Isolation and Characterization of the Lytic *Enterococcus faecium* Phage φEc-ZZ2

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Abstract: *Infections with vancomycin-resistant Enterococcus (VRE) have been increasingly reported, especially in immunocompromised patients. Bacteriophages (phages) are often considered as potential therapeutic candidates in treating infectious diseases. To search for therapeutic phages as antimicrobial agents for the treatment of Enterococcus faecium infections, eight E. faecium phages were isolated from the environments. One of these, phage φEc-ZZ2, which was capable of eliciting efficient lysis of E. faecium, was selected for analysis. Transmission electron microscopy revealed its resemblance to members of the family Siphoviridae, with a tail 200 nm long and an icosahedral head 45 nm in diameter. Phage φEc-ZZ2 exhibited rapid adsorption (> 99% adsorbed in 10 min), a short latency period of 20 min and a large burst size of 180 PFU/cell. Host range determination shows that φEc-ZZ2 has a relatively broader host range (specificity for 54.5% of tested E. faecium strains). Furthermore, when φEc-ZZ2 alone was incubated at different pH, the phage was stable over a wide pH range (5 to 9). These characteristics of φEc-ZZ2 will provide useful information for further research on the interaction between Enterococcus phages and their hosts. Moreover, in consideration of its pH stability and broader host range, φEc-ZZ2 may be a good candidate as a therapeutic/disinfectant agent to control nosocomial infections caused by E. faecium.*

Keywords: *Bacteriophage, Phage therapy, Enterococcus faecium.*

Introduction

The frequent use of effective antibiotics has led to the development of new antibiotic-resistant pathogenic and opportunistic bacteria. Currently, with respect to the growing costs of research on new antimicrobials and increasing bacterial resistance, some researchers have turned their attention to new, convenient and inexpensive bioagents for the control of bacterial infections.

Phage therapy employs a live prokaryotic virus to infect and kill bacterial cells. This method has been documented to be a practicable alternative therapy due to its long history of successful use in East Europe [1, 5]. Recently published experimental findings reveal that phage therapy is proven superior to conventional chemotherapy for certain uses [4, 11, 21].

Enterococci are Gram-positive facultative anaerobic microorganisms which are members of the gut normal microbiota of humans and animals. They do not produce disease in their normal setting but can be the cause of dangerous infections (e.g., bacteremia, endocarditis, and intra-abdominal, wound, and urinary tract infections) when spreading into the bloodstream and tissues [6, 16]. As early as 1989, clinical isolates of vancomycin-resistant *E. faecium* (VRE) from patients in the United States were first documented [23]. *E. faecium* and *E. faecalis* are recognized as the most commonly clinically isolated *Enterococcus* sp. [23]. Individuals with compromised immune systems, such as AIDS patients, cancer patients receiving chemotherapy and radiotherapy, transplant recipients, and the elderly, are particularly prone to developing VRE infections. With respect to the threat from VRE and the increased incidence reported worldwide, the development of novel therapies and bioagents against VRE infections is of great concern. For this purpose, virulent or lytic bacteriophages are highly desirable due to their ability to kill target host cells.

In the present study, clinical isolates of *E. faecium* were collected and used as target cells to screen lytic phages from environments. A novel lytic phage (designated φEc-ZZ2) specifically infecting *E. faecium* isolates was isolated and characterized, and these basically biological features including morphology, host range spectrum, adsorption rate, one-step growth and stability were investigated.

Materials and methods

Bacterial strains and growth conditions

Twenty-two strains of *E. faecium* (non-VRE strains) were isolated at the Clinical Laboratory, Affiliated Hospital of Changchun University of Chinese Medicine between 12 February and 30 June 2018. These clinical isolates were identified by biochemical methods (API 20 Strep, bio Mérieux, Craponne, France). All bacterial strains were grown in Brain heart infusion (BHI) broth obtained from Qingdao Hope Bio-Technology Co. (Qingdao, China). Bacterial growth was monitored turbidimetrically by measuring optical density at 600 nm (OD_{600}). An OD_{600} of 0.4 corresponded to 2×10^{10} CFU/ml. For the phage plaque formation assay, solid medium (containing 1.5% agar) and semisolid medium (containing 0.5% agar) were used for the lower and upper layers, respectively.

