



RESEARCH ARTICLE



# Recombination of T4-like Phages and Its Activity against Pathogenic *Escherichia coli* in Planktonic and Biofilm Forms

Min Li<sup>1,2,3</sup> · Donglin Shi<sup>1,2,3</sup> · Yanxiu Li<sup>1,2,3</sup> · Yuyi Xiao<sup>1,2,3</sup> · Mianmian Chen<sup>1,4</sup> · Liang Chen<sup>5</sup> · Hong Du<sup>6</sup> · Wei Zhang<sup>1,2,3</sup>

Received: 9 October 2019 / Accepted: 10 March 2020 / Published online: 25 May 2020  
© Wuhan Institute of Virology, CAS 2020

## Abstract

The increasing emergence of multi-drug resistant *Escherichia coli* (*E. coli*) has become a global concern, primarily due to the limitation of antimicrobial treatment options. Phage therapy has been considered as a promising alternative for treating infections caused by multi-drug resistant *E. coli*. However, the application of phages as a promising antimicrobial agent is limited by their narrow host range and specificity. In this research, a recombinant T4-like phage, named WGqlae, has been obtained by changing the receptor specificity determinant region of gene 37, using a homologous recombination platform of T4-like phages established by our laboratory previously. The engineered phage WGqlae can lyse four additional hosts, comparing to its parental phages WG01 and QL01. WGqlae showed similar characteristics, including thermo and pH stability, optimal multiplicity of infection and one-step growth curve, to the donor phage QL01. In addition, sequencing results showed that gene 37 of recombinant phage WGqlae had genetically stable even after 20 generations. In planktonic test, phage WGqlae had significant antimicrobial effects on *E. coli* DE192 and DE205B. The optical density at 600 nm (OD<sub>600</sub>) of *E. coli* in phage WGqlae treating group was significantly lower than that of the control group ( $P < 0.01$ ). Besides, phage WGqlae demonstrated an obvious inhibitory effect on the biofilm formation and the clearance of mature biofilms. Our study suggested that engineered phages may be promising candidates for future phage therapy applications against pathogenic *E. coli* in planktonic and biofilm forms.

**Keywords** T4-like phages · *Escherichia coli* (*E. coli*) · Homologous recombination · Gp37 · Planktonic · Biofilm

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s12250-020-00233-2>) contains supplementary material, which is available to authorized users.

✉ Hong Du  
hong\_du@126.com

✉ Wei Zhang  
vszw@njau.edu.cn

<sup>1</sup> College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China

<sup>2</sup> Key Laboratory of Animal Bacteriology, Ministry of Agriculture, Nanjing 210095, China

<sup>3</sup> MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, China

## Introduction

Pathogenic *Escherichia coli* (*E. coli*) is an important human and animal pathogen (Jenkins 2018; Kaper *et al.* 2004). Pathogenic *E. coli* strains were categorized into many pathotypes. Six defined *E. coli* pathotypes can cause

<sup>4</sup> Department of Veterinary Medicine, College of Animal Science and Technology, Zhejiang A&F University, Lin'an, Hangzhou 311300, China

<sup>5</sup> Hackensack-Meridian Health Center for Discovery and Innovation, Nutley, NJ 07110, USA

<sup>6</sup> Department of Clinical Laboratory, The Second Affiliated Hospital of Soochow University, Suzhou 215004, China

intestinal infections, including enterotoxigenic (ETEC), Shiga toxin-producing/enterohemorrhagic (STEC/EHEC), enteropathogenic (EPEC), enteroaggregative (EAEC), enteroinvasive (EIEC), and diffusely adherent (DAEC) (Kaper *et al.* 2004). By contrast, four defined *E. coli* pathotypes, *i.e.* avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), neonatal meningitis causing or meningitis-associated (NMEC/MAEC) and sepsis *E. coli* (SEPEC), were able to cause extra intestinal infections (Moulin-Schouleur *et al.* 2007).

Avian pathogenic *E. coli* (APEC) is the primary cause of colibacillosis, characterized by acute fatal septicemia or sub-acute fibrinous pericarditis, airsacculitis, salpingitis, and peritonitis, in poultry (Lutful Kabir 2010). Antibiotics have been not only used as antibacterial agents for the treatment of bacterial infections, but also as prophylactic and growth-promoting feed additives in breeding industry for a long time. Consequently, the wide usage of antibiotics has inevitably led to the emergence of multidrug-resistant bacteria (Suresh *et al.* 2018). Recent study showed that sixty-eight percent of APEC isolates carried at least 5 out of 8 antimicrobial resistant genes from broiler chickens in Jordan (Ibrahim *et al.* 2019). In a study in China, Li *et al.* reported that 87 APEC isolates showed 100% multi-drug resistant phenotypes, particularly against ampicillin, kanamycin, ciprofloxacin, levofloxacin, streptomycin, gentamycin, ofloxacin, norfloxacin, and ceftriaxone (Li *et al.* 2015). In addition, Liu *et al.* reported that APEC strains demonstrated high pathogenicity (15/26) and multidrug-resistant abilities (26/26) (Liu *et al.* 2019). Nowadays, multi-drug resistant *E. coli* has become a significant problem in veterinary, food and medical fields (Diarra and Malouin 2014).

Due to the multi-drug resistance among these *E. coli* stains, antimicrobial treatment became difficult. Some *E. coli* vaccines, including whole inactivated vaccine and subunit vaccine, have also been used to control colibacillosis. However, the vaccines were usually only effective against single serotype of strains. In fact, it is difficult to develop effective vaccines to prevent and control all the serotype pathogenic *E. coli* (Ebrahimi-Nik *et al.* 2018). Broadly protective vaccines against all or multi-pathotypes *E. coli* are still not available (Nesta and Pizza 2018).

Development of eco-friendly, effective and safe alternatives to antibiotics is imperative. In comparison to other treatment solutions, phages have multiple advantages, including ubiquity (Johnson *et al.* 2008), specificity, broad antimicrobial effects on several different serotypes strains and less interference to the normal flora (Clark and March 2006). Phages have been reconsidered as an alternative option against bacterial infections. For example, phages were used as alternatives to antibiotics for treating human infected with *Pseudomonas aeruginosa* and *Acinetobacter*

*baumannii* (Chan *et al.* 2018; Schooley *et al.* 2017) and animals infected with *E. coli* (Chibani-Chennoufi *et al.* 2004). In addition, engineered mycobacteriophage cocktail have been used for the treatment of a patient infected with drug-resistant *Mycobacterium abscessus* (Dedrick *et al.* 2019).

However, narrow host-range and phage-resistant bacteria remain to be the main problems for phage therapy (Haq *et al.* 2012; Hermoso *et al.* 2007). Currently, two main approaches were used to solve the problems by developing a phage cocktail or generating genetic recombinant phages (Chan *et al.* 2013; Mahichi *et al.* 2009; Yu *et al.* 2018). For example, T4-like phages can recombine with a recombination plasmid containing the gene 37–38 segment from T2-like or T6-like phages (Tetart *et al.* 1998). These engineered phages thus acquired host range of the donor phages and can infect genetically distant host bacteria. In T4-like phages, the receptor-binding tip of long tail fiber determined the specificity and host range, which is encoded by the C-terminal domain of gene 37 (Bartual *et al.* 2010; Chen *et al.* 2017). Our previous research also showed that the recombinant T4-like phages can infect eight additional bacteria, suggesting the expansion of host range (Chen *et al.* 2017).

