environment and the bacterium causes many severe infections of animals and humans. The bacterium can cause food poisoning, gas gangrene (clostridial myonecrosis), enteritis necroticans and non-foodborne gastrointestinal infections in humans and is a veterinary pathogen causing enteric diseases in both domestic and wild animals (Smedley *et al*., 2004; Sawires & Songer, 2006). Spores of the pathogen can persist in soil, feces, and in the environment causing many severe infections in humans and animals. Clinical symptoms and pathogenesis of the infection is determined by enterotoxins produced by *C. perfringens* strains of type A (CPE strains). If a sufficient number of pre-formed *C. perfringens* cells are ingested from contaminated food, these cells are capable of passage from the stomach to the intestinal tract where upon sporulation (spore formation) CPE is released causing the disease state of *C. perfringens* food poisoning (Smedley *et al*., 2004; Sawires & Songer, 2006). Many heat processes are incapable of inactivating the *C. perfringens* endospores. Survival of spores in these products allows the subsequent outgrowth where spores can germinate and commence growth at temperatures of 43 to 47˚C. In foods such as meats with gravy, heating reduces the oxygen tension (lowered redox) to cause sufficient anaerobiosis in which greater numbers of *C. perfringens* will rapidly divide. Importantly, *C. perfringens* has been documented to have very rapid doubling times, in some cases as low as 7 to 9 minutes in beef broth (Smedley *et al*., 2004; Sawires & Songer, 2006).

Clostridium perfringens plays a significant role in food-borne human disease and is among the most common food-borne illnesses in industrialized countries (Brynestad & Granum, 2002; Lindström *et al.,* 2011). It can be the second or third most frequent cause of bacterial foodborne illness in the United States and is responsible for approximately one million domestic cases annually (Mead *et al*., 1999; Scallan *et al.,* 2011). Outbreaks are frequently associated with temperature-abused meat or poultry dishes and typically involve a large number of victims (Lindström *et al.,* 2010). If a sufficient number of *C. perfringens* cells are ingested from contaminated food, these cells are capable of passage from the stomach to the intestinal tract where, upon sporulation, CPE is released causing the disease state of *C. perfringens* food poisoning (Wen & McClane, 2004). In addition to food poisonings, CPE-positive *C. perfringens* type A has been implicated in other diseases such as antibiotic-associated and sporadic diarrhea in humans that also may be food-related or non-food sources (Lindström *et al.,* 2010). The Centers for Disease Control and Prevention (CDC) collects data on food-borne disease outbreaks (FBDOs) from all states and territories through the Food-borne Disease Outbreak Surveillance System (FBDSS). The 12 June 2009 issue of Morbidity and Mortality Weekly Report (http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5822a1.htm) states that one of the pathogen-commodity pairs responsible for the most outbreak-related cases was *C. perfringens* in poultry (902 cases). Although *C. perfringens* is considered in the "medium" risk category, it can become a high risk pathogen/product combination with temperature abused poultry-meat products during extended shelf life or when cross-contaminated by *Listeria monocytogenes* (Mataragas *et al.,* 2008). It was reported that improper retail and consumer refrigeration accounted for approximately 90% of the *C. perfringens* illnesses (Crouch *et al*., 2009) and poultry meat can be a frequently implicated food vehicle during outbreaks (Gormley *et al.,* 2010; Nowell *et al*., 2010).

Necrotic enteritis is a peracute disease syndrome and the subclinical form of *C. perfringens* infection in poultry are caused by *C. perfringens* type A producing the alpha toxin, and some strains of *C. perfringens* type A produce an enterotoxin at the moment of sporulation that are responsible for food-borne disease in humans. The mechanisms for colonization of the avian small intestinal tract and the factors involved in toxin production are largely unknown. Unfortunately, few tools and strategies are available for prevention and control of *C. perfringens* in poultry. Vaccination against the pathogen and the use of probiotic or prebiotic products has been suggested, but are not available for practical use in the field at the present time (Van Immerseel *et al.,* 2004). Since most poultry harbor intestinal *C. perfringens* commensally as a component of the gut microflora, these issues lend credence to the hypothesis that as subtherapeutic usage of antibiotics is discontinued during poultry production, food-borne illness associated with *C. perfringens* will likely increase. This could potentially become a greater problem for the U.S. poultry industry as antibiotics are withdrawn from animal feeds as has been done in the European Union (Casewell *et al.,* 2003; Van Immerseel *et al.,* 2004). Control of clostridia in commercial poultry has traditionally been accomplished by feeding sub-therapeutic amounts of antibiotics in feed (Jones & Ricke, 2003; Collier *et al*, 2003). Antibiotics have been utilized for over thirty years (Maxey and Page, 1977; George *et al.,* 1982; Engberg *et al.,* 2000; Brennan *et al.,* 2003) and resistance of *C. perfringens* to growth-enhancing antibiotics has been detected among isolates from poultry

(Diarra *et al.*, 2007). Consequently, there is a need for developing on-farm interventions to reduce populations of this bacterial pathogen that lead to peracute flock disease and possibly greater numbers of CPE+ isolates of *C. perfringens* entering the human food chain.

4. Early literature reporting bacteriophages of *Clostridium perfringens*

There is a paucity of genomics data for *C. perfringens* bacteriophages, but it has been known that both temperate and lytic phages are associated with the pathogen, while the Russian literature compiled by Spencer (1953) reported the use of clostridial bacteriophages to treat gas gangrene. Investigators at the Institute Pasteur reported bacteriophages that could be induced from lysogeny among isolates of *C. perfringens* that were long-tailed viruses of the *Siphoviridae* (Kreguer *et al.,* 1947; Guelin & Kreguer, 1950; Guelin, 1953; Elford *et al.,* 1953; Hirano & Yonekura, 1967). Subsequently, a member of the *Podoviridae* designated bacteriophage 80 was isolated with a distinct tail structure that was considered morphologically different from previously reported viruses of anaerobic bacteria (Vieu *et al.,* 1965). Intracellular replication of this virus was examined by Bradley & Hoeniger (1971) who reported that the bacteriophage had a head of approximately 40 nm in size with a 30 nm tail. Intact viruses could be detected within the bacterial cell by 75 minutes post-infection (p.i.) with cell lysis beginning at 105 to 115 min p.i.

