Isolation of Three Coliphages and the Evaluation of Their Phage Cocktail for Biocontrol of Shiga Toxin-Producing *Escherichia coli* O157 in Milk

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) O157 is a well-knownfoodborne pathogen and a leading cause of many intestinal diseases. In this study, we explore the use of a phage cocktail to help control STEC O157 in broth and milk. We isolated three virulent phages from sanitary sewages using a STEC O157 as the indicator bacterium. Phenotypical characterizations revealed that these three phages belong to the *Myoviridae* family and were stable at different temperatures and pH. They displayed a short latent period between 10 and 20 min, and a burst size (32–65 per infected cell). No virulence factors and drug resistance genes were found in their genomes. Bacterial lysis assays showed that a phage cocktail comprising these three phages was more effective (at least 4.32 log reduction) against STEC O157 at 25 °C with multiplicity of infection (MOI) = 1000 in broth medium. At 4 °C, a 3.8 log reduction in the number of viable STEC O157 after 168-h treatment with phage cocktail at MOI = 1000 was observed in milk, compared to phage-free bacterial control group. Characterizations of phages suggest they could be developed into novel therapeutic agents to control STEC O157 in milk production.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is a worldwide leading foodborne and zoonotic pathogen responsible for watery or bloody diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome [1]. Although more than 100 serogroups have been assigned to STEC strains based on their O antigens, several serogroups including O157, O26, O104, O45, O103, O111, O121, and O145 are frequently associated with severe illness in humans worldwide and the outbreaks of these serogroups have resulted in huge mobility, mortality, and economical losses [2]. For example, The Germany's 2011 STEC O104 caused US\$ 1.3 billion in losses for farmers and industries in Germany and US\$ 236 million in emergency aid payments to 22 European Union Member States [3]. In the US, 390 STEC O157 outbreaks during 2003–2012 resulted in 4928 illnesses, 1272 hospitalizations, 299 physician-diagnosed HUS cases, and 33 deaths [4]. Recently, STEC O157:H7 (rank the 6th) and STEC non-O157 (rank the 13th) have been ranked as the leading cause of foodborne disease as measured by the combined cost of illness and quality-adjusted life-year [5]. The spread and outbreak of STEC is frequently associated with food contamination. Human taking in poorly cooked food, such as uncooked beef and unpasteurized milk, rather than human-to-human routine, is proposed to be responsible for most cases of STEC infections [6]. In this regard, taking active actions to completely clean up the possible residual bacteria in food is of course beneficial for food safety.

Bacteriophages (also known as phages) were first described by Felix d'Herelle at the early 1900s [7]. Due to their capacity of killing pathogenic bacteria with high specificity, phage therapy was popular in the 1920s and 1930s to treat multiple types of infections and conditions.

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However, the voice of doubt about ambiguous efficacy of phage therapy and the success of emerging antibacterial led to a decline in interest in phage therapy in the late 1930s [7]. In recent year, the emergence of antibiotic-resistant pathogens particularly the multidrug-resistant bacteria due to antibiotic overuse, horizontal gene transfer, and/or bacterial evolution requires new classes of anti-microbials to treat these pathogens but the drug development pipeline is dry. Under this background, there has been a renewed interest in bacteriophage therapy [8]. Several animal studies have highlighted the effectiveness of phages on killing multidrug-resistant bacteria, including extended spectrum beta-lactamase (ESBL) *E. coli* [9], *Klebsiella pneumoniae* [10], *Acinetobacter baumannii* [11], and *Pseudomonas aeruginosa* [12]. In general, phages are usually specific for bacterial species without infecting mammalian cells; thus, they can be safely used in foods or animals [13]. Indeed, there are many studies on the assessment of lytic phages and their potential and efficacy for reduction of *E. coli* O157 in food, and most of these studies suggest a good potential of lytic phages and/or their cocktails on reducing the contamination of *E. coli* O157 in food. For example, a previous study found treatment of a phage cocktail containing three different concentrations of phages (10^{10}, 10^9, and 10^8 PFU/ml) lytic for *E. coli* O157:H7 resulted in statistically significant reductions ($P = < 0.05$) in the number of *E. coli* O157:H7 organisms on the surface of different types of food (tomato, spinach, broccoli, and ground beef) [14]. A more recent study also found immersion of lettuce in 9.8 log PFU/ml of a cocktail of three lytic phages specific for *E. coli* O157:H7 for 2 min significantly ($P < 0.05$) reduced *E. coli* O157:H7 populations after 24 h when stored at 4 °C compared with controls [15]. A notable information is that the US Food and Drug Administration (FDA) and Department of Agriculture (USDA) have approved the use of phages for decreasing *Listeria monocytogenes*, *E. coli* O157:H7 or *E. coli* contamination, and *Salmonella* in the field of livestock and food safety [16].

While phages harbor capacity of killing bacteria with high efficacy, their host specificity might limit their effectiveness on cleaning bacteria completely. Therefore, phage cocktails composed of many different phages were adopted for controlling pathogens [17–19]. In the present study, we isolated three virulent phages from pig farm-sewages in Hubei Province in China by using STEC O157 as an indicator. By performing a series of laboratory tests, the biological and genomic characteristics of these three phages were determined. We also evaluated the potential ability of a cocktail composed of these three phages on controlling *E. coli* O157 in broth and milk at different multiplicity of infection (MOI) values (1000, 100, 10, and 1) at refrigeration temperature (4 °C) and room temperature (25 °C).

