Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

REVIEW ARTICLE

Bacteriophages and bacteriophage-derived endolysins as potential therapeutics to combat Gram-positive spore forming bacteria

A. Nakonieczna[1], C.J. Cooper[2] and R. Gryko[1]

1 Biological Threats Identification and Countermeasure Center of the Military Institute of Hygiene and Epidemiology, Pulawy, Poland
2 Department of Molecular Biosciences, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden

Keywords
Bacillus, bacteriophage, *Clostridium*, endolysin, phage therapy.

Correspondence
Romuald Gryko, Biological Threats Identification and Countermeasure Centre of the Military Institute of Hygiene and Epidemiology, 2 Lubelska St., 24-100 Pulawy, Poland.
E-mail: romgryko@gmail.com

2015/0428: received 2 March 2015, revised 28 May 2015 and accepted 11 June 2015

doi:10.1111/jam.12881

Summary

Since their discovery in 1915, bacteriophages have been routinely used within Eastern Europe to treat a variety of bacterial infections. Although initially ignored by the West due to the success of antibiotics, increasing levels and diversity of antibiotic resistance is driving a renaissance for bacteriophage-derived therapy, which is in part due to the highly specific nature of bacteriophages as well as their relative abundance. This review focuses on the bacteriophages and derived lysins of relevant Gram-positive spore formers within the *Bacillus cereus* group and *Clostridium* genus that could have applications within the medical, food and environmental sectors.

Bacteriophages and their derived lysins

Bacteriophages are believed to be the most abundant biological entities on the planet (estimated at 10^{31} plaque forming units) and are present in nearly every environment. They are well known to play important roles in the control of bacterial populations and bacterial evolution (Stern and Sorek 2011). During the early part of the 20th century, bacteriophages were first used as a therapeutic agent under a variety of different commercial names (*Bacte-intesti-phages, Bacte-pyo-phages, Bacte-coli-phages, Bacte-rhino-phages and Bacte-staphy-phage*; Abedon *et al.* 2011). Bacteriophage preparations were also successfully deployed to treat wounded Red Army soldiers during the Soviet invasion of Finland in 1938 (Chanishvili *et al.* 2001). With the discovery and subsequent success of antibiotics, bacteriophage research and clinical use has been primarily confined to the former Soviet Union due to concerns over safety and efficacy (Hanlon 2007).

Although renewed Western interest has stimulated the commercialization of some phage cocktails (Listex P100, SalmoFresh, ListShield etc.), these have been confined to the food industry where bacterial contamination is an increasing burden (Żaczek *et al.* 2015). The success of phage-based products within the food industry is not only driven by their relative ease of application (Żaczek *et al.* 2015) but also due to negative public perception of chemically treated food and positive perception of phages (Naanwaab *et al.* 2014) together with increasing levels of legislation restricting the use of antibiotics (Seal 2013). The improper prescription or nonprescription sale of antibiotics in some countries, and also poor patient compliance, has contributed to the rapid spread of multidrug resistance in human pathogens. Phages and their derived endolysins offer a number of advantages over conventional antibiotic therapy, ranging from their high degree of specificity, to relative abundance in the environment which should allow for any developed resistance to be overcome, simply by changing the phage.

Although the simplest explanation of bacteriophage replication focuses on two lifecycles, the lytic and lysogenic cycles (Fig. 1), there are multiple variations on these cycles including pseudolysogeny (continual infection) and chronic infection (progeny release through budding) which take place particularly when hosts lack nutrients (Golais *et al.* 2013). Endolysins are produced at the end of the lytic phage lifecycle, accumulated within the host cell cytoplasm until they are activated releasing progeny phage by the

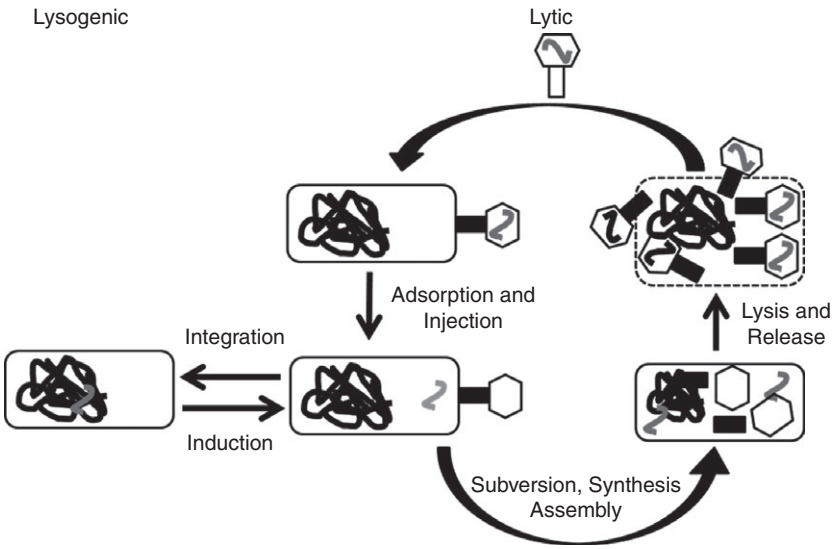

Figure 1 Simplified schematic representation of bacteriophage lifecycle. Adapted from (Sulakvelidze *et al.* 2001).

cleavage of peptidoglycan bonds (Fischetti 2010). Endolysins possess a globular or modular structure (Walmagh *et al.* 2013). Endolysins of Gram-negative bacteria are primarily globular in structure possessing only a catalytic domain (Callewaert *et al.* 2011), while lysins of Gram-positive bacteria are usually 25–40 kDa large (Fischetti 2010), modular in structure and exhibit variety in their architecture that goes beyond the classical two domain structure of endolysins (Yuan *et al.* 2012a).

The highly conservative N-terminal domain has catalytic activity and is responsible for enzymatic function. Endolysins may demonstrate several types of enzymatic activity, and therefore may be divided into five different classes according to the identity of their N-terminal domain: 1) N-acetylmuramidases (lysozymes), 2) endo-β-N-acetylglucosaminidases, 3) lytic trans-glycosylases, 4) endopeptidases, and 5) N-acetylmuramyl-L-Ala-amidases. Classes 1–3 cleave the sugar moieties; class 4 cleaves peptide moieties, while class 5 cleaves peptide bonds between these two moieties. The most populated classes include amidases and muramidases; all endolysins, except for transglycolases, are hydrolases (Schmelcher *et al.* 2012).

The C-terminal domain is capable of specifically recognizing ligands (usually carbohydrate ligands) within the bacterial wall, and is responsible for tethering the endolysin molecule to peptidoglycan. The number of binding domains varies between endolysins (Yuan *et al.* 2012a). The affinity is so strong that it may be compared to antigen-antibody binding (Loessner *et al.* 2002).

The importance of *Bacillus* and *Clostridium* spore formers

Members of both the *Bacillus cereus* group and *Clostridium* genus possess the ability to form endospores upon starvation (Leggett *et al.* 2012). This allows the bacterium to remain dormant for long periods of time. Among the most notable feature of bacterial endospores is the increased level of resistance to decontamination efforts ranging from biocides (Leggett *et al.* 2015) to ionizing radiation (Moeller *et al.* 2014; Setlow *et al.* 2014) which present challenges to those seeking to remediate spore contamination.