This study aims to customize chimeric T4-like phages for specific clinical pathogenic *E. coli* using a T4-like phages homologous recombination platform established by our laboratory (Chen *et al.* 2017). The basic biological characteristics and genetic stability of recombinant phage were then examined and compared with the parental donor phages. Our study suggested that the engineered phage WGqlae had a significant antimicrobial effect against clinical *E. coli* strains in planktonic and biofilm forms *in vitro*.

## Materials and Methods

### Bacteria, Bacteriophages and Growth Conditions

All 113 *E. coli* strains used in this study are listed in Supplementary Table S1. Among them, 104 *E. coli* strains isolated from the brains of ducks with septicemia and neurological clinical symptoms in Eastern China in previous study, were included (Chen *et al.* 2017). The remaining 9 strains were selected from the collection in our laboratory. These *E. coli* strains were used to determine the host range of phages. Phages WG01 and QL01 were used as the parental phages for homologous recombination, while *E. coli* strains DE017 and DE205B were used as host strains of the two phages for amplification, respectively (Chen *et al.* 2017).

Luria–Bertani (LB) liquid medium were used to cultivate bacterial strains. When necessary, 100 µg/mL ampicillin was added. LB 0.5% soft agar plates and LB 1.5% agar plates were used for phages plaque assay. Tryptic soy broth liquid medium (TSB, Merck, Germany) were used to culture *E. coli* biofilm. SM buffer (50 mmol/L Tris–HCl [pH 7.5], 100 mmol/L NaCl, 10 mmol/L MgSO<sub>4</sub> and 0.01% gelatin) was used for the dilution and storage of the phages. Phage cultures were clarified by centrifugation at 5000 ×g, 4 °C for 10 min, then the supernatants were filtered through 0.22 µm filters (Merck Millipore, Germany) and stored at 4 °C. Phosphate Buffer Saline (0.1 mol/L Na<sub>2</sub>HPO<sub>4</sub>, 0.15 mol/L NaCl<sub>2</sub>, pH 7.2) was used to wash and dilute *E. coli* strains.

### Construction of the Plasmids with Diverse Recombination Regions

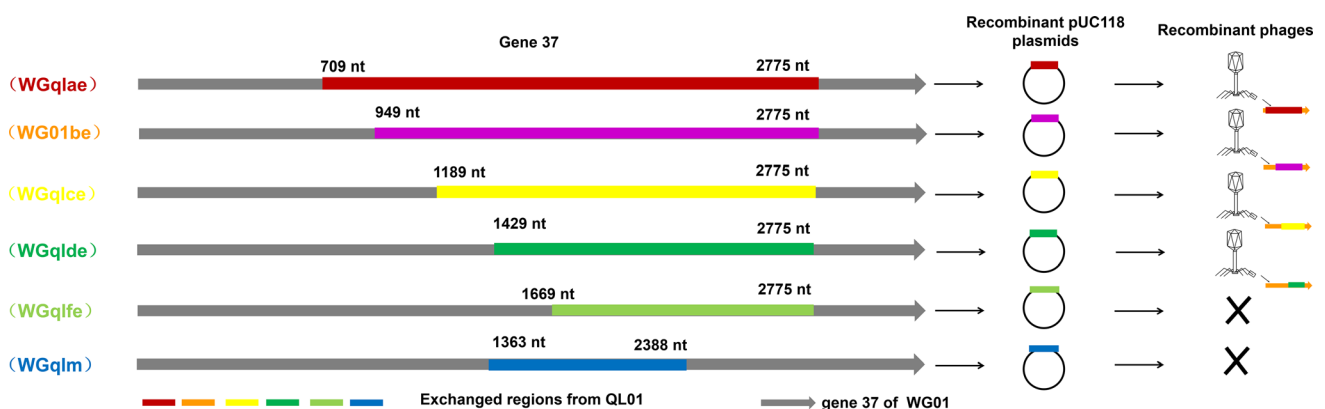
We selected six diverse DNA fragments in gene 37 from QL01 as the recombination regions to replace the corresponding fragments in WG01 (Bartual *et al.* 2010; Chen *et al.* 2017). Six different fragments of gene 37 (*i.e.*, QLae, QLbe, QLce, QLde, QLfe and QLm) were amplified (Fig. 1) and fused using the primers listed in Supplementary Table S2, with WG01 and QL01 phages as the DNA templates. Plasmids construction was performed as described previously (Yoichi *et al.* 2005). In brief, the PCR products were digested with *Bam*H I and *Eco*R I, followed by insertion into plasmid vector pUC118 (Takara, Shiga, Japan), generating six different recombinant plasmids, pUC118ae, pUC118be, pUC118ce, pUC118de, pUC118fe and pUC118m.

### Homologous Recombination and Isolation of Recombinant Phages

Homologous recombination of T4-like phages was performed as described previously (Chen *et al.* 2017; Yoichi *et al.* 2005) with minor modifications. Briefly, competent strain DE017 were transformed with the recombinant plasmid, pUC118ae, pUC118be, pUC118ce, pUC118de, pUC118fe and pUC118m, respectively. The DE017 transformant strains were cultured in 5 mL LB liquid medium with 50 µg/mL ampicillin at 37 °C until OD<sub>600</sub> 0.4, then the phage WG01 was added. After purification and filtration, recombinant phages were isolated using *E. coli* DE205B (susceptible to QL01, but resistant to WG01) as host bacteria by double-layer plate method (Adams 1959). Recombinant phages were purified by plaque assay. PCR and Sanger sequencing analysis of gene 37 from the recombinant phages was carried out using primers Fcheck/Rcheck (Chen *et al.* 2017).

### Host Ranges of Recombinant Phages

The recombinant phages, WGqlae, WGqltbe, WGqltce, WGqlde, and the parent phages WG01 and QL01 were prepared by double-layer plate method (Adams 1959). A total of 113 avian *E. coli* strains were used as the host bacteria to determine the host ranges (Supplementary Table S1) using spot test with minor modifications (Chen *et al.* 2016). In brief, bacterial cultures at exponential phase (100 µL) were spread on an LB agar plates. Each of six phage suspensions (10 µL at 10<sup>9</sup> PFU/mL) was spotted on the surface of the bacteria lawn. The plates were examined for lysis after 8 h incubation at 37 °C.



**Fig. 1** Sketch map of gene 37 in QL01 and the results of the isolated chimeric phages. Different color fragments from QL01 for WG01 recombination. Four types of recombinant phages, namely, WGqlae, WGqlbe, WGqlce and WGqlde, were isolated from DE205B, the host

of QL01. The symbol X indicate that no chimeric phages were isolated with DE205B by infecting the plasmid-containing cell with phage WG01.