Gaspar & Tolnai (1959) published isolation of a virulent *C. perfringens* phage, while Ionesco *et al.* (1974) reported isolation of lysogenic bacteriophages. Lysogenic cultures could be induced by UV irradiation, nitrogen mustard [mechlorethamine; 2-chloro-N-(2-chloroethyl)-N-ethyl-ethanamine, a nonspecific DNA alkylating agent] and to a lesser extent by mercaptoacetic acid. Twelve bacteriophages were induced from type A *C. perfringens* strains, ten from type B and 26 from type C strains of the bacterium, many of the phages were highly host specific with a high proportion of the *C. perfringens* strains resistant to infection by the viruses (Smith, 1959). Smith (1959) also reported that several viruses were apparently unable to enter into lysogeny and hence those were classified as 'virulent' bacteriophages. Following UV irradiation one lysogenic strain of *C. perfringens* resulted in isolation of a long-tailed, DNA-containing bacteriophage with a non-contractile tail, designated CPT1 that produced turbid plaques. This phage had an eclipse phase of approximately 45 min with a maximum rise in titer 45 min following initial release of progeny virus (Mahony and Kalz, 1968). A second bacteriophage designated CPT4 with similar characteristics, but with a shorter tail as compared with CPT1, was also isolated by these investigators (Mahony & Easterbrook, 1970). However, U.V. irradiation did not result in release of viruses from the indicator strain and it was reported that spontaneous release of the virus occurred with all resultant plaques that were clear.

Lysogenic bacteriophages were isolated specifically from *C. perfringens* type C that were induced using mitomycin C treatment on specific isolates of the bacterium (Grant & Riemann, 1976). All the viruses had a similar morphology with polyhedral heads of 55 nm and long flexible tails of 130 to 190 nm. Paquette & Fredette (1977) reported four lysogenic phages from *C. perfringens* type A that were induced with UV irradiation for 5 sec and had 0.5 mm plaques with outer lysis rings. One phage was a podovirus, while the others were siphoviruses (Paquette & Fredette, 1977). Stewart & Johnson (1977) reported that lysogenic phages can have a positive effect on *C. perfringens* sporulation and Canard & Cole (1990) demonstrated that two different lysogenic phages had separate attachment sites that did not share sequence similarity. Also, Shimizu *et al*. (2002) reported at least 20 phage-related sequence elements in the complete *C. perfringens* Strain 13, a gas gangrene isolate.

A bacteriophage isolated from a *C. perfringens* fecal strain was adapted to a number of host strains from clinical swab and fecal isolates to develop a typing scheme using nine host modified phages (Yan, 1989). Of 109 strains, the phage types of 57 (52.3%) were identified, while nine (8.2%) other strains were sensitive to the phages at varying degrees. The remaining 43 (39.4%) strains were resistant and eleven of the 57 typable strains yielded cell-surface mutants which belonged to different phage types from their parent strains (Yan, 1989). Another phage-typing method for the bacterium was developed, but little or no information was available from the report (Satija & Narayan, 1980).

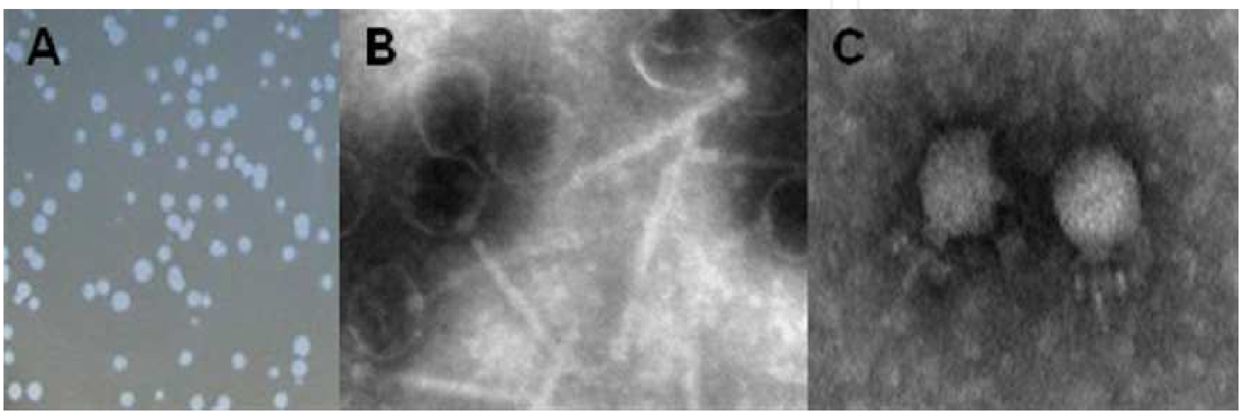


Fig. 1. Plaques and bacteriophages of *Clostridium perfringens* isolated from a joint Russian Federation-USA collaborative research project. (A) Clear plaques produced by bacteriophages from a series of isolates reported in the text and references. (B) Long-tailed phages of the *Siphoviridae*. (C) Short-tailed phages of the *Podoviridae*.

Initial screening for bacteriophages lytic for *C. perfringens* at the Poultry Microbiological Safety Research Unit, of the ARS, USDA and at the State Research Center for Applied Microbiology and Biotechnology in the Russian Federation was performed using filtered samples obtained from poultry (intestinal material), soil and processing drainage water (Figure 1). Bacterial viruses capable of lysing strains of *C. perfringens* type A and producing clear plaques were identified by spot-testing and titration of strains susceptible to the isolated phages (Fig. 1A). Lytic phage preparations were initially characterized morphologically utilizing plaque purified (3X) phage by electron microscopy using the modified method of Horne (1973) where both siphoviruses (Fig. 1B) and podoviruses (Fig. 1C) have been discovered that are virulent for *C. perfringens* (Seal *et al.*, 2011; Volozhantsev *et al.*, 2011).