The *Bacillus cereus* group is a highly homogenous subdivision of the *Bacillus* genus, and its primary members comprise of *B. cereus, Bacillus anthracis* and *Bacillus thuringiensis*. *Bacillus mycoides, Bacillus pseudomycoides* and *Bacillus weihenstephanensis* are minor members of this group (Priest *et al.* 2004; Rasko *et al.* 2005). Although most members of this group are considered to be nonpathogenic (Ceuppens *et al.* 2013), *B. cereus* and *B. anthracis* are known to cause serious human disease. *Bacillus anthracis* is perhaps the most widely known, causing three forms of anthrax, cutaneous, gastrointestinal and inhalational. Cutaneous anthrax is the most commonly exhibited form in humans resulting from close contact with contaminated environments and contaminated animal products (Doganay and Metan 2009), while gastrointestinal and inhalational anthrax are considerably rarer but often more severe. *Bacillus cereus* is a common soil dwelling bacterium, which easily spreads to foodstuffs and exhibits two types of disease in humans as the result of toxin production. The two forms of *B. cereus* poisoning are diarrhoeal, the result of bacterial growth within the small intestine and the emetic form which results from growth on foodstuffs (Granum and Lund 1997). Both forms of *B. cereus* poisoning have been implicated in 235 suspected or confirmed outbreaks between 1998 and 2008 (Bennett *et al.* 2013). The high degree of genetic similarity between the major members of this

group complicates diagnosis with many PCR-based assays unable to consistently distinguish between them (Rasko *et al.* 2005). Indeed, plasmid-associated genes encoding virulence factors such as toxin production are also shared between strain as is the case with *B. anthracis and B. cereus* G9241 (Hoffmaster *et al.* 2004).

The *Clostridium* genus contains approx. 100 species which are spore forming obligate anaerobes. This genus contains species which are useful to industrial applications, *Clostridium thermocellum* which is used in bioethanol production and *Clostridium beijerinckii* involved in biohydrogen production (Roberts *et al.* 2010; Seelert *et al.* 2015) as well as human pathogens *Clostridium difficile*, *Clostridium perfringens* (Seal 2013; Hargreaves and Clokie 2014). Despite existing as a minor component of the gut commensal microflora, *Cl. difficile* has emerged as an important source of healthcare-associated infections within the developed world (Hargreaves and Clokie 2014) with an estimated economic impact of $4·8 billion in excess medical costs within the USA alone (Dubberke and Olsen 2012). *Clostridium perfringens* and *Clostridium botulinum* are both commonly found contaminants of food products that are able to cause gastrointestinal diseases resulting from toxin production, usually type A toxin (Lund and Peck 2013; Seal 2013), with *Cl. perfringens* being confirmed to be or suspected to being implicated in 536 foodborne disease outbreaks between 1998 and 2008 in the USA (Bennett *et al.* 2013).

Bacteriophages of *Bacillus* and *Clostridium*

Although virulent phages have been isolated and characterized against multiple *Bacillus* species (Table 1), *Cl. difficile* phages appear to be wholly temperate in nature (Table 2) and when induced show instability and a tendency to reintegrate into the host genome following the removal of inducers such as mitomycin C (Meessen-Pinard *et al.* 2012).

The use of the lytic γ bacteriophage against *B. anthracis* has long been established as a diagnostic method for anthrax due to its high specificity (≥95%) (Abshire *et al.* 2005). However, the application of phages to therapy requires a wider host range and maintenance of activity. The *B. cereus* Bc431v3 phage, a member of the Myoviridae, was able to cause different levels of lysis within multiple strains of *B. cereus*, *B. anthracis*, *B. licheniformis* and *B. weihenstephanensis* based upon plaque formation (El-Arabi *et al.* 2013).

The presence of Tectiviridae within the *B. cereus* group is at a lower prevalence (3% from 2000 *B. cereus* group isolates) compared to members of the Caudovirale (Gillis and Mahillon 2014). Analysis of the host range of phages from the Tectiviridae showed that the host range was divided into two clusters, with phages that infect *B. anthracis* in one cluster and those isolated from other *B. cereus* group members in the other and that within these two groups, a greater DNA diversity exists than previously thought (Gillis and Mahillon 2014).

The majority of the published literature focuses on *Cl. difficile* phages due to its impact on human health and as such has been reviewed in greater detail elsewhere (Hargreaves and Clokie 2014). These phages are primarily members of the Myoviridae with some members of the Siphoviridae also present (Nale *et al.* 2012; Sekulovic *et al.* 2014). The study by Sekulovic *et al.* (2014) also suggested that Siphoviradae phages isolated from human *Cl. difficile* isolates possessed a wider host rage than their animal counterparts.

Clostridium perfringens phage ϕ3626 was shown to be lytic against 11 strains of 51 *Cl. perfringens* strains that were assessed. This phage has also shown the presence of a sporulation associated sigma factor within ORF32, it was hypothesized that this may have an effect on the sporulation of *Cl. perfringens*, as the curing of a lysogenic strain resulted in decreased heat resistance, sporulation efficacy and potentially the expression of the Cpe enterotoxin (Zimmer *et al.* 2002).

Although phages isolated from *Cl. difficile* are lysogenic in nature, some phages isolated from *Cl. perfringens* exhibit lytic tendencies (Volozhantsev *et al.* 2012). The ΦCPV1 phage is a lytic member of the Podoviridae is distantly related to Φ29-like viruses when compared using protein sequences. However, ΦCPV1 would likely be classified to this group based on the number of protein sequences and the presence of a phage–encoded DNA polymerase. Sequence analysis also revealed the presence of two genes that encode for a N-acetylmuramoyl L-alanine amidase and a lysozyme-endopeptidase. These sequences shared no homology to other known holins (Volozhantsev *et al.* 2012).

Comparative genomic analysis between four *Cl. perfringens* phages (ΦCP9O, ΦCP13O, ΦCP26F and ΦCP34O) showed significant genomic diversity between phages particularly within endolysin regions that could be split into one of two distinct genotypes (Oakley *et al.* 2011).

Endolysins of *Bacillus* and *Clostridium*

Produced during the late stages of phage infection, endolysins are designed to attack the peptidoglycan that holds the bacterial cell together, to release phage progeny. The cleavage site is within or between the sugar or peptide moieties of peptidoglycan (Elbreki *et al.* 2014). Summaries on phage-derived endolysins for *Bacillus* and *Clostridium* can be found in Tables 3 and 4 respectively.

Table 1 Summary of the bacteriophages of the *Bacillus* group

Bacteriophage	Morphology	Host	Genome size (kbp)*	GC%	References
B4	Myoviridae	*Bacillus cereus*	162·6	37·71	Lee *et al.* (2013)
BPS13	Myoviridae	*B. cereus*	158·3	38·75	Shin *et al.* (2014)
BPS10C	Myoviridae	*B. cereus*	159·6	38·74	Shin *et al.* (2014)
12826	Not classified	*B. cereus*	–†	–†	Loessner *et al.* (1997)
Bc431v3	Myoviridae	*B. cereus*	158·6	39·98	El-Arabi *et al.* (2013)
PBC1	Siphoviridae	*B. cereus*	41·2	41·68	Kong *et al.* (2012)
BCP78	Siphoviridae	*B. cereus*	156·2	39·86	Lee *et al.* (2012)
Bastille	Myoviridae	*B. cereus*	127·0	n.d	Loessner *et al.* (1997)
γ	Siphoviridae	*Bacillus anthracis*	37·3	35·22	Fouts *et al.* (2006)
Fah	Siphoviridae	*B. anthracis*	38·0	34·94	Minakhin *et al.* (2005)
Cherry	Siphoviridae	*B. anthracis*	36·6	–†	Oey *et al.* (2009)
AP50	Tectiviridae	*B. anthracis*	14·4	38·65	Sozhamannan *et al.* (2008)
vB_BanS-Tsamsa	Siphoviridae	*B. anthracis*	168·9	34	Ganz *et al.* (2014)
BtCS33	Siphoviridae	*Bacillus thuringiensis*	42·0	35·22	Yuan *et al.* (2012b)

*Genome size has been rounded to the nearest 100 bp.
†No data available. Bacteriophage structure has been reviewed in greater detail elsewhere (Ackermann 2012).