## Thermo and pH Stability of WG01 Derivatives

Thermo and pH stability of phages was evaluated using a previously described protocol with minor modifications (Laemmli 1970). Phage suspensions (100  $\mu$ L) were mixed with SM buffer (900  $\mu$ L) in 1.5 mL sterile Eppendorf tubes, and were kept at different temperatures (*i.e.* 30–60 °C) for 1 h. The survival rates of phages were determined by double-layer method as described above.

To determine the pH stability of phages, 100  $\mu$ L phage suspension was added to 900  $\mu$ L SM buffer with a gradient of pH values (range from 4 to 11), followed by incubation at 37 °C for 1 h. Similarly, double-layer plating method was used to examine the survival rates of phages.

## Optimal Multiplicity of Infection

Multiplicity of infection (MOI) was defined as the ratio of viral particles to potential host cells. Optimal MOIs of phage WG01, QL01 and WGq1ae were determined by using standard protocol with minor modifications (Lu *et al.* 2003). Phages were diluted to  $10^4$ – $10^9$  PFU/mL. Log phase host bacteria were adjusted to  $1 \times 10^8$  CFU/mL (Table 1). Phages at different titer (100  $\mu$ L, MOI = 0.0001, 0.001, 0.01, 0.1, 1, 10) and 100  $\mu$ L  $1 \times 10^8$  CFU/mL log phase host bacteria were added to 1.5 mL sterile Eppendorf tubes containing 800  $\mu$ L LB medium, followed by incubation at 37 °C for 3.5 h with shaking (60 rpm). Bacteria cultures (phage-free) and phage cultures (cell-free) were used as control groups in this experiments. All assays were conducted in duplicate. The MOI that generated the highest phage titer within 3.5 h was considered as optimal MOI.

## One Step Growth Curve of Recombinant Phage WGq1ae

Latent time and burst size of phage WGq1ae were determined by one-step growth curve as previous described (Pajunen *et al.* 2000). 10 mL Mid-exponential phase *E. coli*

DE205B culture ( $2 \times 10^8$  CFU/mL, OD<sub>600</sub> = 0.4) were mixed with 10 mL  $1 \times 10^7$  PFU/mL bacteriophage suspensions. Allow the phages to specifically adsorb to the host bacteria for 10 min at 37 °C. Then, after removing unabsorbed phages by centrifuging at 5000 rpm for 10 min, the precipitated pellet was resuspended in 20 mL fresh LB medium, followed by incubation at 37 °C. Samples were taken out at 10-min intervals for 100 min, and 1% chloroform (final concentration) was added to release the intracellular phage for phage titration using the double-layer agar plate method.

## Genetic Stability of Recombinant Phage WGq1ae

To evaluate the genetic stability of recombinant phage WGq1ae, the 1st, 10th and 20th generation WGq1ae were collected. Gene 37 was amplified by primers Fcheck/Rcheck (Chen *et al.* 2017). PCR products were sequenced by Suzhou genewiz biotechnology Co., Ltd. (Suzhou, China).

## Lytic Capacity of Phages WGq1ae against *E. coli* in Planktonic Form

Lytic activity of phages WGq1ae was evaluated by OD<sub>600nm</sub> values of the host bacteria every 2 h at the MOI of 0.1, 1 and 10 using 96-well microtiter plates. Each of phage group was comprised of 100  $\mu$ L host bacteria ( $2 \times 10^8$  CFU/mL, DE205B or DE192) and 100  $\mu$ L diluted phage lysate ( $2 \times 10^7$ – $2 \times 10^9$  PFU/mL), and the assay was conducted in quadruplicates. Control group was consisted of equal volume of host bacteria suspension and fresh LB liquid medium. The 96-well microtiter plates were incubated at 37 °C for 24 h, and the OD<sub>600</sub> values were measured at 2 h intervals using an ELISA microplate reader (Biotek, VT, USA).

**Table 1** Comparison of MOIs between recombinant WGq1ae and parental phage WG01 and QL01.

MOI	Phages (PFU/mL)	Bacteria (CFU/mL)	Bacteriophage titer after 3.5 h (PFU/mL)			Proliferation times		
			WG01	QL01	WGq1ae	WG01	QL01	WGq1ae
0.0001	$1 \times 10^3$	$1 \times 10^7$	$2.82 \times 10^5$	$3.27 \times 10^5$	$1.77 \times 10^6$	282	327	1770
0.001	$1 \times 10^4$	$1 \times 10^7$	$3.86 \times 10^6$	$4.64 \times 10^7$	$1.53 \times 10^6$	386	4640	153
0.01	$1 \times 10^5$	$1 \times 10^7$	$5.2 \times 10^6$	$2.47 \times 10^7$	$1.31 \times 10^7$	52	247	131
0.1	$1 \times 10^6$	$1 \times 10^7$	$3.57 \times 10^7$	$4.58 \times 10^7$	$2.21 \times 10^7$	35.7	45.8	22.1
1	$1 \times 10^7$	$1 \times 10^7$	$2.87 \times 10^7$	$3.26 \times 10^7$	$3.29 \times 10^7$	2.87	3.26	3.29
10	$1 \times 10^8$	$1 \times 10^7$	$2.4 \times 10^7$	$2.03 \times 10^7$	$2.91 \times 10^7$	0.24	0.20	0.29

## Quantification of Bacterial Biofilm

Quantitative detection of bacterial biofilm was performed as described previously with minor modifications (Stepanovic *et al.* 2000, 2004). Briefly, *E. coli* DE192 and DE205B were incubated in TSB liquid medium containing 1%, 2% and 3% glucose at 37 °C for 24 h, 48 h, 72 h, 96 h and 120 h, respectively. Supernatants were then slowly decanted, and washed three times with 200  $\mu$ L PBS. After air drying, each well was added 200  $\mu$ L methanol to fix for 15 min. After discarding methanol and air drying, each well was added 200  $\mu$ L 1% crystal violet and incubated for 5 min. After being washed three times with PBS, each well was added 100  $\mu$ L acetic acid (33% v/v) to dissolve the crystal violet, and was placed in 37 °C incubator for 30 min. Subsequently, the OD<sub>595nm</sub> value was measured by a microplate reader.

## Inhibiting Efficacy of Phage WGqlae, Polymyxin B, and Their Combination on Biofilm Formation

Fresh host bacteria DE192 and DE205B (100  $\mu$ L,  $2 \times 10^8$  CFU/mL) were added to 96 well plates. Before culture, WGqlae group was added 100  $\mu$ L phage WGqlae ( $2 \times 10^8$  PFU/mL); polymyxin B group was added 100  $\mu$ L polymyxin B (4 mg/L); and WGqlae + Polymyxin B group was added 50  $\mu$ L phages ( $4 \times 10^8$  PFU/mL) and 50  $\mu$ L polymyxin B (8 mg/L, minimum inhibitory concentration was 2 mg/L for DE192 and DE205B). Control group were added equivoluminal TSB medium. Culture condition and quantitative detection of bacterial biofilm were performed as mentioned above.

