Characterization of bacteriophages virulent for *Clostridium perfringens* and identification of phage lytic enzymes as alternatives to antibiotics for potential control of the bacterium[1]

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ABSTRACT There has been a resurgent interest in the use of bacteriophages or their gene products to control bacterial pathogens as alternatives to currently used antibiotics. *Clostridium perfringens* is a gram-positive, spore-forming anaerobic bacterium that plays a significant role in human foodborne disease as well as non-foodborne human, animal, and avian diseases. Countries that have complied with the ban on antimicrobial growth promoters in feeds have reported increased incidences of *C. perfringens*-associated diseases in poultry. To address these issues, new antimicrobial agents, putative lysins encoded by the genomes of bacteriophages, are being identified in our laboratory. Poultry intestinal material, soil, sewage, and poultry processing drainage water were screened for virulent bacteriophages that could lyse *C. perfringens* and produce clear plaques in spot assays. Bacteriophages were isolated that had long noncontractile tails, members of the family *Siphoviridae*, and with short noncontractile tails, members of the family *Podoviridae*. Several bacteriophage genes were identified that encoded *N*-acetylmuramoyl-L-alanine amidases, lysozyme-endopeptidases, and a zinc carboxypeptidase domain that has not been previously reported in viral genomes. Putative phage lysin genes (*ply*) were cloned and expressed in *Escherichia coli*. The recombinant lysins were amidases capable of lysing both parental phage host strains of *C. perfringens* as well as other strains of the bacterium in spot and turbidity reduction assays, but did not lyse any clostridia beyond the species. Consequently, bacteriophage gene products could eventually be used to target bacterial pathogens, such as *C. perfringens* via a species-specific strategy, to control animal and human diseases without having deleterious effects on beneficial probiotic bacteria.

Key words: enzybiotic, antibiotic alternative, bacterial virus, food safety, animal/human health

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INTRODUCTION

Clostridium perfringens is a gram-positive, spore-forming, anaerobic bacterium that is commonly present in the intestines of people and animals. *Clostridium perfringens* is classified into 1 of 5 types (A, B, C, D, or E) based on the toxin production (Smedley et al., 2004; Sawires and 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 it is a veterinary pathogen causing enteric diseases in both domestic and wild animals (Smedley et al., 2004; Sawires and Songer, 2006; Scallan et al., 2011). Necrotic enteritis is a peracute disease syndrome and the subclinical form of *C. perfringens* infections in poultry is caused by *C. perfringens* type A producing the α toxin. Some strains of *C. perfringens* type A produce an enterotoxin at the moment of sporulation that is responsible for foodborne 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 these are not available for practical use in the field at the present time (Van Immerseel et al., 2004). Consequently, there is a need for developing on-farm interventions to reduce populations of this bacterial pathogen that lead to peracute flock disease and potentially greater numbers of animal-borne *Clos-*

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tridium perfringens enterotoxin+ *C. perfringens* entering the human food chain.