LysB4, isolated from *B. cereus* phage B4, differs from many of the other *B. cereus* phage-derived endolysins due to its endopeptidase (cleaving the bonds between L-Ala and D-Glu), rather than amidase activity (Son *et al.* 2012) although this lysin is not unique in possessing a nonamidase mechanism of action. LysB4 also requires the presence of zinc or manganese ions to maintain proper activity (Son *et al.* 2012). This endolysin possessed a wide range of activity against Gram-positive organisms (including *B. subtilis* and *Listeria monocytogenes* strains) as well as activity against some Gram-negative pathogens including *Pseudomonas aeruginosa* and *Salmonella typhimurium* that had been pretreated with EDTA (Son *et al.* 2012). The same *B. cereus* strain was the host for the phage BPS13. Isolation and subsequent analysis using BLASTP software have shown that endolysin LysBPS13 belongs to a class of N-acetylmuramyl-L-Ala-amidases, which generates free N-acetylmuramic acid from murein in treated bacterial cells. LysBPS13 is built of a peptidoglycan recognition protein domain with catalytic properties and a zinc-binding capability resulting from a conserved motif of three amino acid moieties, His29, His129 and Cys137. However, the C/N terminus of this endolysin is still to be fully characterized (Park *et al.* 2012). LysBPS13 is also characterized by a relatively broad spectrum of activity, including several strains of *B. cereus*, *B. thuringiensis*, *B. licheniformis, B. megaterium* and *Bacillus pumilus* (Park *et al.* 2012). LysBPS13 is thermally stable and thermal stability can be further enhanced in combination with 30% (v/v) glycerol. Only a few endolysins with high temperature resistance have been reported to date (Lavigne *et al.* 2004).

The PlyG endolysin, encoded by the *B. anthracis* γ phage, has a narrow spectrum of activity that encompasses only the anthrax bacterium, therefore testing the resistance of a strain to this particular lysin is a simple and quick method for the identification of this pathogen. Yang *et al.* (2012) demonstrated that different PlyG domains are responsible for binding to vegetative cells and spores. A spore binding domain was identified, consisting of 60 amino acids, with binding sites most probably located within the exosporium of a spore. However, the tests led to only a moderate reduction (approx. 1–2 $\log_{10}$) in spore numbers following a 30-min exposure to endolysin, even in the presence of L-alanine as the germination inducer. When the N-terminal structure of PlyG was modified (via point mutations), a histidine residue at position 29 and a glutamic acid residue at position 90 were required for catalytic activity (Kikkawa *et al.* 2008). In addition, the binding domain of PlyG was studied, showing that elimination of its central part completely deprives the enzyme of its lytic activity, suggesting that both domains are equally important for the enzyme to function properly (Kikkawa *et al.* 2008). The thermal stability of PlyG has been investigated and it has been shown that PlyG maintains ≥80% relative lytic activity at temperatures up to 60°C in the presence of a tris(2-carboxyethyl)phosphaline (TCEP) reducing agent after a 10 min exposure time. However, without the TCEP, this activity dropped to approx. 40% at 50°C. The endolysin exhibited a varied half-life depending on the storage temperature. At 50°C this was approx. 35 h while at 55°C this decreased to 3·5 h (Heselpoth *et al.* 2015). PlyG for therapeutic has recently been patented and demonstrates increased levels of survival in *B. anthracis* infected mice that were subsequently treated with PlyG (Fischetti *et al.* 2013). However, the requirement to induce spore germination prior to the addition of PlyG could limit its

Table 2 Summary of the bacteriophages of the *Clostridium* group

Bacteriophage	Morphology	Host	Genome size (kbp)*	GC%	References
ΦC2	Myoviridae	*Clostridium difficile*	56·5	28·7	Goh *et al.* (2007)
ΦC5	Myoviridae	*Cl. difficile*	45·9	–†	Goh *et al.* (2005)
ΦC6	Siphoviridae	*Cl. difficile*	36·3	–†	
ΦC8	Myoviridae	*Cl. difficile*	54·5	–†	
ΦCD27	Myoviridae	*Cl. difficile*	50·9	29·4	Mayer *et al.* (2008)
ΦMMP01	Myoviridae	*Cl. difficile*	–†	–†	Meessen-Pinard *et al.* (2012)
ΦMMP02	Myoviridae	*Cl. difficile*	48·4	29·6	
ΦMMP03	Myoviridae	*Cl. difficile*	–†	–†	
ΦMMP04	Myoviridae	*Cl. difficile*	31·7	30·3	
ΦCP24R	Podoviridae	*Clostridium perfringens*	18·9	27·8	Morales *et al.* (2012)
ΦCPV1	Podoviridae	*Cl. perfringens*	16·7	30·5	Volozhantsev *et al.* (2011)
Φ3626	Siphoviridae	*Cl. perfringens*	33·5	28·4	Zimmer *et al.* (2002)
ΦCPV4	Podoviridae	*Cl. perfringens*	18·0	–†	
ΦZP2	Podoviridae	*Cl. perfringens*	18·1	–†	Volozhantsev *et al.* (2012)
ΦCP7R	Podoviridae	*Cl. perfringens*	18·4	–†	
Φ8701-B1	Siphoviridae	*Cl. sporogenes*	47·6	35·4	Mayer *et al.* (2012)
ΦCTP1	Siphoviridae	*Cl. tyrobutyricum*	51·9	–†	Mayer *et al.* (2010)

*Estimated and rounded to the nearest 100 bp.
†No data available. Bacteriophage structure has been reviewed in greater detail elsewhere (Ackermann 2012).

Table 3 Summary of the enzymatic activity and physical characteristics of bacteriophage-derived endolysisns of members of the *Bacillus* group

Endolysin	Bacteriophage	Enzymatic activity	Mass (kDa)	Length (aa)	References
LysB4	B4	Endopeptidase	28	260	Son *et al.* (2012)
LysBPS13	BPS13	Amidase	37	277	Park *et al.* (2012)
Ply12	12826	Amidase	27·7	257	Loessner *et al.* (1997)
Ply21	TP21	Amidase	29·5	263	
PlyBa	Bastille	Glycosidase	41·1	364	
PlyG	γ	Amidase	26·2	233	Kikkawa *et al.* (2007, 2008), Yang *et al.* (2012)
PlyB	Bcpl	Muramidase	–*	n.d.	Porter *et al.* (2007)
Phage AP50c lysine	AP50c	Amidase	27·7	252	Sozhamannan *et al.* (2008)
PlyBT33	BTCS33	Amidase	33	272	Yuan *et al.* (2012a)
PlyPH	–*	Amidase	–*	n.d	Yoong *et al.* (2006)
PlyL	Ba02	Amidase	–*	263	Low *et al.* (2005)
AmiBA2446	–*	Amidase	*c.* 24	245	Mehta *et al.* (2013)
PlyBt33	BtCS33	Amidase	33	–*	Yuan *et al.* (2012a)

*No data available.

potential application in human therapy. The PlyB endolysin (derived from phage Bcpl) has a different mechanism of action compared to that of PlyG, cleaving the bond between the N-acetylmuramic acid and N-acetylglucosamine. This lysin could also potentially be applied as a human therapeutic for those with an active *B. anthracis* infection as it has been shown that administration of 2·5 μmol l^{-1} of the endolysin resulted in the complete lysis of an exponentially growing culture of *B. anthracis* ATCC 4342 (Porter *et al.* 2007).