5. Characteristics of *Clostridium perfringens* bacteriophage and prophage genomes

Zimmer *et al.* (2002a) isolated two temperate bacteriophages by UV irradiation (phi3626 and phi8533) from lysogenic *C. perfringens*. The linear, double-stranded DNA genome of phi3626 was reported to be 33.5 kb with nine nucleotide 3′ protruding cohesive ends and a G+C content of 28.4% (Zimmer *et al.*, 2002a) which is essentially equivalent to its host DNA of 28.6% (Shimizu *et al.*, 2002; Myers *et al.*, 2006). The phage phi3626 had a 55 nm diameter isometric capsid with a 170 nm flexible, non-contractile tail (Zimmer *et al*., 2002a) that conformed to the *Siphoviridae* phage family in the order *Caudovirales* (Ackermann, 2006). Phage phi3626 was reportedly easier to propagate, so no genomics data were provided for phi8533 (Zimmer *et al*., 2002a). Physical characteristics of other *C. perfringens* bacteriophages are similar to phi3626 in that they were reported to have polyhedral heads of 55 nm in diameter with long flexible tails (Grant & Riemann, 1976; Paquette & Fredette, 1977) that also presumably had double-stranded DNA genomes. Only nineteen gene products could be assigned to the phage phi3626 genome based on bioinformatics analyses. Those were identified as encoding DNA-packaging proteins, structural components, a dual lysis system, a putative lysogeny switch, and proteins involved with replication, recombination, and modification of phage DNA. Several of the genes potentially influence cell spore-formation due to availability of the phage genes in the bacterial genome. Also, the phi3626 attachment site, *attP*, lies in a non-coding region immediately downstream of *int* encoding the integrase protein. Integration of the viral genome occurred into the bacterial attachment site *attB*, which is located within the 3' end of a *C. perfringens guaA* gene homologue. Subsequently, a phage-specific enzyme, a murein hydrolase, was expressed which had lytic activity against forty-eight test cultures of *C. perfringens*, but was not active against other clostridial species or bacteria belonging to other genera (Zimmer *et al*., 2002a).

Bacteriophage genomes from viruses isolated from broiler chicken offal washes (O) and poultry feces (F), designated phiCP39O and phiCP26F, respectively, produced clear plaques on host strains (Seal *et al*., 2011). Both bacteriophages had isometric heads of 57 nm in diameter with 100-nm non-contractile tails characteristic of members of the family *Siphoviridae* in the order *Caudovirales*. The double-strand DNA genome of bacteriophage phiCP39O was 38,753 base pairs (bp), while the phiCP26F genome was 39,188 bp, with an average GC content of 30.3%. Both viral genomes contained 62 potential open reading frames (ORFs) predicted to be encoded on one strand of the DNA (Table 1). Among the ORFs, 29 predicted proteins had no known similarity to other reported proteins while others encoded putative bacteriophage capsid components such as a pre-neck/appendage, tail, tape measure and portal proteins. Other genes encoded a predicted DNA primase, single-strand DNA-binding protein, terminase, thymidylate synthase and a potential transcription factor. Lytic proteins such as a fibronectin-binding autolysin, an amidase/hydrolase and a holin were encoded in the viral genomes. Several ORFs encoded proteins that gave BLASTP matches with proteins from *Clostridium* spp. and other Gram-positive bacterial or bacteriophage genomes as well as unknown putative *Collinsella aerofaciens* proteins that were detected in the virion. Proteomics analysis of the purified viruses resulted in the identification of the putative pre-neck/appendage protein and a minor structural protein encoded by large open reading frames. Variants due to potential phosphorylation of the portal protein were identified in the virion, and several mycobacteriophage gp6-like protein variants were detected in large amounts relative to other virion proteins. The predicted amino acid sequences of the pre-neck/appendage proteins had major differences in the central portion of the protein between the two phage gene products indicating that it may be the potential anti-receptor for the virus. Based on phylogenetic analysis of the large terminase protein, these phages were predicted to be *pac*-type phages, using a head-full DNA packaging strategy. Table 1 summarizes the gene products common to currently known *C. perfringens* siphoviral bacteriophages.

Function ID	Function Name
COG0629	Single-stranded DNA-binding protein
COG0860	N-acetylmuramoyl-L-alanine amidase
COG4722	Phage-related protein
COG5412	Phage-related protein
COG1351	Predicted alternative thymidylate synthase
COG2333	Predicted hydrolase (metallo-beta-lactamase superfamily)
COG3561	Phage anti-repressor protein
COG3645	Uncharacterized phage-encoded protein
COG5545	Predicted P-loop ATPase and inactivated derivatives
COG5546	Small integral membrane protein
COG3617	Prophage antirepressor
COG3747	Phage terminase, small subunit
COG4626	Phage terminase-like protein, large subunit
COG4695	Phage-related protein
COG0175	3'- PAPS reductase/FAD synthetase and related enzymes
COG2369	Uncharacterized protein, homolog of phage Mu protein gp30
COG2755	Lysophospholipase L1 and related esterases
COG4926	Phage-related protein
COG4974	Site-specific recombinase XerD
COG0338	Site-specific DNA methylase
COG0740	Protease subunit of ATP-dependent Clp proteases
COG1476	Predicted transcriptional regulators
COG3757	Lyzozyme M1 (1,4-beta-N-acetylmuramidase)
COG5283	Phage-related tail protein
COG5614	Bacteriophage head-tail adaptor
COG0305	Replicative DNA helicase
COG1191	DNA-directed RNA polymerase specialized sigma subunit
COG1783	Phage terminase large subunit
COG3064	Membrane protein involved in colicin uptake
COG3740	Phage head maturation protease
COG4824	Phage-related holin (Lysis protein)

Note: Domains are observed in all *Clostridium perfringens* siphoviral genomes.