Bacterial viruses were first reported in 1915 by Fredrick William Twort when he described a transmissible glassy transformation of micrococcus cultures that resulted in lysis of the bacterium (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). Prior to the discovery and widespread use of antibiotics, bacterial infections were treated by administering bacteriophages and were marketed by L'Oreal in France. Although Eli Lilly Co. sold phage products for human use up until the 1940s, early clinical studies with bacteriophages were not extensively undertaken in the United States and Western Europe after that time. 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 electron microscopy, 95% of those reported are tailed with only 3.7% being filamentous, polyhedral, or pleomorphic (Ackermann, 2007). The tailed bacteriophages contain linear, double-stranded DNA genomes that can vary from 11 to 500 kb in the order Caudovirales, which is further divided into 3 families based on tail morphology. These bacterial viruses have icosohedral heads, and those phages with contractile tails are placed in the *Myoviridae*, phages with a long noncontractile tail are placed in the *Siphoviridae*, whereas phages with short noncontractile tail structures are members of the *Podoviridae* (Ackermann, 2003, 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 bacteriophages, their lytic enzymes, or both has been of considerable interest for veterinary and human medicine, as well as the bioindustry worldwide due to antibiotic resistance issues. Recently, the US 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 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 routine use of antibiotics in animal feeds became effective on January 1, 2006 (regulation 1831/2003/EC). Removal of these antimicrobials will induce changes within the chicken gastrointestinal 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 (Cotter et al., 2005; Ricke et al., 2005). There has been a limited number of new antibiotic drugs marketed recently with only 2 developed since 2000: linezolid, which targets bacterial protein synthesis, and daptomycin, wherein the mechanism of action is unknown. 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 these agents (Projan, 2008). The view that there is no compelling reason to pursue development of novel therapeutic agents is unwise (Projan and Youngman, 2002), especially considering the emergence of pan-resistant or multiple-antibiotic-resistant strains of gram-positive bacteria (French, 2010). Consequently, bacteriophages 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.

MATERIALS AND METHODS

Bacteriophages were isolated from chicken offal, chicken feces, poultry production run-off, and raw sewage in both the Moscow region of the Russian Federation and the southeastern United States by standard methods (Ackermann, 2007; Van Twest and Kropinski, 2009; Wommack et al., 2009). Additionally, the viruses were purified by isopycnic centrifugation and analyzed by SDS-PAGE followed by mass spectrometry of the proteins. Subsequent identification of the phage genomic open reading frame (**ORF**) descriptive bacteriophage structure and identification of potential lytic proteins was completed by full-genome nucleotide sequencing, genome annotations, and BLAST analyses as described in detail by the investigators (Seal et al., 2011; Oakley et al., 2011; Volozhantsev et al., 2011, 2012; Morales et al., 2012). Recombinant proteins were produced by cloning bacteriophage genes encoding putative lysins such as *N*-acetylmuramoyl-L-alanine amidases and expression in *E. coli* (Simmons et al., 2010, 2012).

RESULTS AND DISCUSSION

Poultry intestinal material, sewage, and poultry processing drainage water were screened for virulent *Clostridium perfringens* bacteriophages that produced clear plaques on the host bacterium (Figure 1A). The first viruses isolated from broiler chicken offal washes and poultry feces were designated ΦCP39O and ΦCP26F that produced clear plaques on host strains (Seal et al., 2011). Both bacteriophages had isometric heads of 57 nm in diameter with 100-nm noncontractile tails characteristic of members of the family *Siphoviridae* in the order *Caudovirales* (Figure 1B). The double-strand DNA genomes of the siphoviral bacteriophages ranged from approximately 37,000 to 40,000 bp with average

guanine-cytosine (**GC**) contents of 30.3%. The viral genomes contained 60 to 64 potential ORF predicted to be encoded on one strand of the genome (Figure 2A). Among the ORF, 29 predicted proteins had no known similarity, whereas others encoded putative bacteriophage capsid components such as a pre-neck/appendage, tail, tape measure, and portal proteins. Other genes encoded were predicted DNA primases, single-strand DNA-binding proteins, terminases, thymidylate synthases, and potential transcription factors. Potential lytic enzymes such as a fibronectin-binding autolysin, an amidase/hydrolase, and a holin were encoded in the viral genomes. Several ORF-encoded proteins that gave BLASTP matches with proteins from *Clostridium* spp. and other gram-positive bacterial and bacteriophage genomes. Proteomic analysis of the purified viruses resulted in the identification of the putative pre-neck/appendage protein and a minor structural protein encoded by large ORF. Variants of the portal protein were identified, 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 2 phage gene products and are most likely the viral attachment protein. Based on phylogenetic analysis of the large terminase protein, these phages are predicted to be pac-type, using a head-full DNA packaging strategy. Importantly, no toxin-encoding genes were identified among the bacteriophages and the phage genomes probably do not integrate into the host genome (Seal et al., 2011).