PlyBt33 is classified as an N-acetylmuramyl-L-Ala amidase and was obtained from bacteriophage BtCS33, possessing lytic activity against *B. thuringiensis kurstaki* strain CS-33. It showed high lytic activity against *B. cereus* group members, including *B. cereus* and *B. anthracis*. Specific activity was also observed in *B. pumilus* and *B. subtilis* strains, whereas the degree of lysis achieved in *B. thuringiensis* was quite low (Yuan *et al.* 2012a). This particular endolysin is also separated from other endolysins by relatively high thermal stability.

The CD27L lysin derived from the *Cl. difficile* ΦCD27 phage has been shown to possess zinc dependent N-acetylmuramoyl-L-alanine amidase activity against multiple *Cl. difficile* strains but possessed no activity against other *Clostridium* species (Mayer *et al.* 2008). A follow up study by the same group showed that lytic activity resided in the N-terminal domain (amino acids 1–179) and that truncation of the endolysin could increase activity

Table 4 Summary of the enzymatic activity and physical characteristics of bacteriophage-derived endolysins of members of the *Clostridium* group

Endolysin	Bacteriophage	Enzymatic activity	Mass (kDa)	Length (aa)	References
CD27L	ΦCD27	Amidase	32	270	Mayer *et al.* (2012, 2008)
CS74L	ATCC 8074-B1	Amidase	31·1	–*	
Psm-his	ΦSM101	Muramidase	40	–*	Nariya *et al.* (2011)
Ctp11	ΦCTP1	–*	33	274	Mayer *et al.* (2010)
Ct		–*	24	197	
Ply3626	Φ3626	Amidase	38·8	347	Zimmer *et al.* (2002)
CP25L	–*	Amidase	45	–*	Gervasi *et al.* (2014)

*No data available.

and host range with the maintenance of some degree of target specificity (Mayer *et al.* 2011). The C-terminal domain was unable to produce specific lysis (Mayer *et al.* 2011). The combination of the CD27L –terminal with the CS74L N-terminal domain resulted in a decrease in the activity against *Clostridium tyrobutyricum* while chimeric CS74L was still unable to target CD27L sensitive bacteria (Mayer *et al.* 2012).

The Psm-his-derived endolysin isolated from *C. perfringens* showed specific activity and was unable to lyse *C. difficile* or *Clostridium histolyticum* (Nariya *et al.* 2011). A great difference was observed between the various toxin types of *C. perfringens* with toxin type E being the most resistant when assessed using mid log phase cultures (22 400 U ml^{-1}) compared to toxin type C (8000 U ml^{-1}).

Application of *Bacillus* and *Clostridium*-derived phages and endolysins

There is a pressing need to differentiate between the members of *B. cereus* group, particularly for the identification of *B. anthracis*. As stated previously, the high degree of genetic similarity between the primary members of this group makes differentiation difficult and has been demonstrated for the identification of *B. anthracis*, where 35 PCR-based assays were evaluated and only four shown to be 100% specific. Of these four assays, three targeted a prophage associated region within the chromosome (Ågren *et al.* 2013).

The use of γ phage for the confirmation and diagnosis of *B. anthracis* is well established due to the high specificity for *B. anthracis,* while most *B. cereus* and *B. thuringiensis* strains are resistant to γ phage infection (Abshire *et al.* 2005). The use of bioluminescence integrated into both γ and Wβ phages has been developed for clinical diagnostics (Schofield *et al.* 2013) to reduce the time taken to detect *B. anthracis*. Schofield *et al.* (2013) demonstrated that a bioluminescent reporter phage can detect *B. anthracis* within 5 h in clinically relevant samples and also provide information on the antibiotic susceptibility profile of that organism, fulfilling a need for rapid diagnostic and treatment assessment in the event of an emergency.

Phage-based diagnostics are not only limited to using replicating phage particles. The use of phage endolysin-derived cell binding domains (CBD) combined with paramagnetic beads have been shown to be able to detect *L. monocytogenes* that was both in suspension and immobilized on contaminated foodstuffs (Kretzer *et al.* 2007). The same study also demonstrated that the procedure could be expanded to include other common contaminants of foodstuffs (*B. cereus* and *Cl. perfringens)* using CBDs that were derived from specific endolysins.

Although limited information exists on the efficacy of phages and their derived endolysins in *in vivo* testing these studies are concerned with nonspore forming organisms such as *Staphylococcus aureus* (Yang *et al.* 2014; Schmelcher *et al.* 2015). Endolysins have been shown to successfully treat infected mice that were challenged with an intraperitoneal dose of *B. cereus* RSVF1 (Schuch *et al.* 2002). This study showed that the administration of 50 U of PlyG within 15 min of infection resulted in a 68·4% full recovery rate and the remainder of the mice (6 of 19) survived up to 21 h. Increased PlyG admission resulted in a higher recovery rate (76·9%), while control animals (PBS treated) died within 24 h (Schuch *et al.* 2002). Another study showed a 40% complete survival of mice infected with an intraperitoneal injection of *B. cereus* RSVF1 that were subsequently treated with a second endolysin PlyPH (Yoong *et al.* 2006). Use of an experimental colon model of *Cl. difficile* infection has shown that although numbers of vegetative cells can be reduced through the application of a single bacteriophage (ΦCD27) compared to an untreated control (in 66% of cases), the number containing the ΦCD27 prophage (t = 35 days) was approx. 90% by the conclusion of the experiment (Meader *et al.* 2013). This study also suggested that infection and integration by ΦCD27 precludes the production of *Cl. difficile* associated toxins. A

Table 5 Summary of nonbroth or agar-based studies using *Clostridium* and *Bacillus* bacteriophages or lysins

Species	Endolysin\Phage	*In vitro**vivo*	Results	References
Bacillus	PlyG	*In vivo*- mouse i.p. injection	• 68·4% of mice fully recovered (>72 h) • 100% survival (≤21 h)	Schuch *et al.* (2002)
	PlyPH		• 40% survival of mice (>35 h)	Yoong *et al.* (2006)
Clostridium	ΦCD27	*In vitro* human colon model	• Reduction in *Clostridium difficile* vegetative and spore numbers • Reduction in toxin production	Meader *et al.* (2013)
	CD140	*In vivo* hamster	All hamsters succumbed to *Cl. difficile* infection within 3 days of the second challenge	Ramesh *et al.* (1999)
	INT-401 cocktail	*In vivo* chicken	• Chickens challenged with *Clostridium perfringens* • Challenged control animals showed 64% mortality due to necrotic enteritis • Mortality decreased to 0% when phage administered in water and <1% when administered in feed	Miller *et al.* (2010)

summary of *Bacillus* and *Clostridium* phages and endolysins that have been evaluated under nonlaboratory conditions can be found in Table 5.

The application of a multivalent phage cocktail (INT-401) to broiler chickens, showed that mortality due to *Cl. perfringens*-induced necrotic enteritis was reduced by 92% when the cocktail was administered orally (Miller *et al.* 2010). A second study performed by the same group also demonstrated that the cocktail could be successfully administered via their drinking water or feed. The expression of a *Cl. perfringens* endolysin (CP25L) within a probiotic *Lactobacillus lactis* strain presents an interesting alternative to the use of a replicating phage cocktail (Seal 2013; Gervasi *et al.* 2014) and has demonstrated activity against multiple *Cl. perfringens* strains *in vitro* and a limited level of cross species activity against a strain of *B. cereus* and *B.* subtilis (Gervasi *et al.* 2014).