## Efficacy of Phage WGqlae, Polymyxin B and Their Combination on the Clearance of Formed Biofilm

*Escherichia coli* DE192 and DE205B were incubated to form biofilm in TSB liquid medium at 37 °C for 48 h and 72 h, respectively. Then, WGqlae group was added 200  $\mu$ L phage WGqlae ( $1 \times 10^8$  PFU/mL); polymyxin B group was added 200  $\mu$ L polymyxin B ( $1 \times 10^8$  PFU/mL), and WGqlae + Polymyxin B group was added 100  $\mu$ L phages ( $2 \times 10^8$  PFU/mL) and 100  $\mu$ L polymyxin B (8 mg/L). Control group was added equivoluminal TSB medium. Culture condition and quantitative detection of bacterial biofilm were performed as mentioned above.

## Statistical Analysis

All statistical analyses in this study were carried out with the GraphPad Prism 5 software package. Mean difference of control and treat groups of planktonic or biofilm assay

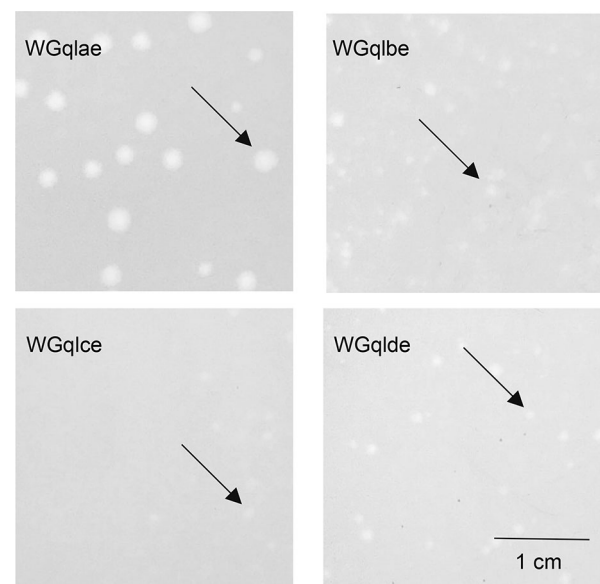
were analyzed by student's *t* test. *P* value  $\leq 0.05$  was considered statistically significant.

## Results

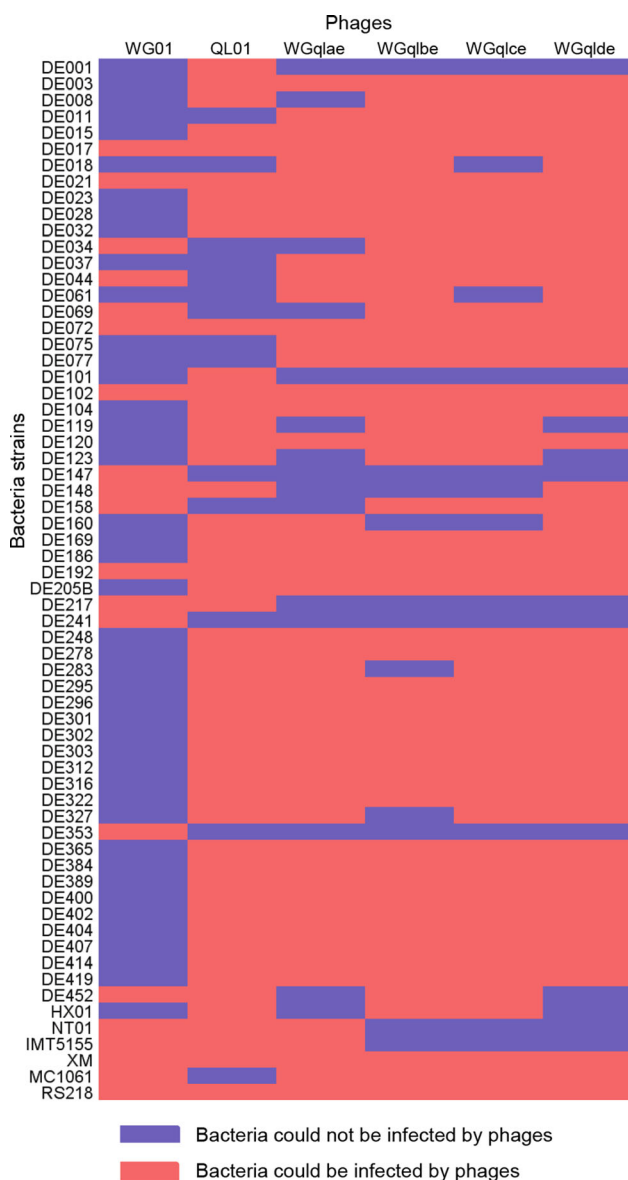
### Recombination of Phage WG01 with Different Regions of Gene 37 of QL01

We randomly selected six different DNA fragments, QLae (709–2775 nt), QLbe (949–2775 nt), QLce (1189–2775 nt), QLde (1429–2775 nt), QLfe (1669–2775 nt) and QLM (1363–2383 nt) of gene 37 in QL01 to replace the corresponding fragments of WG01 using a gene manipulation experiment (Fig. 1).

Four recombinant phages (WGqlce, WGqlbe, WGqlae and WGqlde) were isolated from clear plaques on double-layer plates with *E. coli* DE205B, one of host bacteria of phage QL01 (but not the host of WG01) (Fig. 2). Sequencing analysis of gene 37 confirmed the generation of chimeric phages WGqlce, WGqlbe, WGqlae and WGqlde. The gene 37 of WGqlce did not mutate in the process of homologous recombination. However, nucleic acid mutations occurred in gene 37 of WGqlae, WGqlTbe and WGqlTde, causing 14, 29 and 1 amino acid substitutions, respectively (Supplementary material S3).



**Fig. 2** Plaque morphology of recombination phages. Phages WGqlae, WGqlbe, WGqlce and WGqlde were grown on *E. coli* DE205B in double agar LB plates for 6 h. WGqlce and WGqlbe formed clear and round plaques approximately 0.1 cm in diameter. WGqlae formed clear and round plaques approximately 0.3 cm in diameter. WGqlde formed clear and round plaques approximately 0.2 cm in diameter.

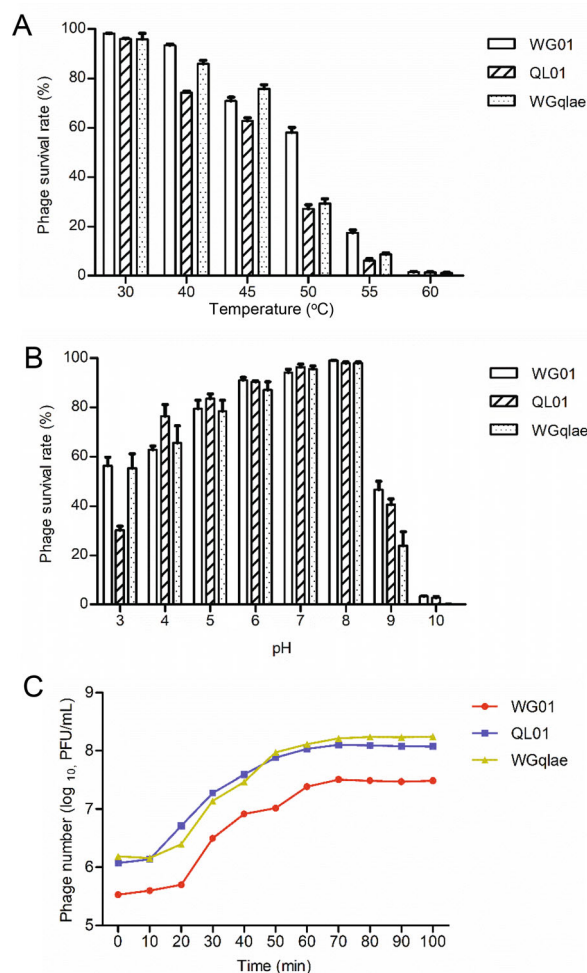


**Fig. 3** Host ranges analysis of recombinant and their parental phages. Phages derivatives WGqlae, Wgqlbe, Wgqlce and Wgqlde lysed 49, 52, 52 and 52 strains of the 113 *E. coli* strains, respectively (Only 64 strains were showed in this map, because other 47 strains were not susceptible to any of these phages). WGqlae can infect 4 additional host bacteria DE011, DE037, DE061 and DE077 in comparison to the parental phages.