Phage whole-genome tetra-nucleotide signatures and proteomic tree topologies correlated closely with host phylogeny. The shared or core genome was comprised of genes with multiple sequence types belonging to 5 protein families (**pfam**) and genes belonging to 12 pfam families, including the holin genes, were almost identical (Oakley et al., 2011). Comparisons of our siphoviral phage genomes to 26 other bacteriophage genomes revealed 3 shared clusters of orthologous groups, 2 of particular interest within this core genome was an endolysin (PF01520, an *N*-acetylmuramoyl-L-alanine amidase) potentially capable of digesting the *C. perfringens* peptidoglycan and a holin (PF04531) that disrupts the bacterial cell wall. Comparative analyses of the evolutionary history and genomic context of these common phage proteins revealed 2 important results: 1) strongly significant host-specific sequence variation within the endolysin, and 2) a protein domain architecture apparently unique to our phage genomes in which the endolysin is located upstream of its associated holin. This is the case relative to the only other siphoviral bacteriophage genome sequenced to date from a lysogenic phage (Zimmer et al., 2002a) wherein a lysin was identified and expressed for digestion of the *C. perfringens* peptidoglycan (Zimmer et al., 2002b). Endolysin sequences from our phages were 1 of 2 very distinct genotypes distinguished by variability within the putative enzymatically active domain. Holins and endolysins represent conserved functions across divergent phage genomes and endolysins can have significant variability and host-specificity even among closely related genomes. Endolysins in our phage genomes may be subject to different unknown selective pressures than the rest of the genome, and these findings could have important implications for potential biotechnological applications of phage gene products to control pathogenic bacteria (Oakley et al., 2011).

A virulent short-tailed bacteriophage ΦCPV1 was isolated in the Russian Federation utilizing *C. perfringens* as the host and was classified in the family *Podoviridae* (Volozhantsev et al., 2011). The purified virus had an icosahedral head and collar of approximately 42 and 23 nm in diameter, respectively, with a structurally complex tail of 37 nm lengthwise and a basal plate of 30 nm (Figure 1C). The ΦCPV1 double-stranded DNA genome was 16,747 bp with a GC composition of 30.5%. Twenty-two ORF coding for putative peptides containing 30 or more amino acid residues were identified in the genome (Figure 2B). Amino acid sequences of the predicted proteins from the ΦCPV1 genome ORF were compared with those from the National Center for Biotechnology Information database, and potential functions of 12 proteins were predicted by functional homology. Three putative proteins were similar to hypothetical proteins with unknown functions, whereas 7 proteins did not have similarity with any known bacteriophage or bacterial proteins. Identified ORF formed at least 4 genomic clusters that ac-