Results

Isolation and Characterization of Three Virulent STEC Phages

By using one of our laboratory collected STEC O157 strain HB11 as an indicator [20], three virulent phages designated vB_EcoM_PHB11 (thereafter referred to as PHB11), vB_EcoM_PHB12 (thereafter referred to as PHB12), and vB_EcoM_PHB13 (thereafter referred to as PHB13) were isolated from sewages collected from different pig farms in Hubei Province in China through a double-layer agar methodology [21]. Morphological characterization by using transmission electron microscope (TEM) showed that PHB11, PHB12, and PHB13 had isometric polyhedral heads with long contractile tails ranging from 103 to 107 nm (Fig. 1A–C; Table 1).

To test the thermolability and the pH sensitivity of the three phages isolated, each of the phages were treated for 1 h at different temperatures (4 °C, 20 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C) and/or were treated for 1 h at 37 °C in conditions with different pH values (3, 5, 7, 9, 11), and the titers of the phages under different treatments were determined. Thermolability tests revealed that the three phages displayed a good stability from 4 to 60 °C (Fig. 1D–F). pH sensitivity tests showed that the three phages were stable from pH 3.0 to 9.0, but the titer dropped approximately 8 log units at pH 11.0 (Fig. 1G–I). Infection of the indicator bacteria by using each of the three phages at different MOIs (0.001, 0.01, 0.1, 1, 10, 100) revealed that the best MOI value of the three phages was 0.1; the titers of PHB11, PHB12, and PHB13 were 8×10^8 PFU/ml, 8.20×10^8 PFU/ml, and 8.40×10^8 PFU/ml, respectively. One-step growth curve determination test revealed that the entire life cycles of PHB11, PHB12, and PHB13 consisted of an approximately 80-min infection process and an approximately 20-min, 10-min, and 10-min eclipse period, respectively; their average burst sizes were 68 PFU, 72 PFU, and 70 PFU per infected cell, respectively (Fig. 1J–L). Host range determination by using the spot tests showed that PHB11, PHB12, and PHB13 could kill five, one, and six of the STEC O157 tested, respectively (Table 2). Interestingly, PHB12 and PHB13 could also kill the *Salmonella enterica* serovar Dublin and the *S. enterica* serovar Typhimurium tested, respectively (Table 2).

Genomic and Phylogenetic Analysis

To determine the genomic characteristics of the three phages isolated, the genomic DNA of the phages was extracted by using the phenol–chloroform method [21] and
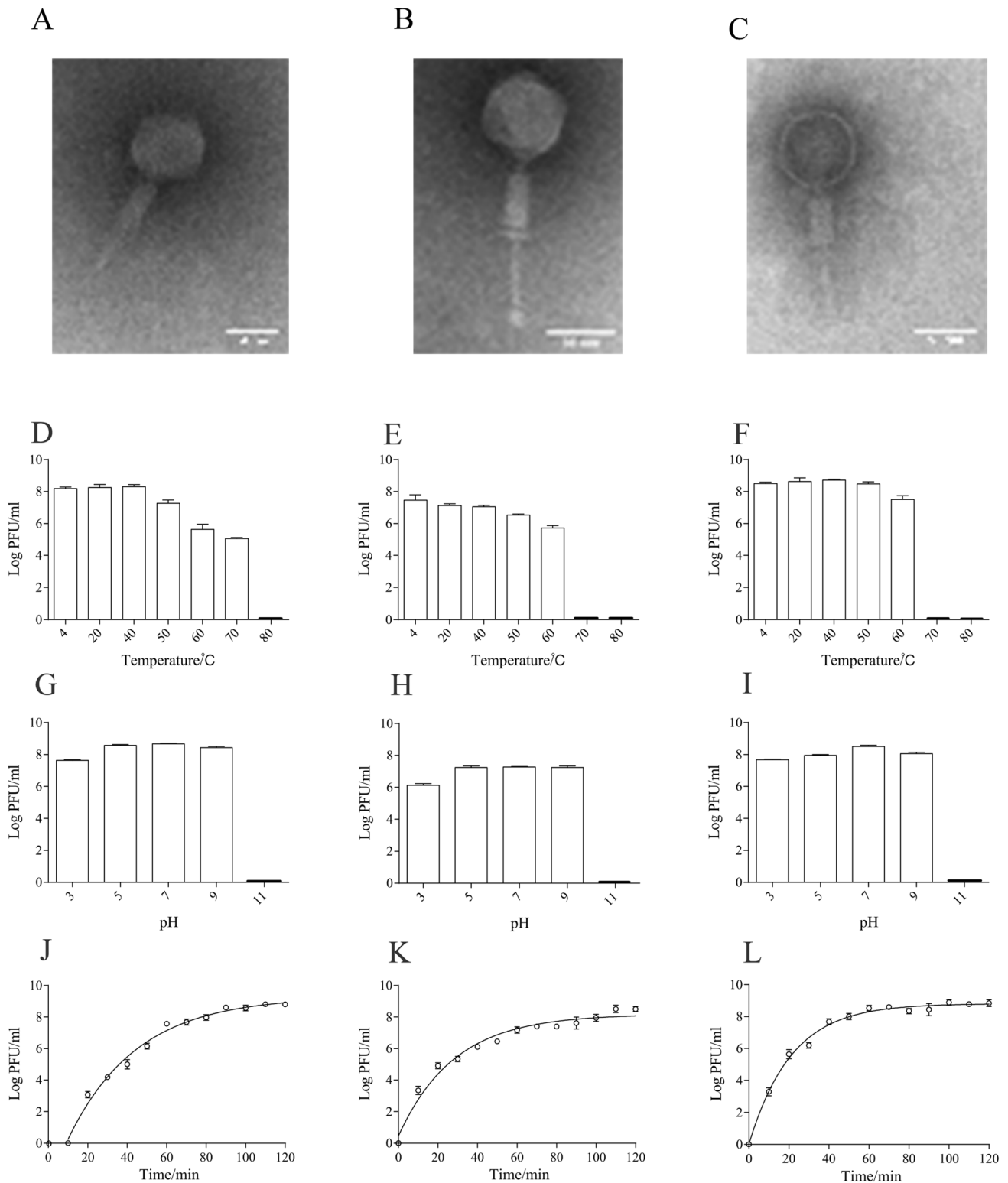
Fig. 1 Phenotypical characteristics of the three coliphages. Panels **A–C** show morphological characteristics of PHB11 (panel **A**), PHB12 (panel **B**), and PHB13 (panel **C**) under transmission electron micrograph (white bars: 50 nm; magnification × 150,000), panels **D–F** show the stability of PHB11 (panel **D**), PHB12 (panel **E**), and PHB13 (panel **F**) at different pH values, panels **G–I** show the stability of PHB11 (panel **G**), PHB12 (panel **H**), and PHB13 (panel **I**) under different temperature conditions, and panels **J–L** show one-step growth curves of PHB11 (panel **J**), PHB12 (panel **K**), and PHB13 (panel **L**); phages were added at MOI = 0.01. Data are presented as mean ± SD from three independent experiments