Table 1. The *Siphoviridae* pan-genome encoded proteins representative of *Clostridium perfringens* bacteriophages. The table shows the union of all COGs present in the genomes of phages SM101, 3626, 9O, 13O, 26F, 34O, and 39O.

Function ID	Function Name
COG0417	DNA polymerase elongation subunit (family B)
COG0739	Membrane proteins related to metalloendopeptidases
COG0860	N-acetylmuramoyl-L-alanine amidase
COG1196	Chromosome segregation ATPases
COG2088	Uncharacterized protein, involved in the regulation of septum location
COG3023	N-acetyl-anhydromuramyl-L-alanine amidase
COG3772	Phage-related lysozyme (muraminidase)
COG5434	Endopolygalacturonase
pfam00246	Peptidase_M14
pfam01391	Collagen
pfam05352	Phage Connector
pfam05894	Podovirus_Gp16 (DNA encapsidation)
pfam12841	YvrJ protein family
PHA00144	major head protein
PHA00148	lower collar protein
PHA00380	tail protein

Note: Domains are observed in all *Clostridium perfringens* podoviral genomes.

Table 2. *Podoviridae* pan-genome protein products representative of *Clostridium perfringens* bacteriophages. The table shows the union of all conserved domains present in the genomes of phages CPV1, CPV4, ZP2, CP7R, and CP24R.

Other bacteriophages lytic for *C. perfringens* were isolated from sewage, feces and broiler intestinal contents and phiCPV1, a virulent bacteriophage, was classified in the family *Podoviridae* (Volozhantsev *et al.,* 2011). The purified virus had an icosahedral head and collar of approximately 42nm and 23nm in diameter, respectively, with a structurally complex tail of 37nm lengthwise and a basal plate of 30nm. The phiCPV1 double-stranded DNA genome was 16,747 base pairs with a GC composition of 30.5%, similar to its host. Twenty-two open reading frames (ORFs) coding for putative peptides containing 30 or more amino acid residues were identified in the genome. Amino acid sequences of the predicted proteins from the phiCPV1 genome ORFs were compared with those from the NCBI database and potential functions of 12 proteins were predicted by sequence homology. Three putative proteins were similar to hypothetical proteins with unknown functions, whereas seven proteins did not have similarity with any known bacteriophage or bacterial proteins. Identified ORFs formed at least four genomic clusters that accounted for predicted proteins involved with replication of the viral DNA, its folding, production of structural components and lytic properties. One bacteriophage genome encoded lysin was predicted to share homology with N-acetylmuramoyl-l-alanine amidases and a second structural lysin was predicted to be a lysozyme-endopeptidase. These enzymes probably digest peptidoglycan of the bacterial cell wall and could be considered potential therapeutics to control *C. perfringens*. Table 2 summarizes the gene products common to currently known *C. perfringens* podoviral bacteriophage genomes.

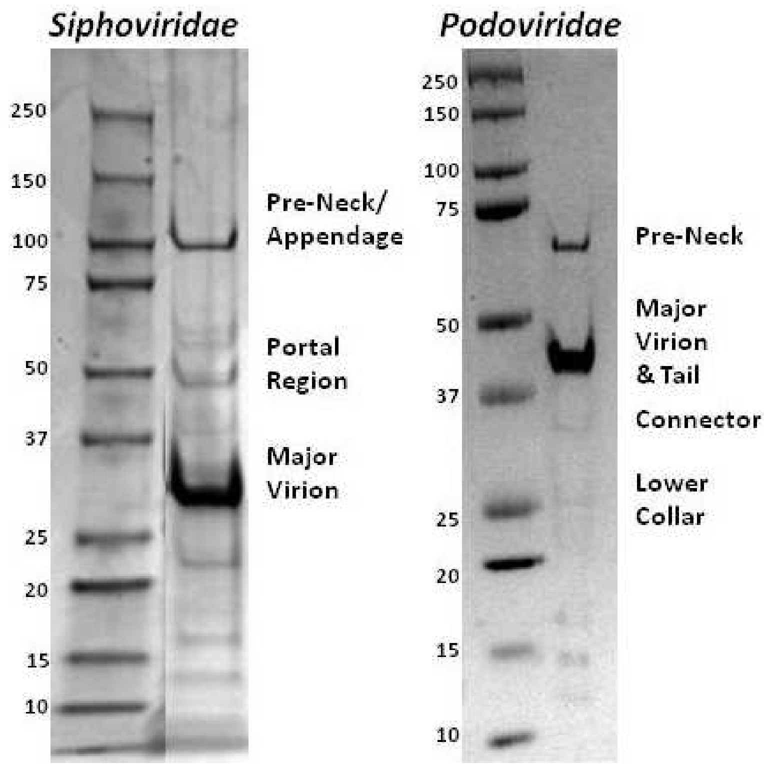


Fig. 2. Protein gel profiles for purified virions from bacteriophages virulent for *Clostridium perfringens* representing the *Siphoviridae* and *Podoviridae* from a joint Russian Federation-USA collaborative research project.