Challenges facing the implementation of bacteriophage or derived endolysins for use against bacterial spore formers

The ability of both *Bacillus* and *Clostridium* members to form endospores represents a challenge to both chemical based decontamination efforts and antibiotic therapy. To force spores to germinate, additional compounds are often added to mimic conditions that would be present within a susceptible host (e.g. L-alanine and inosine in the case of *Bacillus* or bile salts for *Cl. difficile*). It has been suggested that *B. anthracis* endolysin PlyG can bind to the spore itself, and a 1 $\log_{10}$ reduction was observed after 1 h in combination with L-alanine (Yang *et al.* 2012). The ability of *B. anthracis* and *B. thuringiensis* to germinate within a soil matrix has shown that triggering germination can result in a decrease in total number of viable cells over an extended period of time (Bishop 2014).

The presence of other bacterial structures has also been suggested to play a role in resisting bacteriophage infection. The capsule layer produced by *B. anthracis* containing both pXO1 and pXO2 plasmids in the presence of carbon dioxide has been shown to inhibit lytic infection by *B. anthracis*-specific phages (Negus *et al.* 2013). Although not demonstrated this principle should also apply to other *Clostridium* species, in particular *Cl. difficile* due to the presence of capsular polysaccharides (Reid *et al.* 2012).

Bacteriophages are well known to the horizontal transfer of genes between bacterial species and have been shown to be able to move antibiotic resistance markers in *Cl. difficile* (Goh *et al.* 2013). In general lytic phages are regarded as more appropriate for use in therapy as temperate phages have been shown to possess a lower killing potential and can spread genes which enhance bacterial virulence (Kropinski 2006; Fard *et al.* 2011; Loc-Carrillo and Abedon 2011). Therefore, the complete characterization of phage genomes prior to inclusion in therapeutics is vital.

To protect themselves from predation, bacterial populations have developed a number of different mechanisms by which they can become resistant to infection by bacteriophages, such as exclusion or the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas system (Abedon 2012; Barrangou and van der Oost 2015; Goldfarb *et al.* 2015). In brief the CRISPR/Cas system can immunize bacterial cells against foreign nucleic acids as well as providing some degree of lasting immunity. For initial immunization, foreign DNA is cleaved by a Cas complex of proteins producing a novel spacer unit, which is subsequently incorporated into a CRISPR array.

Upon repeat exposure this CRISPR array is transcribed and processed into individual CRISPR RNAs (crRNAs). These crRNAs are then used to guide a Cas complex to target and inactivate the foreign nucleic acid. A more comprehensive review of the CRISPR/Cas system has been produced by Horvath and Barrangou (2010). Although the development of resistance will play an important role in determining the success of any bacteriophage based therapy, the mechanisms have been reviewed in much greater detail elsewhere (Westra *et al.* 2012).

The development of endolysin resistance could be of some concern should they be used in a clinical setting. However, endolysins are targeted towards highly conserved features of the bacterial cell surface and the development of such resistance would require a fundamental alteration of peptidoglycan architecture (Elbreki *et al.* 2014). Although attempts have been made to develop this type of resistance *in vitro* (Briers *et al.* 2014) no lysin resistant bacteria have been isolated to date (Herpers 2015). This hurdle could also potentially be overcome through the creation of chimeric endolysins derived from different parental phages and has been demonstrated in *Staph. aureus* (Mao *et al.* 2013).

Although a recent study suggested that consumers would be willing to pay extra for fresh produce that had been treated with bacteriophage (Naanwaab *et al.* 2014), public reaction to bacteriophage-derived products presented as a therapeutic option either separately or in combination with antibiotics is unknown.

To produce successful phage based therapeutics a number of criteria must be met. These include not only the ability to infect a range of strains as well as the ability to reduce the overall level of infection (Hanlon 2007), but also compliance, both patient and regulatory. The satisfaction of regulatory requirements for topically applied phage products should be relatively simple to attain (i.e. low bacterial content, absence of *Staph. aureus*, *Ps. aeruginosa* and *Escherichia coli*). However, for nontopical applications (e.g. systemic applications), regulatory requirements would be more stringent, particularly endotoxin content. To meet such challenges, current legislation would need to be revised in which acceptable criteria for phage based therapeutics would be established (Verbeken *et al.* 2014) as well as increasing research into effective endotoxin removal without the use of extreme pH or temperature.

Unlike antibiotics, phages and their derived lysins will initiate an immune response in the patient, enhancing clearance from the body and lowering their efficacy (Elbreki *et al.* 2014). To reduce this response, preparations could be combined with additional excipients such as polyethylene glycol that could mask the phage or endolysin from the immune system (Fenton *et al.* 2010). Following the successful lysis of the target bacterium, the accumulation of proinflammatory cellular debris (such as lipopolysaccharide) could also result in an escalation of any immune response produced.

Conclusions and outlook

Bacterial spore formers represent a unique challenge to those seeking to adapt whole bacteriophages and their derived endolysins due to their ability to survive decontamination efforts and remain viable. Currently much of the available literature focuses on the primary isolation and characterization of such entities rather than the development of practical applications. Although derived from virulent bacteriophage, endolysins possess a number of advantages over their parent phages such as a wider host range (Son *et al.* 2012) and ability to interact with spores as well as vegetative cells (Yang *et al.* 2012). The ability to produce such enzymes in recombinant forms may allow for large scale, cost effective production. However, a number of secondary issues need to be addressed to produce effective, safe therapies, particularly the evaluation of testing beyond laboratory conditions.

Conflict of Interest

None declared.

References

Abedon, S.T. (2012) Bacterial 'immunity' against bacteriophages. *Bacteriophage* **2**, 50–54.

Abedon, S.T., Kuhl, S.J., Blasdel, B.G. and Kutter, E.M. (2011) Phage treatment of human infections. *Bacteriophage* **1**, 66–85.

Abshire, T.G., Brown, J.E. and Ezzell, J.W. (2005) Production and validation of the use of gamma phage for identification of *Bacillus anthracis*. *J Clin Microbiol* **43**, 4780–4788.

Ackermann, H.W. (2012) Prokaryote viruses studies by electron microscopy. *Arch Virol* **157**, 1843–1849.

Ågren, J., Hamidjaja, R.A., Hansen, T., Ruuls, R., Thierry, S., Vigre, H., Janse, J., Sundström, A. *et al.* (2013) In silico and in vitro evaluation of PCR-based assays for the detection of *Bacillus anthracis* chromosomal signature sequences. *Virulence* **4**, 671–685.

Barrangou, R. and van der Oost, J. (2015) Bacteriophage exclusion, a new defense system. *EMBO J* **34**, 134–135.

Bennett, S.D., Walsh, K.A. and Gould, L.H. (2013) Foodborne disease outbreaks caused by *Bacillus cereus*, *Clostridium perfringens*, and *Staphylococcus aureus* - United States, 1998–2008. *Clin Infect Dis* **57**, 425–433.

Bishop, A.H. (2014) Germination and persistence of *Bacillus anthracis* and *Bacillus thuringiensis* in soil microcosms. *J Appl Microbiol* **117**, 1274–1282.

Briers, Y., Walmagh, M., Grymonprez, B., Biebl, M., Pirnay, J.-P., Defraine, V., Michiels, J., Cenens, W. *et al.* (2014) Art-175 is a highly efficient antibacterial against multidrug-resistant strains and persisters of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **58**, 3774–3784.

Callewaert, L., Walmagh, M., Michiels, C.W. and Lavigne, R. (2011) Food applications of bacterial cell wall hydrolases. *Curr Opin Biotechnol* **22**, 164–171.