Table 1 Morphological characteristics of the coliphage PHB11, PHB12, and PHB13

	PHB11	PHB12	PHB13
Head length[a] (nm)	63.5 ± 1.3	93.1 ± 0.3	64.4 ± 2.1
Head width[a] (nm)	63.5 ± 1.3	77.1 ± 0.2	64.4 ± 2.1
Tail length[a] (nm)	107.1 ± 0.9	103.4 ± 0.3	107.6 ± 0.8
Tail width[a] (nm)	14.0 ± 0.2	12.6 ± 0.3	18.3 ± 0.4

[a]Five measurements were recorded ($n = 6$); data are expressed as the mean $\pm$ SD

was sequenced on an Illumina MiSeq platform (sequence depth: $5500 \times$ for PHB11; $5233 \times$ for PHB12; $5324 \times$ for PHB13). Sequence assemble by means of SOAPdenovo2.04 [22] yielded a linear double-stranded DNA of 87,553-bp with an average G + C content of 38.9% for the complete genome sequence of PHB11 (GenBank accession no. MK524176), a linear double-stranded DNA of 168,791-bp with G + C content of 37.6% for the complete genome sequence of PHB12 (GenBank accession no. MK524178), and a linear double-stranded DNA of 165,641-bp with a G + C content of 40.4% for the complete genome sequence of PHB13 (GenBank accession no. MK573636). Sequence annotations by using the RAST server [23] determined 125, 268, and 274 putative open reading frames (ORFs) as well as 23, 2, and 0 tRNA genes within the genomes of PHB11, PHB12, and PHB13,

respectively (Fig. 2). These ORFs involved in DNA packing, nucleotide metabolism and replication, structural component, transcription, and/or bacterial lysis (Fig. 2). For example, the terminase large subunit (ORF26 in PHB11, Fig. 2) is a DNA-packaging motor which cleaves concatemeric viral DNA into units of or near the genomic length and pumps each into a preformed capsid precursor termed procapsid powered by ATP hydrolysis [24, 25].

Sequence alignments showed that the whole genome sequences of PHB11, PHB12, and PHB13 shared 92.64%, 94.69%, and 96.37% average nucleotide identities to those of the *Escherichia* phages vB_EcoM_Alf5 (GenBank accession no. KX377933), p000v (GenBank accession no. MK047717), and KFS-EC (GenBank accession no. MH560358), respectively (Fig. 2). Phylogenetic analysis by using either the amino acid sequence of the large terminase subunit or the amino acid sequences of the major capsid protein revealed that PHB11 was a Felixo1virus in *Ounavirinae* subfamily, while PHB12 was a Rb69virus in *Tevenvirinae* subfamily, and PHB13 was a Rb49virus in *Tevenvirinae* subfamily (Fig. 3A, B).