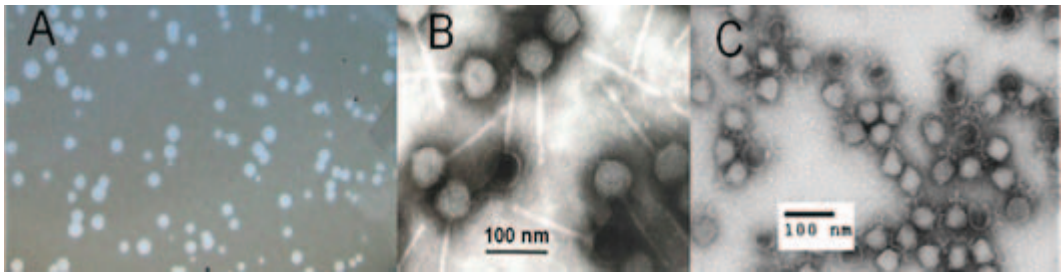
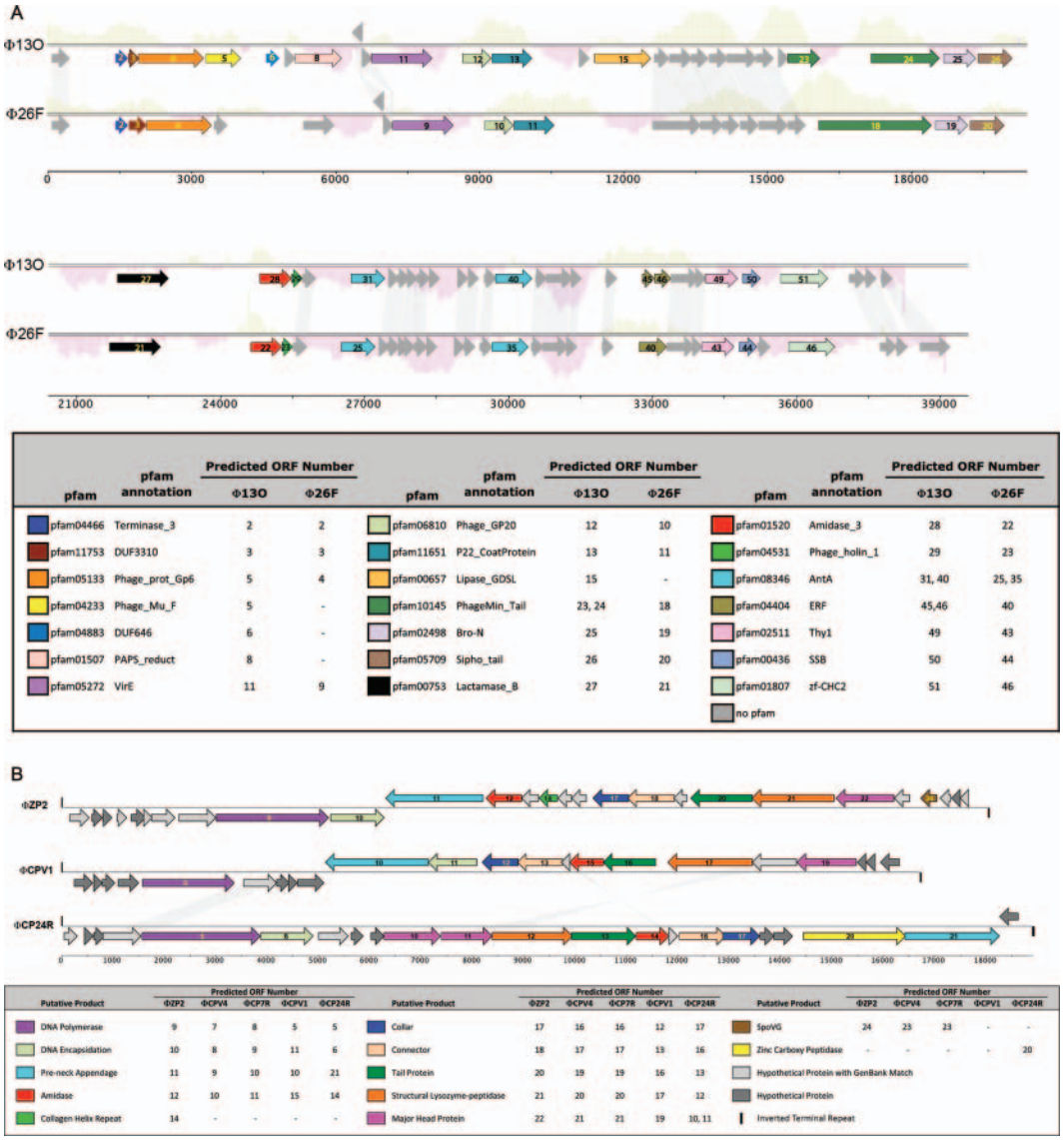


Figure 1. Representative clear plaques and electron micrographs for Russian and US bacteriophages. (A) All phages were plaque-purified at least 3×. (B) Many phages had long noncontractile tails representative of the *Siphoviridae*. (C) Several phages were representative of the *Podoviridae* with short noncontractile tails. Color version available in the online PDF.