### Host Range of WG01 Derivatives and Parental Phages

Host ranges of the phages WG01, QL01 and four WG01 derivatives were determined against 113 *E. coli* strains. Phages WG01 and QL01 lysed 20 (17.70%) and 50 (44.25%) of the 113 *E. coli* strains, respectively. While phages derivatives WGqlae (with AA 237–925 of QL01 gp37), WGqlbe (with AA 317–925 of QL01 gp37), WGqlce (with AA 397–925 of QL01 gp37) and WGqlde

(with AA 477–925 of QL01 gp37) lysed 49 (43.36%), 52 (46.02%), 52 (46.02%) and 52 (46.02%) of the 113 *E. coli* strains, respectively (Fig. 3, Supplementary Table S1). Among them, WGqlde, which was only replaced the downstream 1429–2772 bp in gene 37 of QL01, can lyse the most part of host strains of QL01. This result suggested that this domain of gp37 mainly determined the host range, which is consistent with the finding that C-terminal domain determined the receptor specificity. WGqlae, however, can infect four additional host bacteria, DE011, DE037, DE061 and DE077, in comparison to the parental phages, which suggested the expansion of host-range. Therefore, the recombination phage WGqlae was chosen to analyze the basic characteristic and antibacterial effect.



**Fig. 4** Biological properties of the recombinant phage WGqlae and its parental phages. **A** Thermo stability of recombinant phage WGqlae and the parental phages WG01 and QL01. **B** The pH stability of recombinant phage WGqlae and its parental phages WG01 and QL01. **C** One-step growth curve of the recombinant phage WGqlae and its parental phages WG01 and QL01. All the values are the means of 2 determinations.

## Comparison of Thermo and pH Stability of WG01 Derivatives WGqlae

Thermo stability test was carried out at pH 7 to investigate thermo stability of phages. The results showed that more than 60% of phage WGqlae remained active after incubation at 30 °C, 40 °C and 45 °C for 1 h, which were similar to the results from WG01 and QL01. In contrast, less than 2% of three active phage particles were observed at 60 °C (Fig. 4A). The results showed that the thermo stability of the WG01 derivative WGqlae was nearly the same as donor phage QL01.

The pH stability of phages was tested at 37 °C for 1 h. Phage WGqlae, WG01 and QL01 showed similar pH stability. In detail, they all showed the highest stability at pH 7, and they demonstrated similar survival rate (> 50%) at pH 5–9 (Fig. 4B). For all three phages, the phage titers decreased sharply at the acidic (pH = 4) or alkaline (pH = 10, 11) conditions.

## Optimal Multiplicity of Infection

MOI was defined as the ratio of viral particles to potential host cells. The optimal MOI of parental phage WG01 and QL01 were both 0.001. After incubation for 3.5 h, phage WG01 and QL01 multiplied 386 times and 4640 times, respectively. While the optimal MOI of phage WGqlae was 0.0001, and WGqlae increased by 1770 times after 3.5 h proliferation. The optimal MOI of recombinant phages WGqlae was lower than those of parental phage WG01 and QL01 (Table 2).

## Burst Size and Latent Time Period

One-step growth experiments were performed to compare latent time period and burst size of the phage WGqlae and the parental phages. One-step growth curve consisted of three phases-latent, log and stationary phases. Latent time period of the phage WGqlae was 10 min, and the burst size was about 110 phages/cell (Fig. 4C). In contrast, phage

WGqlae showed similar to phage QL01 in one-step growth experiments.

## Genetic Stability of Recombinant Phage WGqlae

The gene 37 of phage WGqlae in 1st, 10th and 20th generation did not show any mutations, suggesting that recombinant phage WGqlae was genetically stable.

## Efficacy of Phage WGqlae on Inhibiting *E. coli* in Planktonic State

The OD<sub>600</sub> values of the phage groups were significantly lower than that of the control group in 24 h, indicating that the derivative WGqlae inhibited bacteria proliferation. The OD<sub>600</sub> values of the control group of both host bacteria DE192 and DE205B increased continuously in 24 h. All OD<sub>600</sub> values of three phage groups (MOI 10, 1 and 0.1) began to decrease after a short increase in the first 2 h, and it did not increase until 16 h. In the first increased phase, the smaller MOI values were correlated with the more obvious increased trend, but in the second increased phase, the larger MOI values were associated with the more obvious decreased trend (Fig. 5).

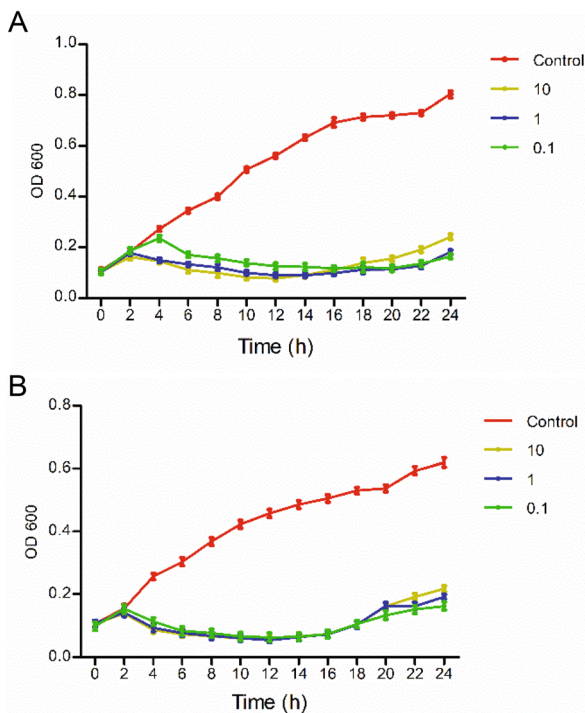
## Efficacy of Phage WGqlae, Polymyxin B and Their Combination to Inhibit Biofilms Formation

Our results showed that optimal medium for biofilm formation of host bacteria DE192 and DE205B was TSB medium without glucose, and optimal culture time of DE192 and DE205B was 48 h and 72 h, respectively. Therefore, the optimal medium and conditions were subsequently used in the following biofilms test.