Efficacy of Individual Phages or Phage Cocktail on Controlling STEC

To explore the lytic activity of individual phages or phage cocktail on controlling STEC, PHB11, PHB12, PHB13, and/or their cocktail at different MOI values (1000, 100, 10,

Table 2 Host range of the coliphage PHB11, PHB12, and PHB13

Strains	Serogroups	Stx1	Stx2	PHB11	PHB12	PHB13
E. coli HB11 (host)	O157	✓	✓	+	+	+
E. coli 18701	O91	✓	×	+	−	+
E. coli 18711	O91	✓	×	+	−	+
E. coli 14049	O102	×	✓	−	−	+
E. coli 14050	O102	×	✓	−	−	−
E. coli 14432	O42	✓	✓	+	−	−
E. coli 14854	O91	✓	×	+	−	+
E. coli 15657	O100	✓	✓	−	−	−
E. coli 15857	O34	✓	✓	−	−	−
E. coli 16129	O92	✓	✓	−	−	−
E. coli 16824	O34	✓	✓	−	−	+
E. coli 18713	O157	✓	✓	−	−	−
E. coli 18714	O97	✓	✓	−	−	−
E. coli 18715	O26	✓	✓	−	−	−
E. coli 18716	O157	✓	✓	−	−	−
E. coli 18717	O97	✓	✓	−	−	−
Salmonella enterica	Enteritidis	×	×	−	−	−
Salmonella enterica	Dublin	×	×	−	+	−
Salmonella enterica	Typhimurium	×	×	−	−	+
Salmonella enterica	Choleraesuis	×	×	−	−	−

✓, Toxin-positive; ×, toxin-negative; +, indicates plaque-formed; −, indicates no plaque-formed
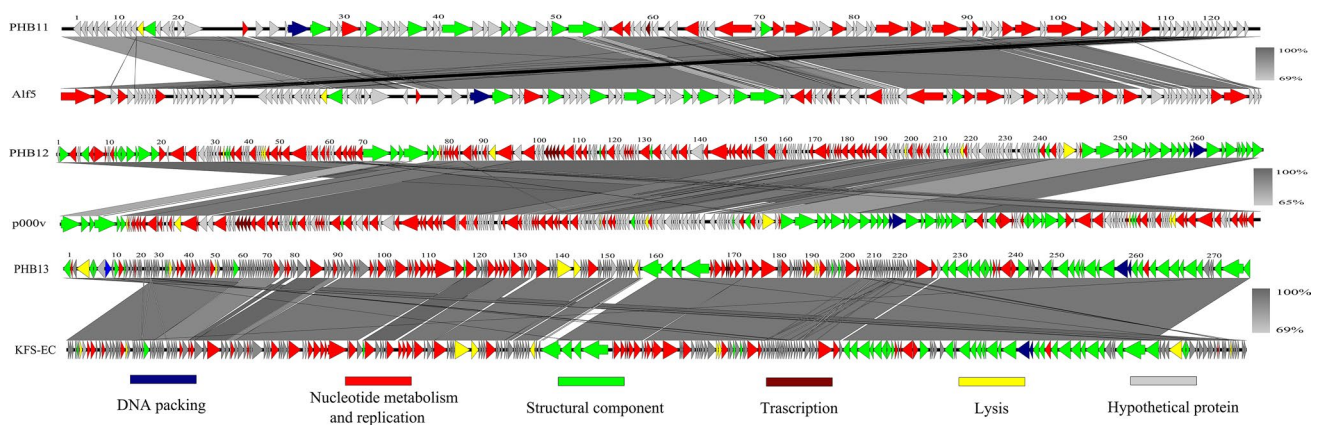
Fig. 2 Alignments of genome sequence maps. Genome sequences were analyzed using Easyfig software. Nucleotide identities between the two genomes (phage PHB11 and Alf5; PHB12 and p000v; PHB13 and KFS-EC) are indicated by gray shading

and/or 1) were incubated with *E. coli* O157 (10^7 CFU/ml; logCFU/ml = 7.0) at 4 °C (generally food storage temperature) or 25 °C (room temperature) for 24-h or 48-h, respectively. The results revealed that different MOIs of individual phages or the phage cocktail displayed capacities of reducing *E. coli* O157 in broth at either 4 °C (Fig. 4A–D) or 25 °C (Fig. 4E–H). However, the phage cocktail overall showed better effect of killing *E. coli* O157 than individual phages (Fig. 4A–C vs. 4D; E, F vs. G). At 4 °C, phage cocktail (MOI = 1000) reduced *E. coli* O157 by 1.21-log and 2.42-log after 24-h and 48-h phage cocktail treatment, respectively (Fig. 4D), while at 25 °C, a reduction of 4.32 log in the number of bacteria was observed at MOI of 1000 after 48-h phage cocktail treatment (Fig. 4H).

We next evaluate the biocontrol of STEC in milk using the phage cocktail. The results revealed that treatments with phage cocktail at different MOIs (1000, 100, 10, and/or 1) resulted in inhibition of *E. coli* O157 growth in milk. At 4 °C, after 168-h treatment of phage cocktail at MOI = 1000, a 3.8-log reduction in the number of *E. coli* O157 was observed, compared to that in phage-free bacterial control group (Fig. 5A). Also, an obvious reduction in surviving *E. coli* O157 (at least reduce 1.15-log) or an inhibition of bacterial growth was observed in phage cocktail treatment group at the MOI of 100, 10, and 1 after 168-h treatment, compared to those in phage-free bacterial control group, respectively. No significant difference of phage titer was observed after between the phage-treated and control groups during this 168-h (Fig. 5B). When the experiment was performed at 25 °C, approximately 6.92-log reduction of *E. coli* O157 was observed after 10-h treatment at the MOI of 1000 in phage cocktail treatment group, compared to that of the phage-free bacterial control group (Fig. 5C). This value was 2.45-log when co-incubation of the bacteria with the phage cocktail at a MOI of 1 (Fig. 5C). A significant difference of phage titers was observed between the phage-treated and control

groups when the phage cocktail was added at a MOI of 10 after 24-h treatment ($P < 0.5$, Fig. 5D).