pfam	pfam annotation	Predicted ORF Number Φ13O	Φ26F	pfam	pfam annotation	Φ13O	Φ26F	pfam	pfam annotation	Φ13O	Φ26F
pfam04466	Terminase_3	2	2	pfam06810	Phage_GP20	12	10	pfam01520	Amidase_3	28	22
pfam11753	DUF3310	3	3	pfam11651	P22_CoatProtein	13	11	pfam04531	Phage_holin_1	29	23
pfam05133	Phage_prot_Gp6	5	4	pfam00657	Lipase_GDSL	15	-	pfam08346	AntA	31, 40	25, 35
pfam04233	Phage_Mu_F	5	-	pfam10145	PhageMin_Tail	23, 24	18	pfam04404	ERF	45,46	40
pfam04883	DUF646	6	-	pfam02498	Bro-N	25	19	pfam02511	Thy1	49	43
pfam01507	PAPS_reduct	8	-	pfam05709	Sipho_tail	26	20	pfam00436	SSB	50	44
pfam05272	VirE	11	9	pfam00753	Lactamase_B	27	21	pfam01807	zf-CHC2	51	46
									no pfam		

Putative Product	Predicted ORF Number ΦZP2	ΦCPV4	ΦCP7R	ΦCPV1	ΦCP24R	Putative Product	ΦZP2	ΦCPV4	ΦCP7R	ΦCPV1	ΦCP24R	Putative Product	ΦZP2	ΦCPV4	ΦCP7R	ΦCPV1	ΦCP24R
DNA Polymerase	9	7	8	5	5	Collar	17	16	16	12	17	SpoVG	24	23	23	-	-
DNA Encapsidation	10	8	9	11	6	Connector	18	17	17	13	16	Zinc Carboxy Peptidase	-	-	-	-	20
Pre-neck Appendage	11	9	10	10	21	Tail Protein	20	19	19	16	13	Hypothetical Protein with GenBank Match					
Amidase	12	10	11	15	14	Structural Lysozyme-peptidase	21	20	20	17	12	Hypothetical Protein					
Collagen Helix Repeat	14	-	-	-	-	Major Head Protein	22	21	21	19	10, 11	Inverted Terminal Repeat					

Figure 2. Genomic maps for bacteriophages virulent for *Clostridium perfringens*. (A) Genomic maps for members of the *Siphoviridae*, bacteriophages with long noncontractile tails. (B) Genomic maps for members of the *Podoviridae*, bacteriophages with short noncontractile tails.

counted 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 digest peptidoglycan of the bacterial cell wall and could be considered potential therapeutics to control *C. perfringens* (Volozhantsev et al., 2011). Another bacteriophage, ΦCP24R, was isolated from raw sewage at a US waste treatment plant and lytic activity was observed against a type A *C. perfringens* isolate (Morales et al., 2012). Electron microscopy revealed a small virion (44-nm-diameter icosahedral capsid) with a short, noncontractile tail, indicative of a member of the family *Podoviridae*. The phage had a linear, double-stranded DNA genome of 18,919 bp with 41 bp inverted terminal repeats that encoded a type B DNA polymerase, which are characteristics of members of the subfamily *Picovirinae*. Out of 22 predicted genes in the genome, 10 had significant sequence similarity to proteins of known functions. Three distinct genes with lytic domains were identified, including a zinc carboxypeptidase domain that has not been previously reported in the viral kingdom (Morales et al., 2012).