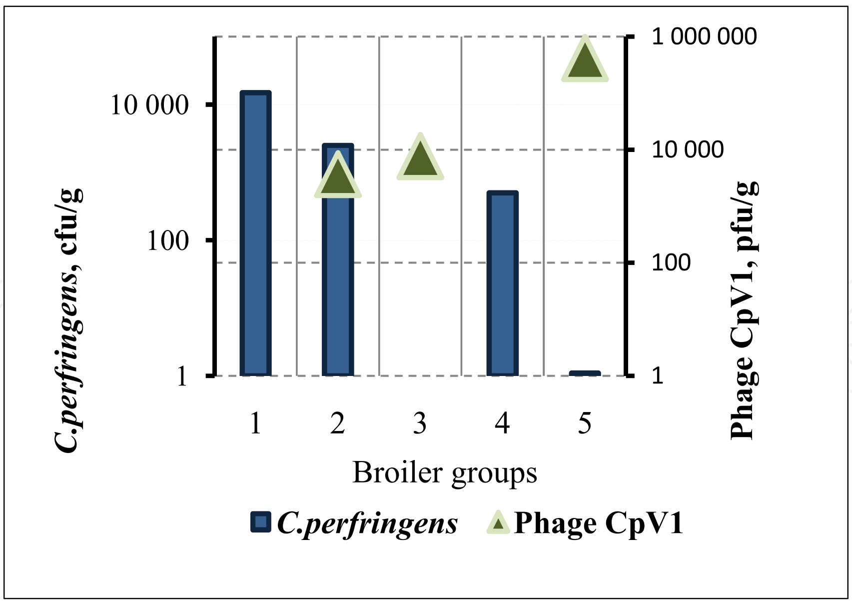
Three structural proteins were identified in the lysogenic phi3626 by N-terminal sequencing of proteins following SDS-PAGE of the purified virions (Zimmer *et al.,* 2002a). The major capsid component was estimated to be 43.3% of total phage protein and was determined to be post-translationally processed resulting in a decrease in size from 47.7 to 34.3 kDa. The major tail protein represented approximately 12.7% of the total protein, with an apparent size of 27 kDa while a minor structural protein composing 2.1% of the virion protein was reported with a predicted size of 55.1kD. More recently the proteins of virulent bacteriophages infecting *C. perfringens* have been described in detail (Seal *et al.,* 2011; Volozhantsev *et al.,* 2011). From the siphoviruses (Seal *et al.,* 2011), four principle virion protein regions were identified (Fig. 2) that included a portal protein, mycobacteriophage gp6-like protein which was the major virion protein, a pre-neck appendage protein and several lower molecular weight minor structural proteins with no known function. The portal protein was identified as a protein that was also highly variable with respect to isoelectric point and size at approximately 50kDa. This was attributed to potential differences in phosporylation and myristilation of the portal protein due to the large number of post-translational modification sites on the molecule. The podoviruses identified to date have virion proteins essentially indicative of those types of bacteriophages (Volozhantsev *et al.,* 2011). These viruses encode for a collar protein with a predicted size of approximately 27kDa and a connector protein with a predicted size of approximately 35.9kDa. The major head or major capsid protein was predicted to have a size of 43.3kDa and was found in the greatest abundance in the purified virus. A large pre-neck protein of 75 kDa and a tail protein of a size similar to the major capsid protein were also identified in *C. perfringens* phages of the *Podoviridae* (Fig. 2).

C. perfringens is an important agricultural as well as human pathogen and because biotechnological uses of bacteriophage gene products as alternatives to conventional antibiotics will require a thorough understanding of their genomic context, we sequenced and analyzed the genomes of four more closely related viruses isolated from the bacterium, then compared the known phage genomes (Oakley *et al.,* 2011). Phage whole-genome tetra-nucleotide signatures and proteomic tree topologies correlated closely with host phylogeny. Comparisons of our phage genomes to 26 others revealed three shared COGs of which one of particular interest within this core genome was an endolysin (PF01520, an N-acetylmuramoyl-L-alanine amidase) and a holin (PF04531). Comparative analyses of the evolutionary history and genomic context of these common phage proteins revealed two important results. One was a strongly significant, host-specific sequence variation within the endolysin and secondly is the protein domain architecture apparently unique to our phage genomes in which the endolysin is located upstream of its associated holin among certain members of the *Siphoviridae* (Oakley *et al.,* 2011). Endolysin sequences from our viruses were one of two very distinct genotypes distinguished by variability within the putative enzymatically-active domain. The shared or core genome was comprised of genes with multiple sequence types belonging to five pfam families, and genes belonging to 12 pfam families, including the holin genes, which were nearly identical.

6. Potential use of bacteriophages or their gene products to control *Clostridium perfringens*

Bacteriophages have been utilized experimentally in an attempt to control a variety of pathogens and there has been increased interest to control disease among poultry (Joerger, 2002). *In vivo* studies were conducted to determine if a cocktail of *C. perfringens* bacteriophages (INT-401) would be capable of controlling necrotic enteritis (NE) caused by *C. perfringens* (Miller *et al*., 2010). The first study investigated the efficacy of INT-401 and a toxoid-type vaccine in controlling NE among *C. perfringens*-challenged broiler chickens reared until 28 days old. Compared with the mortality observed with the bacterium-challenged, but untreated chickens, oral administration of INT-401 significantly reduced mortality of the *C. perfringens*-challenged birds by 92%. Overall, INT-401 was more effective than the toxoid vaccine in controlling active *C. perfringens* infections of chickens. When the phage cocktail was administered via oral gavages, feed, or drinking water it significantly reduced mortality due to the bacterium and weight gain as well as feed conversion ratios were significantly better in the *C. perfringens*-challenged chickens treated with bacteriophages than in the *C. perfringens*-challenged, phage-untreated control birds (Miller *et al*., 2010).