Discussion

In recent year, due to the worldwide concern on the rapid increase of multidrug-resistant bacteria, there is currently a great interest in developing bacteriophages as bio-antibacterial agents or foodborne pathogen biocontrol agents [26–29]. In this study, we isolated three virulent phages and explored the effect of three individual phages or their phage cocktail on removing STEC, a well-known foodborne pathogen that is associated with different types of gastrointestinal diseases and/or may also be transmitted to humans by contact exposure and food chain [26]. Our tests on the heat stability and acidic stability of these three phages demonstrated that they were stable within a wide range of temperature (4 from to 40 °C) and pH values (from 5 to 9). These results are in agreement with the exports of the other studies that phage titers exhibited no significant change with the variation of temperature (from 4 to 40 °C) and pH values (from 5 to 9) [30–32]. These characteristics indicate the good adaptation of the three phages to a variable environment. Life cycle characterization showed that phages PHB11, PHB12, and PHB13 had a short latent period and a medium burst size with 32 to 65 phage particles released from per infected bacteria. Killing the host bacteria by phages in a short period of time and reducing the occurrence frequency of phage-resistant mutant are reported to be essential properties for therapeutic use of phages [33].

During therapy, phage should have a precise dose for clinical use. Ideally, phage cocktail with a wide possible range of hosts is capable of targeting pathogens, and use of phage cocktail might also prevent the development of phage-resistant mutants [34]. Therefore, we explore the use of a phage
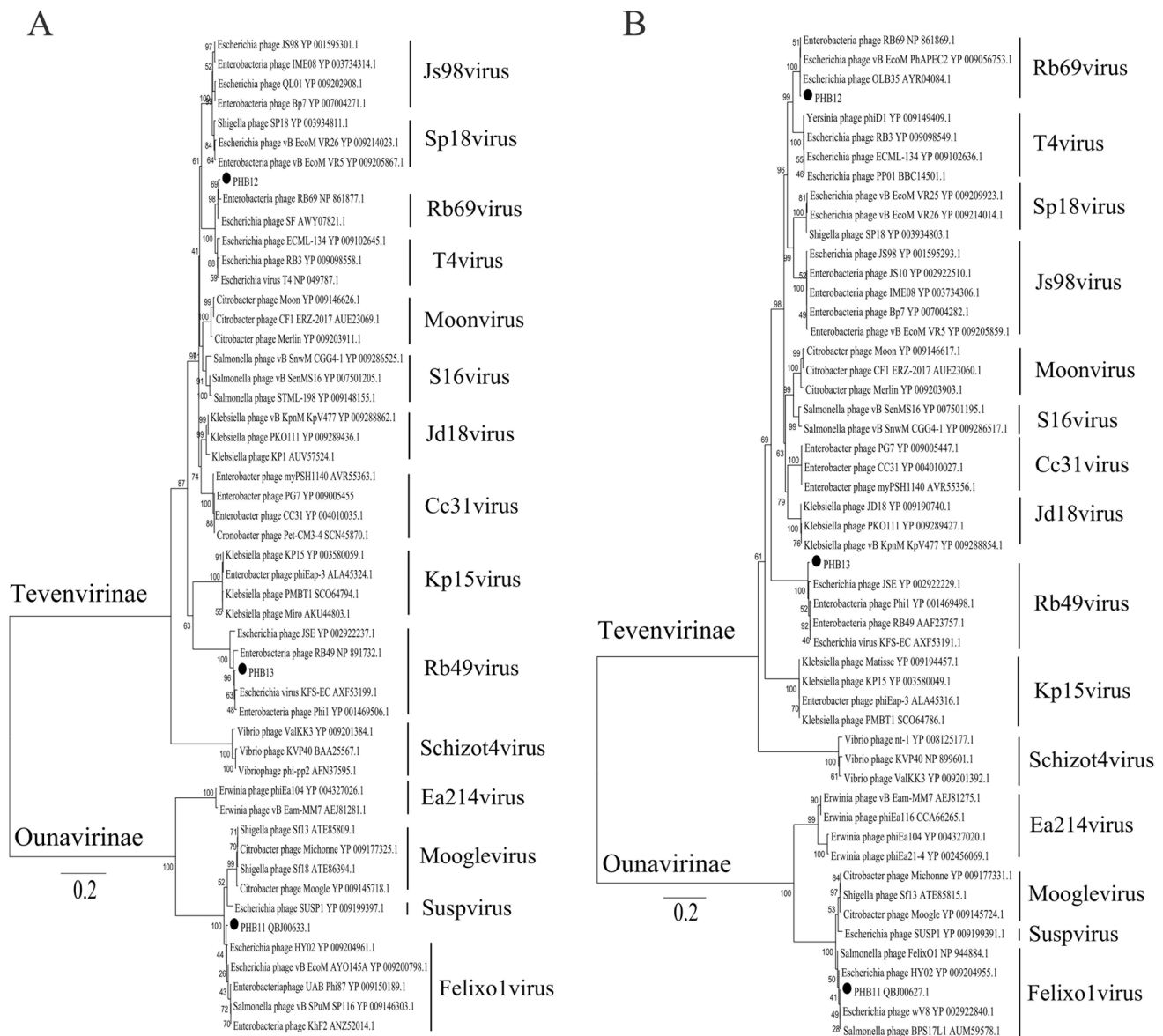
A