Two more short-tailed bacteriophages, designated ΦCPV4 and ΦZP2, were isolated in the Moscow Region of the Russian Federation, whereas another closely related virus, named ΦCP7R, was isolated in the southeastern United States (Volozhantsev et al., 2012). The viruses were identified as members of the order Caudovirales in the family *Podoviridae* with short, noncontractile tails of the C1 morphotype. The genomes of the 3 bacteriophages were 17,972, 18,078, and 18,397 bp, respectively, encoding 26 to 28 ORF with inverted terminal repeats and an average GC content of 34.6% (Figure 2B). Structural proteins identified by mass spectrometry in the purified ΦCP7R virion included a

pre-neck/appendage with putative lyase activity, major head, tail, connector/upper collar, lower collar, and a structural protein with putative lysozyme-peptidase activity. All 3 podoviral bacteriophage genomes encoded a predicted *N*-acetylmuramoyl-L-alanine amidase and a putative stage V sporulation protein. Each putative amidase contained a predicted bacterial SH3 domain at the C-terminal end of the protein, presumably involved with binding the *C. perfringens* cell wall. The predicted DNA polymerase type B protein sequences were closely related to other members of the *Podoviridae* including *Bacillus* phage Φ29. Whole-genome comparisons supported this relationship, but also indicated that the Russian and US viruses may be unique members of the subfamily *Picovirinae* (Volozhantsev et al., 2012).

Bacteriophages have been considered as potentially important alternatives to antibiotics (Sulakvelidze et al., 2001; Lu and Koeris, 2011; Maura and Debarbieux, 2011). However, it is important to emphasize that development of bacterial resistances to their viruses occurs, such as evolution of phage receptors, super-infection exclusion, restriction enzyme-modification systems, and abortive infection systems such as bacterial CRISPR sequences (Labrie et al., 2010). These phenomena all point to the inevitable need for constantly searching for new bacteriophage isolates to use therapeutically. Also, it should be noted that although bacteriophage therapy has been used as a treatment, Smith (1959) reported that a large proportion of *C. perfringens* strains remained resistant to infection by many of the bacteriophages isolated during his investigations. This has been observed during our investigations, wherein most bacteriophages virulent for *C. perfringens* have a restricted host range for a specific isolate of the bacterium (Figure 3). 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; Oakley et al., 2011; Volozhantsev et al., 2011, 2012). Therefore, selection of appropriate bacteriophage cocktails may not necessarily be effective against many of the various bacterial isolates that exist in the environment. This was proven to be the case for *Listeria monocytogenes* where the FDA-approved phage cocktail does not kill many isolates of the bacterium obtained from ready-to-eat foods (Shen et al., 2006).

Several new antimicrobial agents, putative lysins encoded by the genomes of clostridial bacteriophages have been identified in our laboratories (Figure 4). Two putative phage lysin genes (ply) from the clostridial phages ΦCP39O and ΦCP26F were cloned and expressed in *E. coli*, and the resultant proteins were purified to near homogeneity. Gene and protein sequencing revealed that the predicted and chemically determined amino acid sequences of the 2 recombinant proteins were homologous to *N*-acetylmuramoyl-L-alanine amidases. The proteins from those 2 bacteriophages were identical in the C-terminal putative cell-wall binding domain, but had only 55% identity to each other in the presumptive *N*-terminal catalytic domain. Both recombinant lysins were capable of lysing (Figure 5) their parental phage host strains of *C. perfringens* as well as other strains of the bacterium in spot and turbidity reduction assays. The observed reduction in turbidity was correlated with up to a 3 log reduction of viable *C. perfringens* by culturing. Importantly, all other member species of the clostridia were resistant to the lytic activity by both assays proving the species-specificity for *C. perfringens* (Simmons et al., 2010) as was the case for the only other reported bacteriophage murein hydrolase (Zimmer et al., 2002b). Although there has been no reported detection of resistances to bacteriophage lysins (Nelson et al., 2012), investigators are examining the ability to create multi-functional enzymes expressing more than one lytic activity to avoid potential resistance issues as a "hurdle approach" antimicrobial (Schmelcher et al., 2012).