In order to repeat a similar study by Miller *et al.,* (2010) and to determine optimal schemes for application of bacteriophage formulations to cure or prevent disease from *C. perfringens* infection in poultry, investigators at the *State Research Center for Applied Microbiology and Biotechnology* (Obolensk, Moscow Region, Russian Federation) completed a series of experiments to monitor the persistence of *C. perfringens* lytic bacteriophage phiCPV1 in broiler gastrointestinal tracts (GIT). The phage suspension was administered *per os* once to 14-17 days old chicks (6×10^8 pfu/bird). To determine concentrations of the phage, materials from each section of the gastrointestinal tract (the crop, glandular stomach, the upper department of the small intestine, ileum, cecum, and the large intestine) were suspended in



Note: Fourteen-day old broilers were inoculated with a suspension of two phiCPV1-sensitive *C. perfringens* Rif^R-strains in the volume of 0.2ml (3×10^7 - 1×10^8 CFU for broilers) given *per os* to each broiler at day 19 (Groups 1 and 2) and at days 19, 20, 21 and 22 (Groups 4 and 5). The phiCPV1 in the volume of 0.2ml was administered *per os* twice a day to birds of Group 2 (10^8 pfu/bird) when they reached the age of 19 days, and to broilers of Groups 3 and 5 (10^9 PFU/bird) at days 19, 20, 21 and 22.

Fig. 3. Titres of *Clostridium perfringens* and phiCPV1 in lower sections of the gastrointestinal tract (ileum/cecum) of broiler chickens experimentally infected with the bacterium.

phage buffer followed by agar layer titration on a lawn produced by a *C. perfringens* phiCPV1- susceptible strain. Two independent experiments revealed that the highest concentration of the phage titer at 7×10^7 pfu/g was in the crop one hour after the administration. In the glandular stomach its concentration varied between 2×10^3 and 3×10^5 pfu/g. In the interval between 3 and 12 hours after treatment, phage concentration reached 10^7pfu/g both in cecum and ileum of all birds. Such high concentrations of the phage in the GIT are extremely important from the standpoint of the phage therapy for *C. perfringens*-associated infection. Ileum and cecum are known to be main sites for the bacterium to colonize and proliferate. In the ileum and cecum, as well as in the large intestine, the maximal phage concentration (>10^6 pfu/g) was detected 6 hours after the administration of viruses and retained at a rather high level (>10^5 pfu/g) at least for the next 6 hours. The following day after administration of the phage in the GIT, the concentration decreased markedly. However, the phage was not fully eliminated even from the crop and was detected at the concentration of 500 pfu/g 48 hours later. The assessment of therapeutic and prophylactic effects of bacteriophage formulations in broilers during model experiments has demonstrated that phiCPV1 reduced intestinal colonization of the phage-sensitive *C. perfringens* in broiler chickens, with the phage titer being increased (Fig.3). At the same time experiments on phage therapy of broilers carrying natural *C. perfringens* infection by means of a phage cocktail were not successful and this was associated with the narrow lytic spectra of the phages. Consequently, natural *C. perfringens* isolated from the broiler chickens were resistant to the bacteriophages used during the experimentation, demonstrating the need for libraries of bacteriophage isolates to therapeutically eliminate the bacterium in animals.

Zimmer *et al*. (2002b) investigated the cell wall lysis system of *C. perfringens* bacteriophage phi3626, whose dual lysis gene cassette consisted of a holin gene and an endolysin gene. The Hol3626 had two predicted membrane-spanning domains (MSDs) and was designated a group II holin. A positively charged beta turn between the two MSDs indicated that both the amino-terminus and the carboxy-terminus of Hol3626 protein might be located outside the cell membrane which is a very unusual holin topology (Young, 2002). The holin function was experimentally demonstrated by using the ability of the peptide to complement a deletion of the heterologous phage lambda S holin in lambda delta-Sthf. The endolysin gene *ply*3626 was cloned into an *E. coli* expression system. However, protein synthesis occurred only when the *E. coli* were supplemented with rare tRNA(Arg) and tRNA(Ile) genes required for proper codon usage of Gram+ genes in a Gram- system (Kane, 1995). Amino-terminal modification by a six-histidine tag did not affect enzyme activity and enabled purification by Ni-chelate affinity chromatography. The Ply3626 had an N-terminal amidase domain and a unique C-terminal portion that was hypothesized to be responsible for the specific lytic range of the enzyme. A total of 48 *C. perfringens* strains were sensitive to the murein hydrolase, whereas other clostridia and bacteria belonging to other genera were generally not affected by the lysin (Zimmer *et al*., 2002b).

Two putative phage lysin genes (*ply*) from the clostridial phages phiCP39O and phiCP26F were cloned, expressed in *E. coli* and the resultant proteins were purified to near homogeneity (Simmons *et al.,* 2010). Gene and protein sequencing revealed that the predicted and chemically determined amino acid sequences of the two recombinant proteins were homologous to N-acetylmuramoyl-L-alanine amidases. The proteins were identical in the C-terminus cell-wall binding domain, but only 55 per cent identical to each other in the N-terminal catalytic domain. Both recombinant lytic enzymes were capable of lysing both parental phage host strains of *C. perfringens* as well as other type-strains of the bacterium in spot and turbidity reduction assays. The observed reduction in turbidity was correlated with up to a 3 log cfu/ml reduction in viable *C. perfringens* on brain heart infusion agar plates. However other member species of the clostridia were resistant to the enzymes by both assay methods. Interestingly, diversity exists even among closely-related bacteriophages, holins and endolysins represent conserved functions across divergent phage genomes and endolysins can have significant variability with host-specificity even among closely-related genomes. Endolysins of phage genomes in the presented study may be subject to different selective pressures than the rest of the genome and these findings may have important implications for potential biotechnological applications of phage gene products (Oakley *et al.,* 2011). Interestingly, a variety of encoded potential gene products have been detected in the genomes of *C. perfringens* bacteriophages that could potentially be utilized as antimicrobials to control the bacterium (Fig. 4).