Js98virus
Sp18virus
Rb69virus
T4virus
Moonvirus
S16virus
Jd18virus
Cc31virus
Kp15virus
Rb49virus
Schizot4virus
Ea214virus
Mooglevirus
Suspvirus
Felixo1virus

Tevenvirinae

Ounavirinae

0.2

B

Rb69virus
T4virus
Sp18virus
Js98virus
Moonvirus
S16virus
Cc31virus
Jd18virus
Rb49virus
Kp15virus
Schizot4virus
Ea214virus
Mooglevirus
Suspvirus
Felixo1virus

Tevenvirinae

Ounavirinae

0.2

Fig. 3 Phylogenetic tree analysis of the phage-encoded large terminase subunits and major capsid proteins. The amino acid sequences of the major capsid proteins (**A**) and the large terminase subunits (**B**) were compared using MEGA X, and phylogenetic tree was generated using the neighbor-joining method with 1000 bootstrap replicates

cocktail comprising PHB11, PHB12, and PHB13 to control *E. coli* O157. Our study revealed that a better effect of suppressing or killing bacteria was achieved at high MOI than at low MOI within a certain period of time. Although *E. coli* O157 could not be completely removed from milk by phage cocktails, the reduction in *E. coli* O157 was closely related with increase in MOI value. Our results were in accordance with the report that a MOI of at least 1000 was required for the reduction in viable *E. coli* O157 in broth culture at 4 °C or 37 °C [35]. Previous studies have also shown that the reduction in *E. coli* was related to the concentration of phage [36, 37]. Phages require a metabolic process to conduct its

own replication in the host cells [38]. In low-temperature environments, phage replication might be affected. In our study, phage cocktail could reduce pathogen number at low temperature, compared to the control group. The effect of killing or inhibiting bacteria by phage cocktail at high MOI value and low temperature may be related to the mechanism of abortive infection and/or lysis from without [39]. These results agree well with the previous report that phage was able to reduce pathogen number, including *E. coli*, *Salmonella*, and *L. monocytogenes* at low temperature [32, 36, 40].