Listeria monocytogenes is a gram-positive, non-spore forming, catalase-positive rod that is a highly fatal bacterial foodborne disease agent of humans associated with uncooked meats including poultry, uncooked vegetables, soft cheeses, and unpasteurized milk. The bacterium may be carried by animals without signs of disease, can replicate at refrigeration temperatures, and is frequently associated with biofilms. There is a need to discover innovative pathogen intervention technologies for this bacterium due to resistances to antibiotics and bacteriophages (Shen et al., 2006). Consequently, using our phage lysins as query subjects, bioinformatic analyses were used to identify genes encoding lytic protein sequences in the genomes of *L. monocytogenes* isolates (Simmons et al., 2012). The PCR primers were designed that amplified nucleotide sequences of a putative *N*-acetylmuramoyl-L-alanine amidase gene from the genomic DNA of a *L. monocytogenes* strain 4b isolate. Gene and protein sequencing revealed that the predicted and chemically determined amino acid sequence of the recombinant protein, designated PlyLM, was a putative *N*-acetylmuramoyl-L-alanine amidase. The recombinant lytic protein was capable of lysing both the parental *L. monocytogenes* strain as well as other strains of the bacterium in spot and microbial inhibition assays, but was not active against other bacteria beyond the genus. A common microtiter plate assay (Djordjevic et al., 2002) was used to assay for the ability of the recombinant lysin protein to potentially aid with digestion of a *L. monocytogenes* biofilm. Protease or lysozyme digestion alone did not significantly reduce the *L. monocytogenes* biofilm. Although the recombinant lysin protein alone reduced the biofilm by only 20%, complete digestion of the bacterial monolayer was accomplished in conjunction with a protease (Simmons et al., 2012). Recombinant bacteriophage endolysins (e.g., amidases) have also been used to hydrolyze heat-killed staphylococci as well as digest staphylococcal biofilms (Wu et al., 2003; Sass and Bierbaum, 2007; Son et al., 2010).

Enzymes are added to monogastric animal feed for digesting carbohydrates and for metabolizing phytate

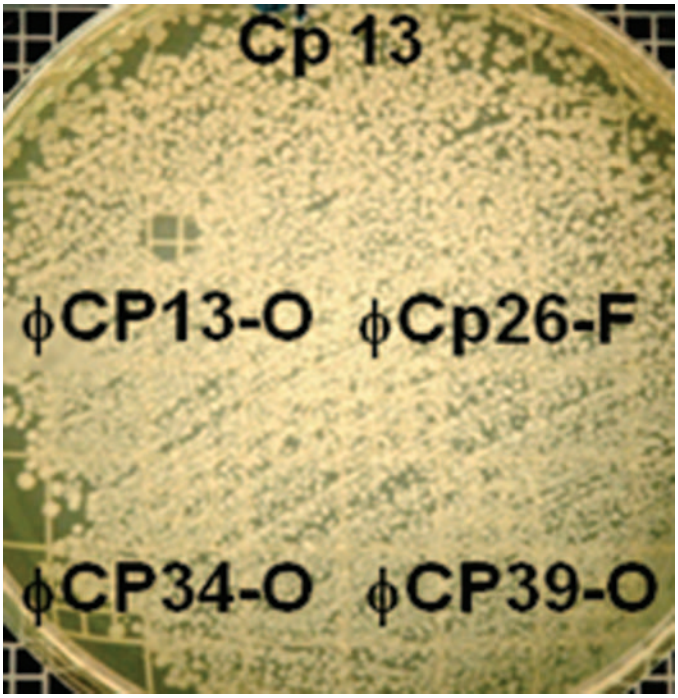


Figure 3. Bacteriophage host restriction for *Clostridium perfringens*. Infection of the parent *Clostridium perfringens* strain Cp13 only supports replication of its individual bacteriophage and not that of other *C. perfringens* bacteriophages demonstrating viral host restriction.

to produce free phosphorus. These are marketed commercially for poultry feed additives, many of which are produced as recombinant proteins in yeast and sold as a lysate (Cowieson et al., 2006; Selle et al., 2012), which argues for the economic feasibility of developing bacteriophage enzymes as feed additives. Production of enzymes by *Pichia pastoris* can serve as a potential