The number of known genes encoding these peptidoglycan hydrolases has increased markedly in recent years, due in large part to advances in DNA sequencing technology. As the genomes of more bacterial species/strains are sequenced, lysin-encoding open reading frames (ORFs) can be readily identified in lysogenized prophage regions such as in the genomes of *C. perfringens* (Shimizu, *et al.,* 2002; Myers *et al.,* 2006). The genomes of nine *C. perfringens* strains were computationally mined for prophage lysins and lysin-like ORFs, revealing several dozen

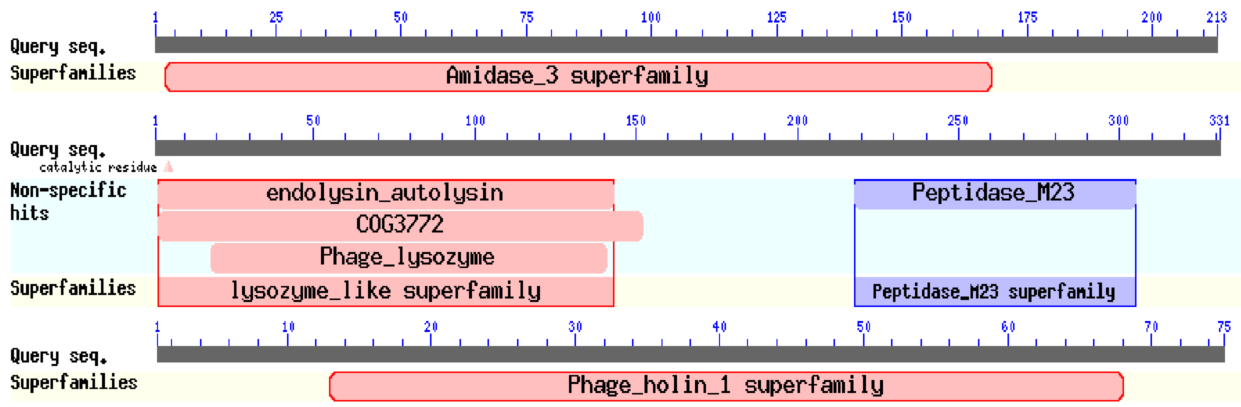


Fig. 4. Lytic proteins discovered in *Clostridium perfringens* bacteriophages from a joint Russian Federation-USA collaborative research project.

proteins of various enzymatic classes (Schmitz *et al.*, 2011). Of these lysins, a muramidase from strain ATCC 13124 (termed PlyCM) was chosen for recombinant analysis based on its dissimilarity to previously characterized *C. perfringens* lysins. Following expression and purification, various biochemical properties of PlyCM were determined *in vitro*, including pH/salt-dependence and temperature stability. The enzyme exhibited activity at low µg/ml concentrations, a typical value for phage lysins. It was active against 23 of 24 strains of *C. perfringens* assayed, with virtually no activity against other clostridial or non-clostridial species (Schmitz *et al.*, 2011). Also, an endolysin predicted to encode an N-acetylmuramidase was identified as encoded by the episomal phage phiSM101 of *C. perfringens* (Nariya *et al.*, 2011). Homologous genes were identified in the genomes of all five *C. perfringens* toxin types and the phiSM101 muramidase gene (psm) was cloned, then expressed in *E. coli* as a protein histidine-tagged at the N-terminus (Psm-his). Similar to other *C. perfringens* phage lysins the purified protein lysed cells of all *C. perfringens* toxin types, but not other clostridial species tested as demonstrated by a turbidity reduction assay (Nariya *et al.*, 2011). Consequently, more potential antimicrobials remain to be discovered utilizing genomics approaches.

Immobilization and separation of bacterial cells by replacing antibodies with cell wall-binding domains (CBDs) of bacteriophage-encoded peptidoglycan hydrolases (endolysins) has been accomplished for use as a potential diagnostic (Kretzer *et al.*, 2007). Paramagnetic beads coated with recombinant phage endolysin-derived CBD molecules and bacterial cells could be immobilized and recovered from diluted suspensions within 20 to 40 min. The CBD-based magnetic separation (CBD-MS) procedure was evaluated for capture and detection of *Listeria monocytogenes* from contaminated food samples and this approach was demonstrated by using specific phage-encoded CBDs specifically recognizing both *Bacillus cereus* and *C. perfringens* cells (Kretzer *et al.*, 2007). Consequently, the use of bacteriophage lysin cell-wall binding domains could be utilized for other applications as well as for improving diagnostic detection of Gram+ bacteria.

7. Conclusions

Bacteriophages have been utilized as potential interventions to treat bacterial infections. However, the development of bacterial resistances to their viruses occurs that include evolution of phage receptors, super-infection exclusion, restriction-modification systems and abortive infection systems such as genomic CRISPR sequences (Labrie *et al.*, 2010). These phenomena substantiate the inevitable need to constantly search for new bacteriophage isolates to use therapeutically. Also, it should be noted that although bacteriophage therapy has been utilized and examined as a treatment, it was pointed out early on by Smith (1959) that a large proportion of *C. perfringens* strains remained insusceptible to many of the bacteriophages isolated during those studies. This has routinely been observed during our investigations wherein most bacteriophages virulent for *C. perfringens* have a restricted host range (Fig. 5). Host specificity has routinely been observed relative to the bacteriophages isolated from various *C. perfringens* isolates that is most likely due to evolution of the receptor and anti-receptor molecules (Seal *et al.*, 2011; Volozhantsev *et al.*, 2011; Oakley *et al.*, 2011). Therefore, selection of appropriate 'bacteriophage cocktails' may not necessarily be effective against many of the various bacterial isolates that exist in the environment and cause disease.