At present, there is an increasing interest in the use of water to rinse or spray antibacterial drugs to reduce
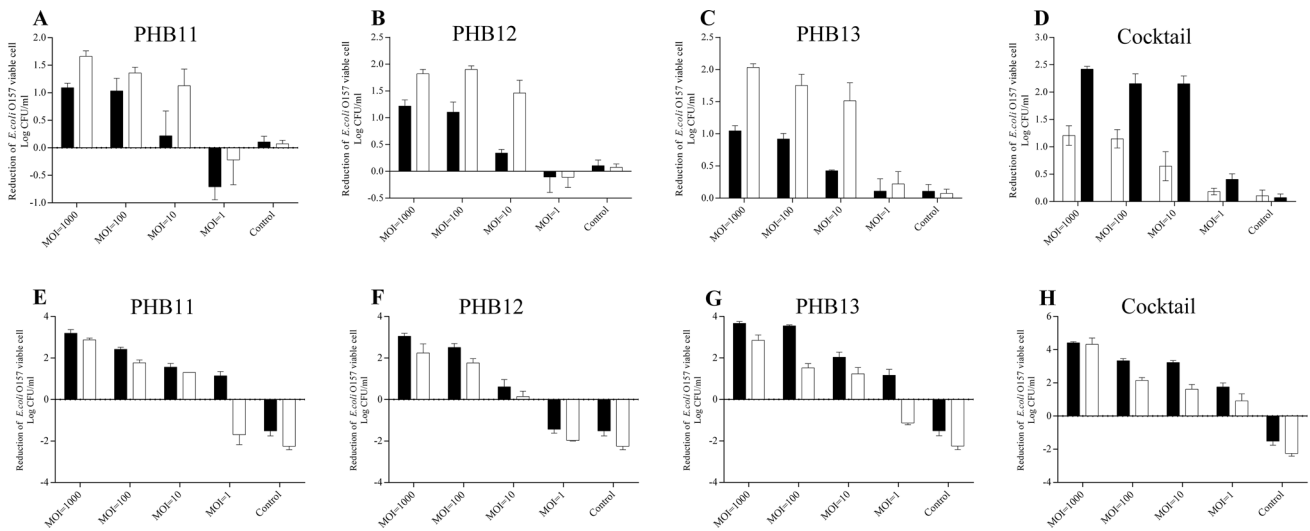
Fig. 4 Reduction in *E. coli* O157 viable cells in broth medium. *E. coli* O157 was treated with different MOIs of individual phage or phage cocktail for 24 h (white bar) and 48 h (black bars) at 4 °C (panels **A**–**D**) and 25 °C (panels **E**–**H**). Bacteria were counted by 10-folds plating serial dilutions of the cultures. Data are presented as mean ± SD from three independent experiments
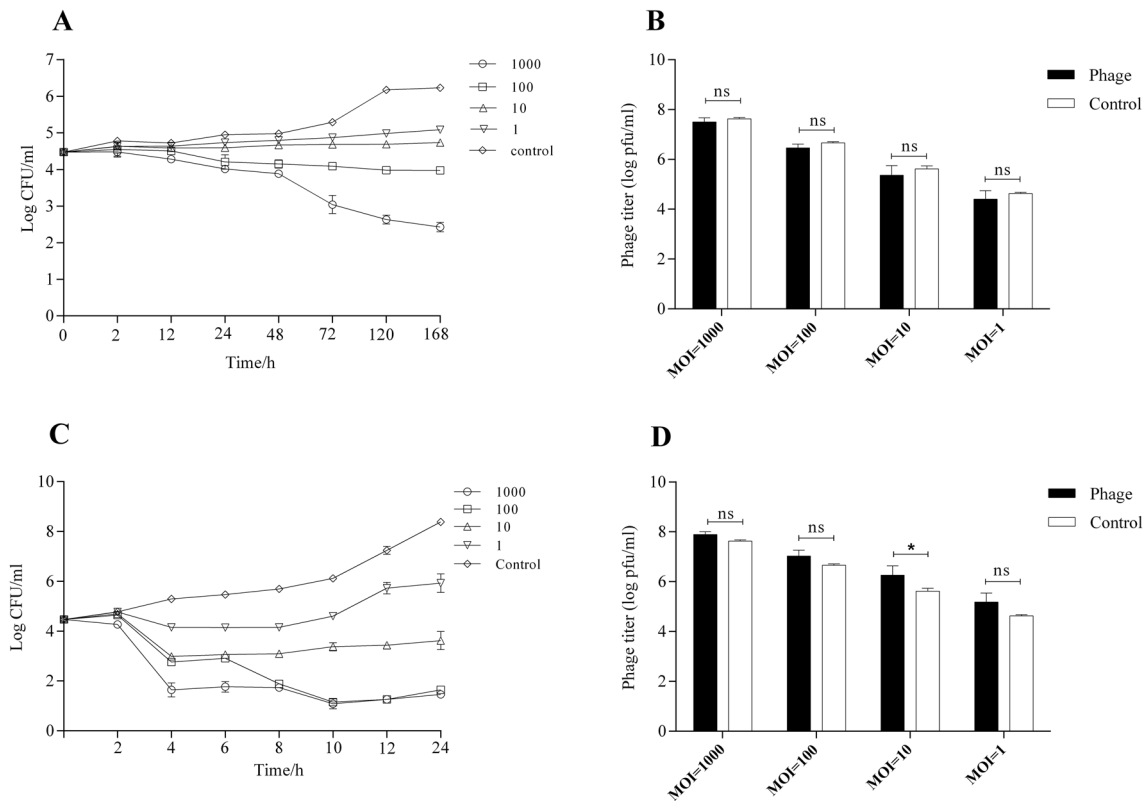

Fig. 5 Reduction in *E. coli* O157 viable cells in milk. *E. coli* O157 was incubated with different MOIs of phage cocktail (*open circles* MOI = 1000; *open squares*: MOI = 100; *up pointing triangles*: MOI = 10; *down pointing triangles*: MOI = 1 and *diamonds* control group) at 4 °C (panel **A**) and/or 25 °C (panel **C**). The phage titers were detected at 4 °C for 168-h treatment (panel **B**) and at 25 °C for 24-h treatment (panel **D**), respectively. Bacteria were counted by plating serial dilutions of the cultures; "ns" represented no significance. Data are presented as mean ± SD from three independent experiments

the pollution of *E. coli* O157 in food. However, there are still many drawbacks with these methods. We simulated the ambient temperature (4 °C and 25 °C) during food storage and transportation. In our study, phage cocktail (MOI = 1000) reduced *E. coli* O157 by 3.8 log and 6.92 log in milk at 4 °C after 168-h treatment and at 25 °C after 10-h treatment, respectively. Our coliphage cocktail has been confirmed to be effective in reducing the bacterial number in milk at refrigerator temperature (4 °C) and room temperature (25 °C). Our findings were supported by the previous studies of other coliphages [31, 36].

To be concluded, this study reveals that phages PHB11, PHB12, and PHB13 have a strong stability and a good sterilization effect in vitro. In addition, no virulence factors and drug resistance genes are identified in the genome of these three phages. Furthermore, phage cocktails effectively lyse *E. coli* O157 in broth and milk at 4 °C and 25 °C. They represent an interesting new drug candidate against STEC O157 to control this pathogen in refrigerated milk.

Materials and Methods

Strains and Phages

STEC O157 strains producing Stx1 and/or Stx2 are our laboratory collective strains and were isolated from clinical samples in Central China [20]. All strains were streaked from the frozen culture stock onto Luria–Bertani (LB) plates (Solarbio, England; 0.75% agar). Single clone from LB plates was cultured in LB broth. Soft LB (0.75% agar) plates were used as upper medium. Phage samples were collected from local sanitary sewage, using a double-layer agar method as described previously [21]. Phage SM buffer [5.8 g of NaCl, 2.0 g of $MgSO_4 \cdot 7H_2O$, 50 ml of Tris–HCl (pH 7.4), 5.0 ml of 2% gelatin] was used to resuspend plaques [21, 41]. Phages were purified by CsCl gradient ultra-centrifugation and stored at 4 °C, as described previously [41].