Isolation and purification of bacteriophages

The procedure of phage isolation was conducted as the previous description [9, 22]. Twenty-two strains of *E. faecium* were chosen as the host cells for the isolation of lytic bacteriophages. Several specimens from raw sewage were centrifuged at 10.000×g for 15 min at 4 °C to remove debris, and the supernatants were filtered through 0.45-μm-pore-size membranes (Millipore, Bedford, MA, USA). The filtrate was mixed with a fresh bacterial culture in BHI at 37 °C overnight. The mixture was centrifuged at 10.000×g for 15 min and filtered through a 0.4-μm-pore-size membrane (Millipore, Bedford, MA, USA) to obtain the initial phage stock. Plaque formation was observed using the standard double-layer agar methods. A pure phage stock was achieved through purified plaque using three consecutive rounds of single-plaque isolation. Finally, phage stocks were stored in SM buffer (10 mM $MgSO_4$, 100 mM NaCl, 0.01% gelatin, and 50 nM Tris/HCl, pH 7.5) at 4 °C.

Lytic analysis of phage

The lytic range of phage was tested using spot assay. Briefly, a log-phase bacterial culture was added to 5 ml of molten BHI (containing 0.5% agar), and the mixture was overlaid on BHI plates (containing 1.5% agar). After waiting 15 min to allow the molten BHI to solidify, phage suspensions were spotted on the surface of the plates, which were incubated for 16 h at 37 °C. The bacterial lawn was examined for clear zones.

Transmission electron microscopy (TEM)

Twenty ml of phage suspension (10^{10} PFU/ml) were placed on a 400-mesh-size copper grid coated with formvar-carbon film for 15 min. Subsequently, the sample was negatively stained with 2% uranyl acetate. Images were obtained using TEM (Hitachi System, Japan) at an acceleration voltage of 80 kV.

Extraction of phage DNA and restriction analysis

The genomic DNA of phage particles was extracted from phage suspension using standard phenol/chloroform extraction protocols [18] with minor modifications. Phage stock solution was incubated with 5 μg/ml *DNase I* and 1 μg/ml *RNase A* at 37 °C for 2 h. The mixture was treated with 20 mM EDTA, 0.5% SDS and 50 μg/ml proteinase K, and then incubated at 56 °C for 1 h. An equal volume of phenol was added to the mixtures. Following centrifugation (5.000×g for 10 min), the aqueous layer was removed to a tube containing an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 6.000×g for 10 min. The aqueous layer was collected and mixed with 3M NaAc and ethanol at –20 °C overnight. The mixture was then centrifuged (10.000×g) at 4 °C for 15 min, and the precipitated DNA was washed with 75% ethanol. Finally, the genomic DNA was solubilized in deionized water. The extracted DNA of phages was treated with the following ten restriction enzymes, *Hind* III, *EcoR* V, *Nde* I, *Pst* I, *Bgl* II, *Xba* I, *Not* I, *EcoR* I, *Xho* I, and *Sac* II, respectively.

Adsorption rate and one-step growth assay

Procedures for the adsorption rate were performed as described previously [14]. Briefly, phage particles were mixed with 10 ml *E. faecium* in the mid-exponential phase. The ratio between phage to host cell was 0.01 based on multiplicity of infection (MOI). The mixture was incubated at 37 °C at 150 rpm. At 1 min intervals for 10 min, 100 μl of the sample was mixed with 450 μl SM buffer (50 mM Tris-Cl, pH 7.5, containing 0.58% NaCl and 0.2% $MgSO_4$) and centrifuged at 10.000×g for 6 min. The percentage of unabsorbed phages in each sample was measured by the double-layer method.

For one-step growth curve experiments, 50 ml *E. faecium* was incubated to the mid-exponential phase (6×10^8 CFU/ml) and harvested by centrifugation. The pellet was resuspended in 20 ml of fresh BHI medium. Phage solution was added to the bacterial suspension at a MOI of 0.01. This mixture was incubated at 37℃ for 5 min to allow adsorption. After 5 min, the mixture was centrifuged at 5.000×g for 5 min, the supernatant was removed and the pellets were resuspended in BHI broth. Subsequently, 100 μl samples were taken at 5 min intervals and phage titer was determined by the double-layer method. Finally, a one-step growth curve was deduced according to the constant phage titer.