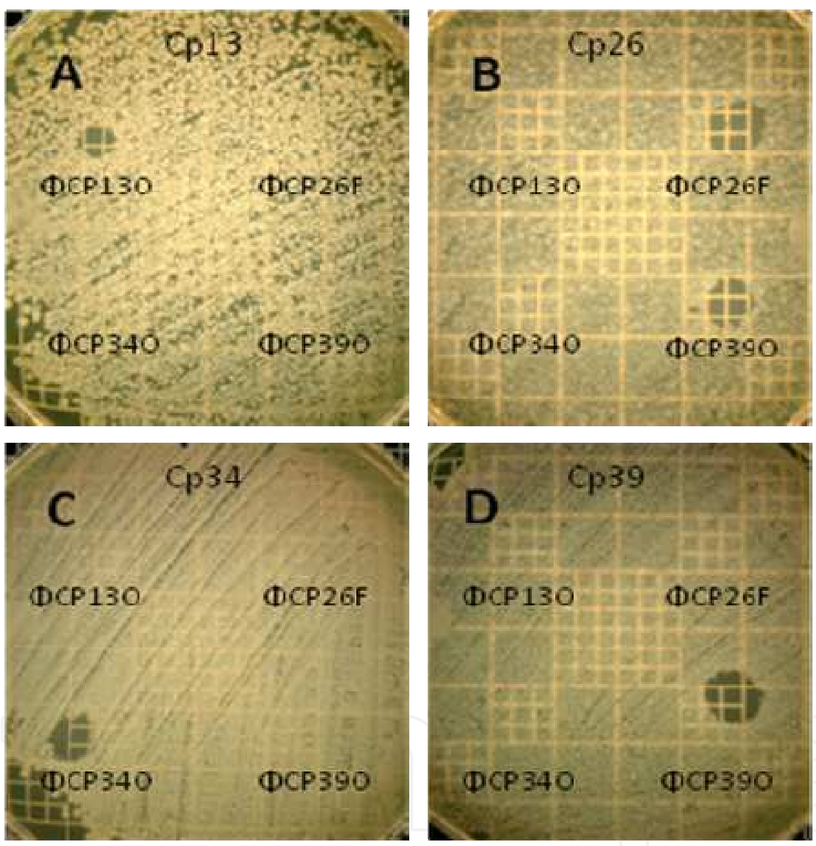


Fig. 5. Spot-assay with *Clostridium perfringens* bacteriophages on their respective hosts. Note that most all phages are restricted in their respective host-ranges.

Many enzymes are added to monogastric animal feeds to increase the digestibility of nutrients, leading to greater feed efficiency during the production of meat animals and eggs. Enzymes are added to monogastric animal feed for digesting carbohydrates and for metabolizing phytate to produce free phosphorus (Cowieson *et al.*, 2006; Olukosi *et al.*, 2010). There are a wide variety of enzymes marketed commercially for poultry feed additives, many of which are produced as a recombinant proteins in yeast and sold as a lysate which argues for the economic feasibility of developing enzyme additives (see DSM:

http://www.dsm.com/en_US/html/dnp/anh_enzymes.htm; Bio-Cat: http://www.bio-cat.com/applicationDetails.php?application_id=8; Ultra BioLogics: http://www.ublcorp.com/; Danisco: http://www.danisco.com/; Novozymes: http://www.novozymes.com/en/solutions/agriculture/animal-nutrition/). Consequently, production of enzymes by *Pichia pastoris* can serve as a potential source for structural or animal feed studies (Johnson *et al.*, 2010) and lysozyme can be encapsulated (Zhong & Jin, 2009) which has been utilized as a feed additive in the diet of chickens to significantly reduce the concentration of *C. perfringens* in the ileum and reduce intestinal lesions due to the organism (Liu *et al.*, 2010). Therefore, it is conceivable that bacteriophage proteins capable of lysing *C. perfringens* could be expressed in yeast and added as lysates to animal feed for reducing the bacterium to improve health and food safety for monogastric animals.

Clostridium perfringens (formerly known as *C. welchii*) is a ubiquitous Gram+ anaerobic, spore-forming bacterium that causes debilitating diseases in both humans and a wide variety of animals resulting in both personal tragedy and economic losses. Although the bacterium can cause severe diseases in most animals including domestic chickens, non-virulent forms of the bacillus are commonly found in the intestinal tracts of warm-blooded species as well as the environment. Several antibiotics can be utilized to treat clostridial diseases and sub-therapeutic amounts of antibiotics have been used in animal feeds as antibiotic growth promoters (AGP). Removal of AGP's from animal feed has resulted in the need for increased use of antibiotics therapeutically to treat diseases among food-producing animals, in particular necrotic enteritis in poultry. Consequently, this situation along with a concern as how to treat antibiotic resistant bacteria has provided the impetus to develop alternative antimicrobials or new antimicrobials that can be used synergistically with antibiotics. Prior to the discovery and widespread use of antibiotics, bacterial infections were often treated with bacteriophages, which were marketed and sold commercially for human use up until the 1940's. Following discovery of antibiotics, the use of phages to treat bacterial diseases was discontinued in Western Europe and the United States. Bacteriophages continue to be sold in the Russian Federation and Eastern Europe as treatments for bacterial infections and there is renewed interest in utilizing bacterial viruses to prevent or treat bacterial infections. Bacteriophages which infect *C. perfringens* that are both lysogenic and virulent have been discovered that have long tails, members of the *Siphoviridae*, and those with short tails, members of the *Podoviridae*. If these bacteriophages or their gene products are to be used as antimicrobials, it is essential to have a blueprint of the genomic machinery underlying phage-mediated bacterial lysis. As genome sequencing costs are reduced in price, genomics-enabled approaches to utilizing bacteriophages, or perhaps more importantly their gene products, as naturally occurring antimicrobials will become increasingly more common.

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Bacteriophages have received attention as biological control agents since their discovery and recently their value as tools has been further emphasized in many different fields of microbiology. Particularly, in drug design and development programs, phage and prophage genomics provide the field with new insights. Bacteriophages reveals information on the organisms ranging from their biology to their applications in agriculture and medicine. Contributors address a variety of topics capturing information on advancing technologies in the field. The book starts with the biology and classification of bacteriophages with subsequent chapters addressing phage infections in industrial processes and their use as therapeutic or biocontrol agents. Microbiologists, biotechnologists, agricultural, biomedical and sanitary engineers will find Bacteriophages invaluable as a solid resource and reference book.

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821