Recombinant phage WGqlae ( $P < 0.01$ ), polymyxin B ( $P < 0.05$ ) and the mixture of WGqlae + Polymyxin B ( $P < 0.01$ ) inhibited the biofilm formation of host bacteria DE192 (Fig. 6A) and DE205B (Fig. 6B), significantly. When phage WGqlae was used alone, the biofilm of host bacteria DE192 and DE205B decreased by 38.59% and

**Table 2** Optimal biofilm formation conditions of DE192 and DE205B.

Time	Control	DE192 (OD <sub>595</sub> )					DE205B (OD <sub>595</sub> )				
		LB	TSB	1% G-TSB	2% G-TSB	3% G-TSB	LB	TSB	1%G-TSB	2%G-TSB	3%G-TSB
24 h	0.29	0.49	0.53	0.53	0.53	0.45	0.63	0.41	0.46	0.38	0.34
48 h	0.34	1.06	3.81	2.02	1.95	1.58	1.76	1.57	1.50	1.18	1.34
72 h	0.32	1.43	2.38	2.37	2.38	1.59	1.37	1.86	1.14	0.90	1.04
96 h	0.31	0.63	0.82	1.06	1.02	1.05	0.92	0.67	0.67	0.68	0.61
120 h	0.33	0.78	1.16	1.47	1.24	1.25	1.21	0.89	0.74	0.78	0.88

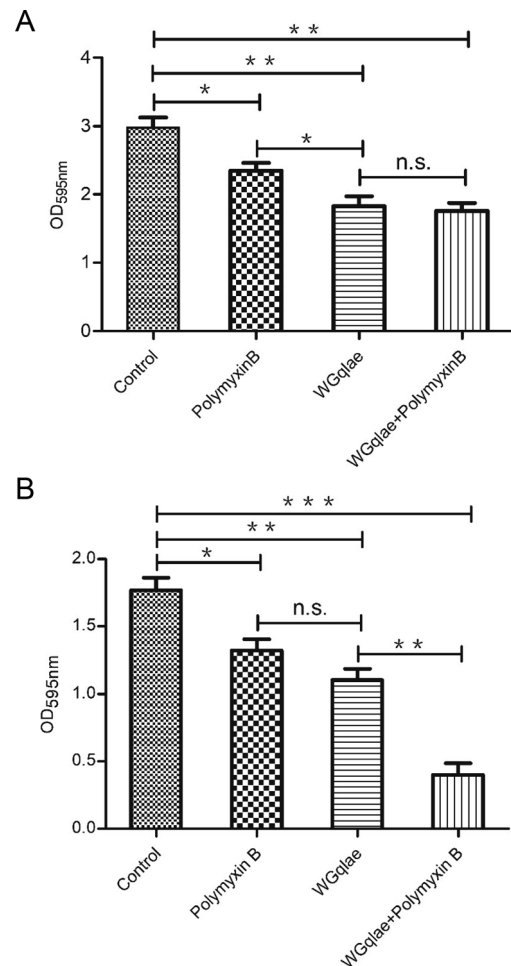


**Fig. 5** Antibacterial activity of phage WGQlae against planktonic *E. coli*. Phages WGQlae can inhibit planktonic *E. coli* DE192 (A) and DE205B (B) at different MOIs (0.1, 1, 10).

38.20% respectively ( $P < 0.01$ ), compared with the control group. As shown in Fig. 6A, phage WGQlae had better antibacterial effect than antibiotic Polymyxin B ( $P < 0.05$ ). As shown in Fig. 6B, when the mixture of WGQlae + Polymyxin B was used, Polymyxin B significantly enhanced the ability of phage WGQlae to inhibit biofilm formation of strain DE205B ( $P < 0.01$ ). Comparing with the control group, the biofilm formation of host bacteria DE192 and DE205B in WGQlae + Polymyxin B group was reduced by 40.94% ( $P < 0.01$ ) and 77.53% ( $P < 0.001$ ), respectively (Fig. 6).

### Efficacy of Phage WGQlae, Polymyxin B and Their Combination to Degrade Mature Biofilm

Comparing with the control group, the formed mature biofilm of *E. coli* DE192 and DE205B in phage WGQlae group decreased by 32.23% (Fig. 7A) and 33.22% (Fig. 7B), respectively ( $P < 0.01$ ). No significant difference was observed between Polymyxin B group and the control group, suggesting Polymyxin B had no effect on established mature biofilm (both Fig. 7A, 7B). There was also no significant difference between WGQlae + Polymyxin B group and the only phage WGQlae group in removing the formed biofilm, which suggested that Polymyxin B can contribute to inhibiting biofilms formation but



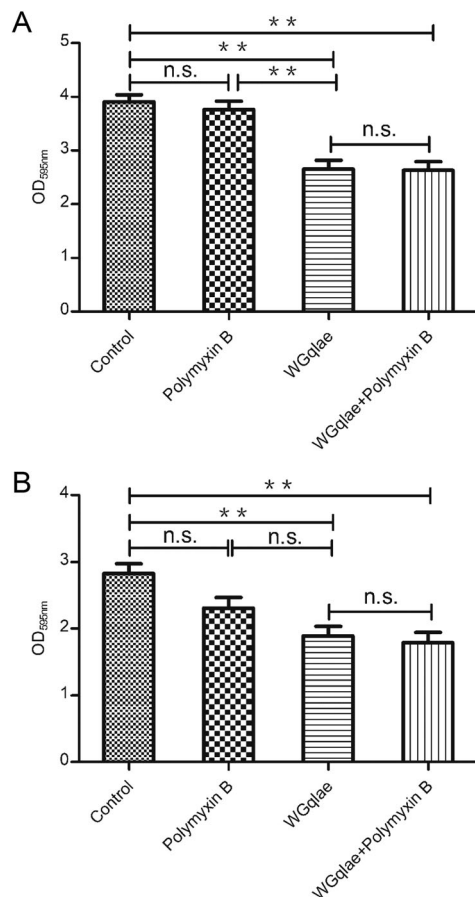
**Fig. 6** Inhibition activity of WGQlae, Polymyxin B and the combination of WGQlae and Polymyxin B on biofilm formation of DE192 (A) and DE205B (B). Each assay was performed in triplicate and repeated three times. Significant differences are indicated (\*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ ).

not to remove the formed mature biofilm of DE192 (Fig. 7A) and DE205B (Fig. 7B).

### Discussion

In this study, four chimeric phages (WGQlce, WGQlbe, WGQlae and WGQlde) with expanded host range were obtained by homologous recombination of gene 37. The four chimeras possessed the phage WG01 backbone with partial replacement of gene 37 from the phage QL01. To our surprise, derivatives WGQlae obtained nearly the entire host range from parental phage QL01 and an extra four host bacteria which was unsusceptible to the parental phages. The chimeric phages, WGQlae (with AA 237–925 of QL01 gp37), WGQlbe (with AA 317–925 of QL01 gp37), WGQlce (with AA 397–925 of QL01 gp37) and WGQlde (with AA 477–925 of QL01 gp37), had similar





**Fig. 7** Degrading activity of phage WGqlae, polymyxin B and the combination of WGqlae and Polymyxin B on formed mature biofilm of DE192 (A) and DE205B (B). Each assay was performed in triplicate and repeated three times. Significant differences are indicated (\*\* $P < 0.01$ ; \* $P < 0.05$ ).

host ranges. Furthermore, our previous research showed that phage WG01 derivatives WQD1 obtained a large proportion of host range of QL01, even though only the AA 714–925 fragment of QL01 gp37 has been replaced (Chen *et al.* 2017). These results indicated that the main receptor binding region of QL01 was located in the C-terminal AA 714–925 of gp37, but not much to do with the N-terminal AA 1–713 region.