Ceuppens, S., Boon, N. and Uyttendaele, M. (2013) Diversity of *Bacillus cereus* group strains is reflected in their broad range of pathogenicity and diverse ecological lifestyles. *FEMS Microbiol Ecol* **84**, 433–450.

Chanishvili, N., Chanishvili, T., Tediashvili, M. and Barrow, P.A. (2001) Phages and their application against drug-resistant bacteria. *J Chem Technol Biotechnol* **76**, 689–699.

Doganay, M. and Metan, G. (2009) Human anthrax in Turkey from 1990 to 2007. *Vector Borne Zoonotic Dis* **9**, 131–140.

Dubberke, E.R. and Olsen, M.A. (2012) Burden of *Clostridium difficile* on the healthcare system. *Clin Infect Dis* **55**, S88–S92.

El-Arabi, T.F., Griffiths, M.W., She, Y.M., Villegas, A., Lingohr, E.J. and Kropinski, A.M. (2013) Genome sequence and analysis of a broad-host range lytic bacteriophage that infects the *Bacillus cereus* group. *Virol J* **10**, 48.

Elbreki, M., Ross, R.P., Hill, C., O'Mahony, J., McAuliffe, O. and Coffey, A. (2014) Bacteriophages and their derivatives as biotherapeutic agents in disease prevention and treatment. *J Viruses* **2014**, 20.

Fard, R.M.N., Barton, M.D. and Heuzenroeder, M.W. (2011) Bacteriophage-mediated transduction of antibiotic resistance in enterococci. *Lett Appl Microbiol* **52**, 559–564.

Fenton, M., McAuliffe, O., O'Mahony, J. and Coffey, A. (2010) Recombinant bacteriophage lysins as antibacterials. *Bioeng bugs* **1**, 9–16.

Fischetti, V.A. (2010) Bacteriophage endolysins: a novel anti-infective to control gram-positive pathogens. *Int J Med Microbiol* **300**, 357–362.

Fischetti, V.A., Schuch, R. and Nelson, D. (2013) Phage-associated lytic enzymes for treatment of *Bacillus anthracis* and related conditions. US Patent **8**, 580, 553.

Fouts, D.E., Rasko, D.A., Cer, R.Z., Jiang, L., Fedorova, N.B., Shvartsbeyn, A., Vamathevan, J.J., Tallon, L. *et al.* (2006) Sequencing *Bacillus anthracis* typing phages gamma and cherry reveals a common ancestry. *J Bacteriol* **188**, 3402–3408.

Ganz, H.H., Law, C., Schmuki, M., Eichenseher, F., Calendar, R., Loessner, M.J., Getz, W.M., Korlach, J. *et al.* (2014) Novel giant siphovirus from *Bacillus anthracis* features unusual genome characteristics. *PLoS ONE* **9**, e85972.

Gervasi, T., Horn, N., Wegmann, U., Dugo, G., Narbad, A. and Mayer, M. (2014) Expression and delivery of an endolysin to combat *Clostridium perfringens*. *Appl Microbiol Biotechnol* **98**, 2495–2505.

Gillis, A. and Mahillon, J. (2014) Prevalence, genetic diversity, and host range of tectiviruses among members of the *Bacillus cereus* group. *Appl Environ Microbiol* **80**, 4138–4152.

Goh, S., Riley, T.V. and Chang, B.J. (2005) Isolation and characterization of temperate bacteriophages of *Clostridium difficile*. *Appl Environ Microbiol* **71**, 1079–1083.

Goh, S., Ong, P.F., Song, K.P., Riley, T.V. and Chang, B.J. (2007) The complete genome sequence of *Clostridium difficile* phage ΦC2 and comparisons to ΦCD119 and inducible prophages of CD630. *Microbiology* **153**, 676–685.

Goh, S., Hussain, H., Chang, B.J., Emmett, W., Riley, T.V. and Mullany, P. (2013) Phage ΦC2 mediates transduction of Tn6215, encoding erythromycin resistance, between *Clostridium difficile* strains. *MBio* **4**, e00840-13.

Golais, F., Hollý, J. and Vítkovská, J. (2013) Coevolution of bacteria and their viruses. *Folia Microbiol* **58**, 177–186.

Goldfarb, T., Sberro, H., Weinstock, E., Cohen, O., Doron, S., Charpak-Amikam, Y., Afik, S., Ofir, G. *et al.* (2015) BREX is a novel phage resistance system widespread in microbial genomes. *EMBO J* **34**, 169–183.

Granum, P.E. and Lund, T. (1997) *Bacillus cereus* and its food poisoning toxins. *FEMS Microbiol Lett* **157**, 223–228.

Hanlon, G.W. (2007) Bacteriophages: an appraisal of their role in the treatment of bacterial infections. *Int J Antimicrob Agents* **30**, 118–128.

Hargreaves, K.R. and Clokie, M.R. (2014) *Clostridium difficile* phages: still difficult? *Front Microbiol* **5**, 184.

Herpers, B. (2015) Endolysins: redefining antibacterial therapy. *Future Microbiol* **1**, 309–311.

Heselpoth, R.D., Owens, J.M. and Nelson, D.C. (2015) Quantitative analysis of the thermal stability of the gamma phage endolysin PlyG: a biophysical and kinetic approach to assaying therapeutic potential. *Virology* **477**, 125–132.

Hoffmaster, A.R., Ravel, J., Rasko, D.A., Chapman, G.D., Chute, M.D., Marston, C.K., De, B.K., Sacchi, C.T. *et al.* (2004) Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. *Proc Natl Acad Sci USA* **101**, 8449–8454.

Horvath, P. and Barrangou, R. (2010) CRISPR/Cas, the immune system of bacteria and archaea. *Science* **327**, 167–170.

Kikkawa, H., Fujinami, Y., Suzuki, S. and Yasuda, J. (2007) Identification of the amino acid residues critical for specific binding of the bacteriolytic enzyme of gamma-phage, PlyG, to *Bacillus anthracis*. *Biochem Biophys Res Commun* **363**, 531–535.

Kikkawa, H.S., Ueda, T., Suzuki, S.-I. and Yasuda, J. (2008) Characterization of the catalytic activity of the γ-phage lysin, PlyG, specific for *Bacillus anthracis*. *FEMS Microbiol Lett* **286**, 236–240.

Kong, M., Kim, M. and Ryu, S. (2012) Complete genome sequence of *Bacillus cereus* bacteriophage PBC1. *J Virol* **86**, 6379–6380.

Kretzer, J.W., Lehmann, R., Schmelcher, M., Banz, M., Kim, K.P., Korn, C. and Loessner, M.J. (2007) Use of high-affinity cell wall-binding domains of bacteriophage

endolysins for immobilization and separation of bacterial cells. *Appl Environ Microbiol* **73**, 1992–2000.

Kropinski, A.M. (2006) Phage therapy – everything old is new again. *Can J Infect Dis Med Microbiol* **17**, 297–306.

Lavigne, R., Briers, Y., Hertveldt, K., Robben, J. and Volckaert, G. (2004) Identification and characterization of a highly thermostable bacteriophage lysozyme. *Cell Mol Life Sci* **61**, 2753–2759.

Lee, J.-H., Shin, H., Son, B. and Ryu, S. (2012) Complete genome sequence of *Bacillus cereus* bacteriophage BCP78. *J Virol* **86**, 637–638.

Lee, J.-H., Shin, H., Son, B., Heu, S. and Ryu, S. (2013) Characterization and complete genome sequence of a virulent bacteriophage B4 infecting food-borne pathogenic *Bacillus cereus*. *Arch Virol* **158**, 2101–2108.