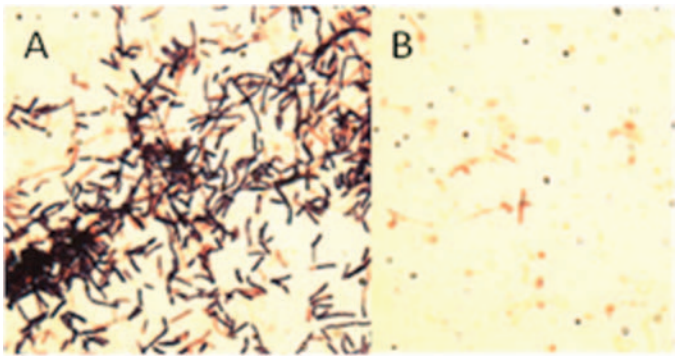


Figure 5. Lysis of *Clostridium perfringens* by a recombinant bacteriophage enzyme. Cells treated without phage lysin (A) or with lysin (B); note that all *C. perfringens* cells were completely digested with the addition of a recombinant bacteriophage-encoded lysin.

source for structural or animal feed studies (Johnson et al., 2010) and lysozyme can be encapsulated (Zhong and Jin, 2009), which has been used 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 feeds for reducing the bacterium to improve health and food safety for monogastric animals during production.

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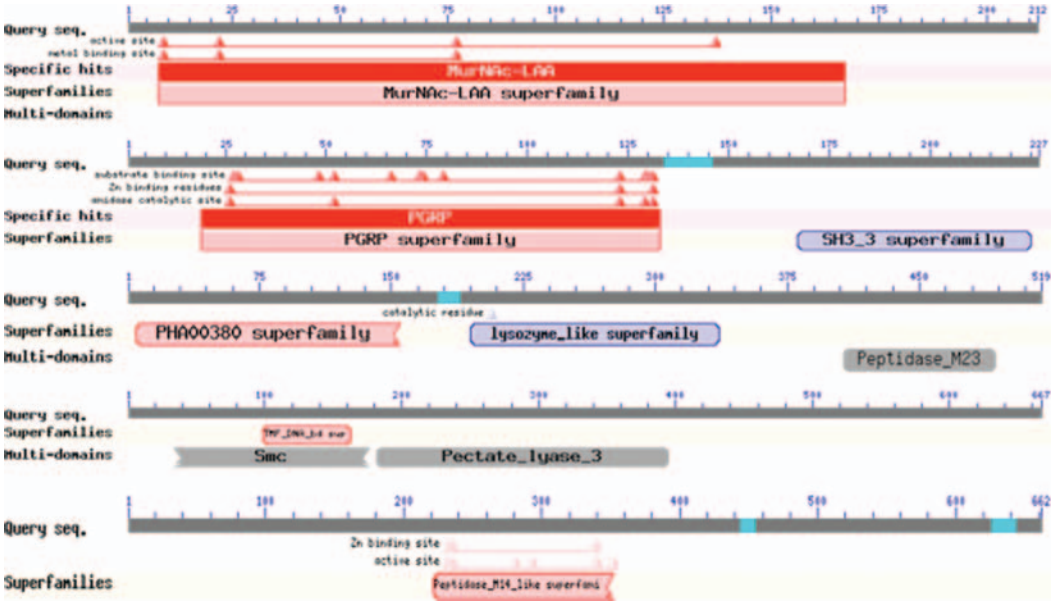

Figure 4. Bacteriophage proteins identified with potential to lyse *Clostridium perfringens*. Potential lytic proteins encoded by bacteriophages included *N*-acetylmuramoyl-L-alanine amidases with C-terminal cell wall binding domains, tail proteins with lysozyme and peptidase activities as well as a pectate lyase and a previously unknown viral Zn-endopeptidase.

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