The distal tail fiber locus specifies the all-important adsorption tip of T4 phages and primarily determines the host range of the phages (Hashemolhosseini *et al.* 1994; Kellenberger *et al.* 1965; Tetart *et al.* 1998; Wais and Goldberg 1969). As early as 1996, Tetart *et al.* reported that T4 phage can recombine with gene 37–38 segment from another T-even phage, thus acquiring donor phage's host range (Tetart *et al.* 1996). Other studies reported that T4 phage can acquire the host range of donor phage T2-like, T6 phages, or even phage  $\lambda$  by recombination of the tail fiber gene (Tetart *et al.* 1998). However, in this study, phages WGqlfe and WGqlm were not successfully isolated,

which indicated that not all subsegments recombination can generate active engineered phage, probably because they changed the three-dimensional structure of gp37 or the defect of screening methods for chimeric phages. As it stands, the mechanism of the exact locus, subsegments module or key amino acids of domain (AA 714–925) in gp37 sequence determining receptor binding specificity remains unclear and will be an important project in future. Further study is required to address how the key domains or amino acid sites in gp37 sequence affect three dimensional structure and the function of gp37, as well as the host range.

Determining the environmental stability of phages is essential for biocontrol of pathogenic bacteria. Our study showed that the biological properties (thermo stability, pH stability and one-step growth curve) of the recombinant phage WGqlae was similar to the donor phage QL01. In addition, the gene 37 of chimeric phage WGqlae did not mutate after 20 generations, which suggested that the receptor binding specificity of the recombinant phage was genetically stable.

Biofilms are generally considered as an essential factor in the pathogenesis of various opportunistic bacterial and fungal infections. In addition, biofilms can colonize medical devices or wounds which were particularly hard to be removed as biofilms are inherently highly antibiotic resistant (Hughes and Webber 2017). Our results showed that the engineered phage WGqlae can inhibit *E. coli* DE192 and DE205B in planktonic state, prevent biofilm formation, and remove mature biofilm. This study further demonstrated that specific host-range engineered phages can be successfully used as antibacterial agents against *E. coli* in planktonic state and biofilm form. Some studies also indicated that phage-based antibacterial testing had significant effect against biofilm formation (Endersen *et al.* 2017; Kelly *et al.* 2012). Once bacteria have formed biofilm, they tended to be less susceptible to antibiotics ( $P > 0.05$ ) (Fig. 7), causing a vital issue that antibiotics become ineffective. However, our study showed that phage WGqlae was highly efficient against formed mature biofilm ( $P < 0.01$ ) (Fig. 7), which is superior to antibiotics in the treatment of biofilm producers. Furthermore, another advantage of phages was that they are protein-based entities totally devoid of metabolic machinery that can actively replicate only in the presence of viable host bacterial cells but have no affinity to eukaryotic cells (Rios *et al.* 2016). Consequently, the phage therapy has been proven to be safe and free from adverse side effects (Rios *et al.* 2016). Currently, several natural or engineered phage products have been being patented and as commercial antimicrobial agents (Kaistha and Umrao 2016).

In conclusion, our study demonstrated that recombinant T4-like phage with expanded host range can inhibit

planktonic pathogenic *E. coli*, prevent biofilm formation and remove mature biofilm. It is highlighted that we can engineeringly obtain phages containing the characteristics of wide host range and strong lytic activity, providing a promising tool for prevention and treatment of infections caused by pathogenic *E. coli* in the future. Further experiments should be performed to evaluate the safety and efficacy of engineered phages for the clinical usage.

**Acknowledgements** This study was supported by Grants from the National Natural Science Foundation of China (U1803109), Key research and development plan of Jiangsu province (BE2019304), National Key R&D Program of China (2018YFC1602500), the Central University Basic Scientific Research Fund-Animal pathogenic bacteria (KYZ201846) and Jiangsu modern agriculture (waterfowl) industrial technology system disease prevention and control innovation team (JATS[2018]222).

**Author Contributions** WZ and HD conceived of the study. ML, DS, YL, YX, MC performed experiment, computational analysis. WZ and ML wrote the paper. ML and LC revised the final manuscript. All authors read and approved the final manuscript.

## Compliance with Ethics standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Animal and Human Rights Statement** This article does not contain any studies with human participants or animals performed by any of the authors.