Leggett, M.J., Mcdonnell, G., Denyer, S.P., Setlow, P. and Maillard, J.Y. (2012) Bacterial spore structures and their protective role in biocide resistance. *J Appl Microbiol* **113**, 485–498.

Leggett, M.J., Schwarz, J.S., Burke, P.A., Mcdonnell, G., Denyer, S.P. and Maillard, J.-Y. (2015) Resistance to and killing by the sporicidal microbicide peracetic acid. *J Antimicrob Chemother* **70**, 773–779.

Loc-Carrillo, C. and Abedon, S.T. (2011) Pros and cons of phage therapy. *Bacteriophage* **1**, 111–114.

Loessner, M.J., Maier, S.K., Daubek-Puza, H., Wendlinger, G. and Scherer, S. (1997) Three *Bacillus cereus* bacteriophage endolysins are unrelated but reveal high homology to cell wall hydrolases from different bacilli. *J Bacteriol* **179**, 2845–2851.

Loessner, M.J., Kramer, K., Ebel, F. and Scherer, S. (2002) C-terminal domains of *Listeria monocytogenes* bacteriophage murein hydrolases determine specific recognition and high-affinity binding to bacterial cell wall carbohydrates. *Mol Microbiol* **44**, 335–349.

Low, L.Y., Yang, C., Perego, M., Osterman, A. and Liddington, R.C. (2005) Structure and lytic activity of a *Bacillus anthracis* prophage endolysin. *J Biol Chem* **280**, 35433–35439.

Lund, B.M. and Peck, M.W. (2013) *Clostridium botulinum*. Guide to Foodborne Pathogens, 2nd edn. pp. 91–111. Oxford: John Wiley & Sons.

Mao, J., Schmelcher, M., Harty, W.J., Foster-Frey, J. and Donovan, D.M. (2013) Chimeric Ply187 endolysin kills *Staphylococcus aureus* more effectively than the parental enzyme. *FEMS Microbiol Lett* **342**, 30–36.

Mayer, M.J., Narbad, A. and Gasson, M.J. (2008) Molecular characterization of a *Clostridium difficile* bacteriophage and its cloned biologically active endolysin. *J Bacteriol* **190**, 6734–6740.

Mayer, M.J., Payne, J., Gasson, M.J. and Narbad, A. (2010) Genomic sequence and characterization of the virulent bacteriophage φCTP1 from *Clostridium tyrobutyricum* and heterologous expression of its endolysin. *Appl Environ Microbiol* **76**, 5415–5422.

Mayer, M.J., Garefalaki, V., Spoerl, R., Narbad, A. and Meijers, R. (2011) Structure-based modification of a *Clostridium difficile*-targeting endolysin affects activity and host range. *J Bacteriol* **193**, 5477–5486.

Mayer, M.J., Gasson, M.J. and Narbad, A. (2012) Genomic sequence of bacteriophage ATCC 8074-B1 and activity of its endolysin and engineered variants against *Clostridium sporogenes*. *Appl Environ Microbiol* **78**, 3685–3692.

Meader, E., Mayer, M.J., Steverding, D., Carding, S.R. and Narbad, A. (2013) Evaluation of bacteriophage therapy to control *Clostridium difficile* and toxin production in an in vitro human colon model system. *Anaerobe* **22**, 25–30.

Meessen-Pinard, M., Sekulovic, O. and Fortier, L.-C. (2012) Evidence of in vivo prophage induction during *Clostridium difficile* infection. *Appl Environ Microbiol* **78**, 7662–7670.

Mehta, K.K., Paskaleva, E.E., Azizi-Ghannad, S., Ley, D.J., Page, M.A., Dordick, J.S. and Kane, R.S. (2013) Characterization of AmiBA2446, a novel bacteriolytic enzyme active against *Bacillus* species. *Appl Environ Microbiol* **79**, 5899–5906.

Miller, R.W., Skinner, E.J., Sulakvelidze, A., Mathis, G.F. and Hofacre, C.L. (2010) Bacteriophage therapy for control of necrotic enteritis of broiler chickens experimentally infected with *Clostridium perfringens*. *Avian Dis* **54**, 33–40.

Minakhin, L., Semenova, E., Liu, J., Vasilov, A., Severinova, E., Gabisonia, T., Inman, R., Mushegian, A. *et al.* (2005) Genome sequence and gene expression of *Bacillus anthracis* bacteriophage Fah. *J Mol Biol* **354**, 1–15.

Moeller, R., Raguse, M., Reitz, G., Okayasu, R., Li, Z., Klein, S., Setlow, P. and Nicholson, W.L. (2014) Resistance of *Bacillus subtilis* spore DNA to lethal ionizing radiation damage relies primarily on spore core components and DNA repair, with minor effects of oxygen radical detoxification. *Appl Environ Microbiol* **80**, 104–109.

Morales, C., Oakley, B., Garrish, J., Siragusa, G., Ard, M. and Seal, B. (2012) Complete genome sequence of the podoviral bacteriophage ΦCP24R, which is virulent for *Clostridium perfringens*. *Arch Virol* **157**, 769–772.

Naanwaab, C., Yeboah, O.-A., Kyei, F., Sulakvelidze, A. and Goktepe, I. (2014) Evaluation of consumers' perception and willingness to pay for bacteriophage treated fresh produce. *Bacteriophage* **4**, e979662.

Nale, J.Y., Shan, J., Hickenbotham, P.T., Fawley, W.N., Wilcox, M.H. and Clokie, M.R. (2012) Diverse temperate bacteriophage carriage in *Clostridium difficile* 027 strains. *PLoS ONE* **7**, e37263.

Nariya, H., Miyata, S., Tamai, E., Sekiya, H., Maki, J. and Okabe, A. (2011) Identification and characterization of a putative endolysin encoded by episomal phage phiSM101 of *Clostridium perfringens*. *Appl Microbiol Biotechnol* **90**, 1973–1979.

Negus, D., Burton, J., Sweed, A., Gryko, R. and Taylor, P.W. (2013) Poly-γ-d-glutamic acid capsule interferes with lytic infection of *Bacillus anthracis* by *B. anthracis*-specific bacteriophages. *Appl Environ Microbiol* **79**, 714–717.

Oakley, B.B., Talundzic, E., Morales, C.A., Hiett, K.L., Siragusa, G.R., Volozhantsev, N.V. and Seal, B.S. (2011)

Comparative genomics of four closely related *Clostridium perfringens* bacteriophages reveals variable evolution among core genes with therapeutic potential. *BMC Genom* **12**, 282.

Oey, M., Lohse, M., Scharff, L.B., Kreikemeyer, B. and Bock, R. (2009) Plastid production of protein antibiotics against pneumonia via a new strategy for high-level expression of antimicrobial proteins. *Proc Natl Acad Sci USA* **106**, 6579–6584.

Park, J., Yun, J., Lim, J.-A., Kang, D.-H. and Ryu, S. (2012) Characterization of an endolysin, LysBPS13, from a *Bacillus cereus* bacteriophage. *FEMS Microbiol Lett* **332**, 76–83.

Porter, C.J., Schuch, R., Pelzek, A.J., Buckle, A.M., Mcgowan, S., Wilce, M.C.J., Rossjohn, J., Russell, R. *et al.* (2007) The 1.6 Å crystal structure of the catalytic domain of PlyB, a bacteriophage lysin active against *Bacillus anthracis*. *J Mol Biol* **366**, 540–550.

Priest, F.G., Barker, M., Baillie, L.W.J., Holmes, E.C. and Maiden, M.C.J. (2004) Population structure and evolution of the *Bacillus cereus* group. *J Bacteriol* **186**, 7959–7970.