Biological Characteristics of the Phages

The morphology of phages was examined by using a 100-kV TEM (Hitachi H-7650, Tokyo, Japan). In addition, thermal stability, pH sensitivity, optimal MOI, and one-step growth curve were tested by double-layer agar method, as described previously [21, 41]. The host range assays were performed by spot testing, as previously described in the same protocol [41].

Preparation of the Phage Cocktail

High titer stocks were prepared for biocontrol studies, as described in a previous study [42]. Phage cocktails including PHB11, PHB12, and PHB13 were prepared by equal concentrations of phage stocks to reach final concentration of approximately 1×10^{10} plaque-forming units (PFU)/ml as McLean et al. (2013) recommended [30]. Phage stocks were stored at 4 °C ready for use.

Frequency Determination of Phage-Insensitive Mutations (BIMs)

The frequency of BIMs was determined in the method reported by O'Flynn et al. (2004) [42]. Phage alone or phage cocktails were mixed *E. coli* O157 strain at a MOI of 10 and then were incubated for 5 min at 37 °C. The mixture added with 6 ml of overlay agar medium was poured onto LB plates (1.5% agar), and then, the plates were incubated for 12 h at 37 °C. Single clone was picked up to be cultured with LB medium at 37 °C for 12 h. The BIM frequency was calculated by the ratio of surviving colonies to the number of original bacteria. This experiment was repeated three times.

DNA Extraction and Analysis of Genome Sequence

Purified phages were treated with DNase I (final concentration, 10 μl/ml) and RNase A (final concentration, 5 μl/ml) in SM buffer at 37 °C for 1 h. Subsequently, the proteinase K (100 mg/ml), SDS (10%, w/v), and EDTA (0.5 mM, pH 8.0) were added at 56 °C for 1 h. Finally, the genomic DNA of phage was extracted according to standard phenol–chloroform extraction protocols, as described previously [21]. Afterward, the quality and quantity of the genomic DNA were checked by electrophoresis on a 1% agarose gel and a NanoDrop2000 (Thermo Scientific, Waltham, USA), respectively. Following this step, DNA libraries with an insert size of 270 bp were constructed using the NEBNext®Ultra™ II DNA Library Prep Kit (NEB, Ipswich, MA, US), and were then sequenced by using the Illumina MiSeq sequencer platform (Illumina, USA). Raw reads with low quality were filtered and eliminated by SOAPnuke (version 1.5.0) software (https://github.com/BGI-flexlab/SOAPnuke) [43]. Following criteria were set for reads filtration: reads with a certain proportion of low quality (20) bases (40% as the default, parameter setting at 20 bp), and/or with a certain proportion of Ns (10% as the default, parameter setting at 1 bp) were removed; adapter contamination (15 bp overlap between the adapter and reads as the default, parameter setting at 15 bp) and duplication contamination were also removed [21]. Finally, phage sequence was assembled by splicing the reads using Newbler version 3.0 software with default parameters. Genome annotations and ORFs were identified using online tool RAST (http://rast.nmpdr.org/). The putative functions of the ORFs were examined using the BLASTP (http://blast.ncbi.nlm.nih.gov/). Phylogenetic trees based on the phage large terminase subunit and major capsid

proteins were constructed using the ClustalW program in MEGA X [44]. Blast comparisons of phage genomes were conducted with Easyfig [45].

Biocontrol in Broth

The effect of killing the host bacteria by phage was investigated in broth by a previously described method with minor modification [41]. Briefly, phage cocktails were added to their common host strain *E. coli* O157 (1×10^7 CFU/ml) at different MOIs of 1000, 100, 10, and 1. And then, the mixture was cultured at 4 °C and 25 °C with constant shaking (180 r/min) for 24 h and 48 h, respectively. The control group was added with an equal volume of sterilized SM buffer. Bacterial counts were performed by plating serial dilutions of the cultures. Three independent experiments were carried out.

Biocontrol in Milk

Biocontrol experiments were carried out at 4 °C and 25 °C as mentioned above. A commercial, reconstituted (10%, w/v) skim milk powder (pH 6.7) added with $CaCl_2$ (0.28 g/l) to make up the nutrient loss caused by the sterilization of milk, as Tomat et al. (2018) described [19]. The skim milk powder infected with the *E. coli* O157 (5×10^4 CFU/ml) was inoculated with the phage cocktails at different MOIs (1000, 100, 10, and 1). Host strains added with the phage cocktails were cultured at 4 °C for 0, 2, 12, 24, 48, 72, 120, and 168 h, and 25 °C for 0, 2, 4, 6, 8, 10, 12, and 24 h, respectively. The control group added with an equal volume of sterilized SM buffer was cultured under the same conditions as mentioned above. The culture was homogenized by shaking before sampling. Bacterial counts were performed by plating serial dilutions of the cultures on LB agars. This experiment was repeated three times.

Statistical Analysis

The "Two-way ANOVA" strategy in GraphPad Prism6.0 was used for statistical analysis. Data represent mean $\pm$ SD. The significance level was set at $P < 0.05$.

Declarations

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