## References

- Adams M (1959) Bacteriophage. Interscience Publishers, New York
- Bartual SG, Otero JM, Garcia-Doval C, Llamas-Saiz AL, Kahn R, Fox GC, van Raaij MJ (2010) Structure of the bacteriophage T4 long tail fiber receptor-binding tip. *Proc Natl Acad Sci U S A* 107:20287–20292
- Chan BK, Abedon ST, Loc-Carrillo C (2013) Phage cocktails and the future of phage therapy. *Future Microbiol* 8:769–783
- Chan BK, Turner PE, Kim S, Mojibian HR, Eleftheriades JA, Narayan D (2018) Phage treatment of an aortic graft infected with *Pseudomonas aeruginosa*. *Evol Med Public Health* 2018:60–66
- Chen M, Xu J, Yao H, Lu C, Zhang W (2016) Isolation, genome sequencing and functional analysis of two T7-like coliphages of avian pathogenic *Escherichia coli*. *Gene* 582:47–58
- Chen M, Zhang L, Abdelgader SA, Yu L, Xu J, Yao H, Lu C, Zhang W (2017) Alterations in gp37 expand the host range of a T4-like phage. *Appl Environ Microbiol*. <https://doi.org/10.1128/AEM.01576-17>
- Chibani-Chennoufi S, Sidoti J, Bruttin A, Kutter E, Sarker S, Brussow H (2004) *in vitro* and *in vivo* bacteriolytic activities of *Escherichia coli* phages: implications for phage therapy. *Antimicrob Agents Chemother* 48:2558–2569
- Clark JR, March JB (2006) Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. *Trends Biotechnol* 24:212–218
- Dedrick RM, Guerrero-Bustamante CA, Garlena RA, Russell DA, Ford K, Harris K, Gilmour KC, Soothill J, Jacobs-Sera D, Schooley RT, Hatfull GF, Spencer H (2019) Engineered bacteriophages for treatment of a patient with a disseminated drug-resistant *Mycobacterium abscessus*. *Nat Med* 25:730–733
- Diarra MS, Malouin F (2014) Antibiotics in Canadian poultry productions and anticipated alternatives. *Front Microbiol* 5:282
- Ebrahimi-Nik H, Bassami MR, Mohri M, Rad M, Khan MI (2018) Bacterial ghost of avian pathogenic *E. coli* (APEC) serotype O78:K80 as a homologous vaccine against avian colibacillosis. *PLoS ONE* 13:888
- Endersen L, Buttner C, Nevin E, Coffey A, Neve H, Oliveira H, Lavigne R, O'Mahony J (2017) Investigating the biocontrol and anti-biofilm potential of a three phage cocktail against *Cronobacter sakazakii* in different brands of infant formula. *Int J Food Microbiol* 253:1–11
- Haq IU, Chaudhry WN, Akhtar MN, Andleeb S, Qadri I (2012) Bacteriophages and their implications on future biotechnology: a review. *Virol J* 9:9
- Hashemolhosseini S, Montag D, Kramer L, Henning U (1994) Determinants of receptor specificity of coliphages of the T4 family. A chaperone alters the host range. *J Mol Biol* 241:524–533
- Hermoso JA, Garcia JL, Garcia P (2007) Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Curr Opin Microbiol* 10:461–472
- Hughes G, Webber MA (2017) Novel approaches to the treatment of bacterial biofilm infections. *Br J Pharmacol* 174:2237–2246
- Ibrahim RA, Cryer TL, Lafi SQ, Basha EA, Good L, Tarazi YH (2019) Identification of *Escherichia coli* from broiler chickens in Jordan, their antimicrobial resistance, gene characterization and the associated risk factors. *BMC Vet Res* 15:159
- Jenkins C (2018) Enterococcal *Escherichia coli*. *Curr Top Microbiol Immunol* 416:27–50
- Johnson RP, Gyles CL, Huff WE, Ojha S, Huff GR, Rath NC, Donoghue AM (2008) Bacteriophages for prophylaxis and therapy in cattle, poultry and pigs. *Anim Health Res Rev* 9:201–215
- Kaistha SD, Umrao PD (2016) Bacteriophage for mitigation of multiple drug resistant biofilm forming pathogens. *Recent Pat Biotechnol* 10:184–194
- Kaper JB, Nataro JP, Mobley HL (2004) Pathogenic *Escherichia coli*. *Nat Rev Microbiol* 2:123–140
- Kellenberger E, Bolle A, Boydelatour E, Epstein RH, Franklin NC, Jerne NK, Reale Scafati A, Sechaud J (1965) Functions and properties related to the tail fibers of bacteriophage T4. *Virology* 26:419–440
- Kelly D, McAuliffe O, Ross RP, Coffey A (2012) Prevention of *Staphylococcus aureus* biofilm formation and reduction in established biofilm density using a combination of phage K and modified derivatives. *Lett Appl Microbiol* 54:286–291
- Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685
- Li Y, Chen L, Wu X, Huo S (2015) Molecular characterization of multidrug-resistant avian pathogenic *Escherichia coli* isolated from septicemic broilers. *Poult Sci* 94:601–611
- Liu C, Diao Y, Wang D, Chen H, Tang Y, Diao Y (2019) Duck viral infection escalated the incidence of avian pathogenic *Escherichia coli* in China. *Transbound Emerg Dis* 66:929–938
- Lu Z, Breidt F Jr, Fleming HP, Altermann E, Klaenhammer TR (2003) Isolation and characterization of a *Lactobacillus plantarum* bacteriophage, phiJL-1, from a cucumber fermentation. *Int J Food Microbiol* 84:225–235
- Lutful Kabir SM (2010) Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Int J Environ Res Public Health* 7:89–114

- Mahichi F, Synnott AJ, Yamamichi K, Osada T, Tanji Y (2009) Site-specific recombination of T2 phage using IP008 long tail fiber genes provides a targeted method for expanding host range while retaining lytic activity. *FEMS Microbiol Lett* 295:211–217
- Moulin-Schouleur M, Reperant M, Laurent S, Bree A, Mignon-Grasteau S, Germon P, Rasschaert D, Schouler C (2007) Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. *J Clin Microbiol* 45:3366–3376
- Nesta B, Pizza M (2018) Vaccines against *Escherichia coli*. *Curr Top Microbiol Immunol* 416:213–242
- Pajunen M, Kiljunen S, Skurnik M (2000) Bacteriophage phiYeO3-12, specific for *Yersinia enterocolitica* serotype O:3, is related to coliphages T3 and T7. *J Bacteriol* 182:5114–5120
- Rios AC, Moutinho CG, Pinto FC, Del Fiol FS, Jozala A, Chaud MV, Vila MM, Teixeira JA, Balcao VM (2016) Alternatives to overcoming bacterial resistances: state-of-the-art. *Microbiol Res* 191:51–80
- Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, Lessor L, Barr JJ, Reed SL, Rohwer F, Benler S, Segall AM, Taplitz R, Smith DM, Kerr K, Kumaraswamy M, Nizet V, Lin L, McCauley MD, Strathdee SA, Benson CA, Pope RK, Leroux BM, Picel AC, Mateczun AJ, Cilwa KE, Regeimbal JM, Estrella LA, Wolfe DM, Henry MS, Quinones J, Salka S, Bishop-Lilly KA, Young R, Hamilton T (2017) Development and use of personalized bacteriophage-based therapeutic cocktails to treat a patient with a disseminated resistant *Acinetobacter baumannii* infection. *Antimicrob Agents Chemother.* <https://doi.org/10.1128/AAC.00954-17>
- Stepanovic S, Vukovic D, Dakic I, Savic B, Svabic-Vlahovic M (2000) A modified microtiter-plate test for quantification of staphylococcal biofilm formation. *J Microbiol Methods* 40:175–179
- Stepanovic S, Cirkovic I, Ranin L, Svabic-Vlahovic M (2004) Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Lett Appl Microbiol* 38:428–432
- Suresh G, Das RK, Kaur Brar S, Rouissi T, Avalos Ramirez A, Chorfi Y, Godbout S (2018) Alternatives to antibiotics in poultry feed: molecular perspectives. *Crit Rev Microbiol* 44:318–335
- Tetart F, Repoila F, Monod C, Krisch HM (1996) Bacteriophage T4 host range is expanded by duplications of a small domain of the tail fiber adhesin. *J Mol Biol* 258:726–731
- Tetart F, Desplats C, Krisch HM (1998) Genome plasticity in the distal tail fiber locus of the T-even bacteriophage: recombination between conserved motifs swaps adhesin specificity. *J Mol Biol* 282:543–556
- Wais AC, Goldberg EB (1969) Growth and transformation of phage T4 in *Escherichia coli* B-4, *Salmonella*, *Aerobacter*, *Proteus*, and *Serratia*. *Virology* 39:153–161
- Yoichi M, Abe M, Miyanaga K, Unno H, Tanji Y (2005) Alteration of tail fiber protein gp38 enables T2 phage to infect *Escherichia coli* O157:H7. *J Biotechnol* 115:101–107
- Yu L, Wang S, Guo Z, Liu H, Sun D, Yan G, Hu D, Du C, Feng X, Han W, Gu J, Sun C, Lei L (2018) A guard-killer phage cocktail effectively lyses the host and inhibits the development of phage-resistant strains of *Escherichia coli*. *Appl Microbiol Biotechnol* 102:971–983