Ramesh, V., Fralick, J.A. and Rolfe, R.D. (1999) Prevention of *Clostridium difficile*-induced ileocecitis with bacteriophage. *Anaerobe* **5**, 69–78.

Rasko, D.A., Altherr, M.R., Han, C.S. and Ravel, J. (2005) Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol Rev* **29**, 303–329.

Reid, C.W., Vinogradov, E., Li, J., Jarrell, H.C., Logan, S.M. and Brisson, J.R. (2012) Structural characterization of surface glycans from *Clostridium difficile*. *Carbohydr Res* **354**, 65–73.

Roberts, S.B., Gowen, C.M., Brooks, J.P. and Fong, S.S. (2010) Genome-scale metabolic analysis of *Clostridium thermocellum* for bioethanol production. *BMC Syst Biol* **4**, 31.

Schmelcher, M., Donovan, D.M. and Loessner, M.J. (2012) Bacteriophage endolysins as novel antimicrobials. *Future Microbiol* **7**, 1147–1171.

Schmelcher, M., Shen, Y., Nelson, D.C., Eugster, M.R., Eichenseher, F., Hanke, D.C., Loessner, M.J., Dong, S. *et al.* (2015) Evolutionarily distinct bacteriophage endolysins featuring conserved peptidoglycan cleavage sites protect mice from MRSA infection. *J Antimicrob Chemother* **70**, 1453–1465.

Schofield, D.A., Sharp, N.J., Vandamm, J., Molineux, I.J., Spreng, K.A., Rajanna, C., Westwater, C. and Stewart, G.C. (2013) *Bacillus anthracis* diagnostic detection and rapid antibiotic susceptibility determination using 'bioluminescent' reporter phage. *J Microbiol Methods* **95**, 156–161.

Schuch, R., Nelson, D. and Fischetti, V.A. (2002) A bacteriolytic agent that detects and kills *Bacillus anthracis*. *Nature* **418**, 884–889.

Seal, B.S. (2013) Characterization of bacteriophages virulent for *Clostridium perfringens* and identification of phage lytic enzymes as alternatives to antibiotics for potential control of the bacterium1. *Poult Sci* **92**, 526–533.

Seelert, T., Ghosh, D. and Yargeau, V. (2015) Improving biohydrogen production using *Clostridium beijerinckii* immobilized with magnetite nanoparticles. *Appl Microbiol Biotechnol* **99**, 4107–4116.

Sekulovic, O., Garneau, J.R., Néron, A. and Fortier, L.-C. (2014) Characterization of temperate phages infecting *Clostridium difficile* isolates of human and animal origins. *Appl Environ Microbiol* **80**, 2555–2563.

Setlow, B., Parish, S., Zhang, P., Li, Y.Q., Neely, W.C. and Setlow, P. (2014) Mechanism of killing of spores of *Bacillus anthracis* in a high-temperature gas environment, and analysis of DNA damage generated by various decontamination treatments of spores of *Bacillus anthracis*, *Bacillus subtilis* and *Bacillus thuringiensis*. *J Appl Microbiol* **116**, 805–814.

Shin, H., Lee, J.-H., Park, J., Heu, S. and Ryu, S. (2014) Characterization and genome analysis of the *Bacillus cereus*-infecting bacteriophages BPS10C and BPS13. *Arch Virol* **159**, 2171–2175.

Son, B., Yun, J., Lim, J.A., Shin, H., Heu, S. and Ryu, S. (2012) Characterization of LysB4, an endolysin from the *Bacillus cereus*-infecting bacteriophage B4. *BMC Microbiol* **12**, 33.

Sozhamannan, S., Mckinstry, M., Lentz, S.M., Jalasvuori, M., Mcafee, F., Smith, A., Dabbs, J., Ackermann, H.-W. *et al.* (2008) Molecular characterization of a variant of *Bacillus anthracis*-specific phage AP50 with improved bacteriolytic activity. *Appl Environ Microbiol* **74**, 6792–6796.

Stern, A. and Sorek, R. (2011) The phage-host arms race: shaping the evolution of microbes. *BioEssays* **33**, 43–51.

Sulakvelidze, A., Alavidze, Z. and Morris, J.G. (2001) Bacteriophage therapy. *Antimicrob Agents Chemother* **45**, 649–659.

Verbeken, G., Pirnay, J.-P., Lavigne, R., Jennes, S., De Vos, D., Casteels, M. and Huys, I. (2014) Call for a dedicated european legal framework for bacteriophage therapy. *Arch Immun Ter Exp* **62**, 117–129.

Volozhantsev, N.V., Verevkin, V.V., Bannov, V.A., Krasilnikova, V.M., Myakinina, V.P., Zhilenkov, E.L., Svetoch, E.A., Stern, N.J. *et al.* (2011) The genome sequence and proteome of bacteriophage PhiCPV1 virulent for *Clostridium perfringens*. *Virus Res* **155**, 433–439.

Volozhantsev, N.V., Oakley, B.B., Morales, C.A., Verevkin, V.V., Bannov, V.A., Krasilnikova, V.M., Popova, A.V., Zhilenkov, E.L. *et al.* (2012) Molecular characterization of podoviral bacteriophages virulent for *Clostridium perfringens* and their comparison with members of the *Picovirinae*. *PLoS ONE* **7**, e38283.

Walmagh, M., Boczkowska, B., Grymonprez, B., Briers, Y., Drulis-Kawa, Z. and Lavigne, R. (2013) Characterization of five novel endolysins from gram-negative infecting bacteriophages. *Appl Microbiol Biotechnol* **97**, 4369–4375.

Westra, E.R., Swarts, D.C., Staals, R.H., Jore, M.M., Brouns, S.J. and van der Oost, J. (2012) The CRISPRs, they are a-changin': how prokaryotes generate adaptive immunity. *Annu Rev Gene* **46**, 311–339.

Yang, H., Wang, D.-B., Dong, Q., Zhang, Z., Cui, Z., Deng, J., Yu, J., Zhang, X.-E. *et al.* (2012) Existence of separate domains in lysin PlyG for recognizing *Bacillus anthracis* spores and vegetative cells. *Antimicrob Agents Chemother* **56**, 5031–5039.

Yang, H., Zhang, Y., Yu, J., Huang, Y., Zhang, X.E. and Wei, H. (2014) Novel chimeric lysin with high-level antimicrobial activity against methicillin-resistant *Staphylococcus aureus* in vitro and in vivo. *Antimicrob Agents Chemother* **58**, 536–542.

Yoong, P., Schuch, R., Nelson, D. and Fischetti, V.A. (2006) PlyPH, a bacteriolytic enzyme with a broad pH range of activity and lytic action against *Bacillus anthracis*. *J Bacteriol* **188**, 2711–2714.

Yuan, Y., Peng, Q. and Gao, M. (2012a) Characteristics of a broad lytic spectrum endolysin from phage BtCS33 of *Bacillus thuringiensis*. *BMC Microbiol* **12**, 297.

Yuan, Y.H., Gao, M.Y., Wu, D.D., Liu, P.M. and Wu, Y. (2012b) Genome characteristics of a novel phage from *Bacillus thuringiensis* showing high similarity with phage from *Bacillus cereus*. *PLoS ONE* **7**, e37557.

Żaczek, M., Weber-Dąbrowska, B. and Górski, A. (2015) Phages in the global fruit and vegetable industry. *J Appl Microbiol* **118**, 537–556.

Zimmer, M., Scherer, S. and Loessner, M.J. (2002) Genomic analysis of *Clostridium perfringens* bacteriophage φ3626, which integrates into guaA and possibly affects sporulation. *J Bacteriol* **184**, 4359–4368.