SDS-page analysis of phage proteins

Phage proteins were analysed using SDS-polyacrylamide gel electrophoresis (PAGE). The phage solution was subjected to an Amicon-100 filter, and the phage particles were washed three times with SM buffer. Purified phage particles were mixed with sample buffer

and heated in a boiling water bath for 6 min. A volume of purified viral particles corresponding to 10^{10} PFU/ml was loaded directly onto a 12.5% SDS-PAGE for 2 h. Protein bands were visualized by staining the gels with 0.1% Coomassie brilliant blue and documented using a gel image system.

pH stability test

Resistance to different pH values at 37 °C was carried out as previously documented [8]. Briefly, the pH of the BHI broth was adjusted with either 1 M HCl or 0.5 M NaOH to obtain a pH ranging from 1 to 14. A total of 100 µl of bacteriophage suspension (5×10^8 PFU/ml) was inoculated into 5 ml of pH-adjusted medium. After incubation for 1 h at 37 °C the surviving phage particles were counted immediately using the double-layer method.

Statistical analysis

All assays were performed in triplicate and data were expressed as means and standard deviation values.

Results

Isolation of lytic phage φEc-ZZ2 and its host range

Eight phages were isolated from raw sewage using 22 *E. faecium* isolates as hosts. A lytic bacteriophage, named φEc-ZZ2, was chosen for host range test and basically biological analysis. Plaques of phage φEc-ZZ2 were about 4-5 mm in diameter. Host range test showed that φEc-ZZ2 could infect 12 out of 22 *E. faecium* isolates tested, suggesting that φEc-ZZ2 have a relatively broader host range (specificity for 54.5% of tested hosts, Table 1). However, φEc-ZZ2 did not infect two *E. faecalis* strains.

Morphologic attributes of phages φEc-ZZ2

Images of phage φEc-ZZ2 were developed using TEM. As shown in Fig. 1, φEc-ZZ2 has an icosahedral head and a long, flexible tail. The head diameter was about 50 nm. The flexible tail length was 200 nm. Based on these morphological characteristics, phage φEc-ZZ2 was assigned to the family *Siphoviridae*, order *Caudovirales* according to the classification of Ackerman [2].

Restriction analysis of phage φEc-ZZ2 genome

Purified viral DNA was tested with ten restriction endonucleases and subsequently subjected to electrophoresis analysis. As shown in Fig. 2, the DNA genome of phage φEc-ZZ2 was digested with *Hind* III, *EcoR* V, *Nde* I, *Xba* I and *EcoR* I, respectively. However, the genome of φEc-ZZ2 appeared resistant to digestion by *Pst* I, *Bgl* II, *Not* I, *Xho* I and *Sac* II. Moreover, the genome of φEc-ZZ2 was completely digested with *DNase* I, while it remained intact with *RNase*A treatment (data not shown), which indicated that phage φEc-ZZ2 was a double-stranded DNA phage.

Adsorption rate and one-step growth curve of phage φEc-ZZ2

The phage exhibited rapid adsorption onto the host cells as shown in Fig. 3. More than 90% of the phage particles were adsorbed in 9 min. The latent period and burst size are the parameters used to measure phage infectivity. The one-step growth curve revealed a latent period (defined as the time interval between the adsorption and the beginning of the first burst) of 20 min and a burst size of 180 PFU/infected cells (Fig. 4).

Table 1. Lysis range analysis of phage φEc-ZZ2. Phage φEc-ZZ2 was tested for host range: lysis (+), no lysis (–). ID: Number used to distinguish between different bacteria.

Species	ID	Source	lysis
E. faecium	ZZ1	Infectious specimen	+
E. faecium	ZZ2	Infectious specimen	+
E. faecium	ZZ3	Infectious specimen	+
E. faecium	ZZ4	Infectious specimen	+
E. faecium	ZZ5	Infectious specimen	–
E. faecium	ZZ6	Infectious specimen	–
E. faecium	ZZ7	Infectious specimen	–
E. faecium	ZZ8	Infectious specimen	+
E. faecium	ZZ9	Infectious specimen	–
E. faecium	ZZ10	Infectious specimen	–
E. faecium	ZZ11	Infectious specimen	–
E. faecium	ZZ12	Infectious specimen	+
E. faecium	ZZ13	Infectious specimen	+
E. faecium	ZZ14	Infectious specimen	–
E. faecium	ZZ15	Infectious specimen	+
E. faecium	ZZ16	Infectious specimen	–
E. faecium	ZZ17	Infectious specimen	–
E. faecium	ZZ18	Infectious specimen	+
E. faecium	ZZ19	Infectious specimen	+
E. faecium	ZZ20	Infectious specimen	–
E. faecium	ZZ21	Infectious specimen	+
E. faecium	ZZ22	Infectious specimen	+
E. faecalis	CC4	Infectious specimen	–
E. faecalis	CC6	Infectious specimen	–

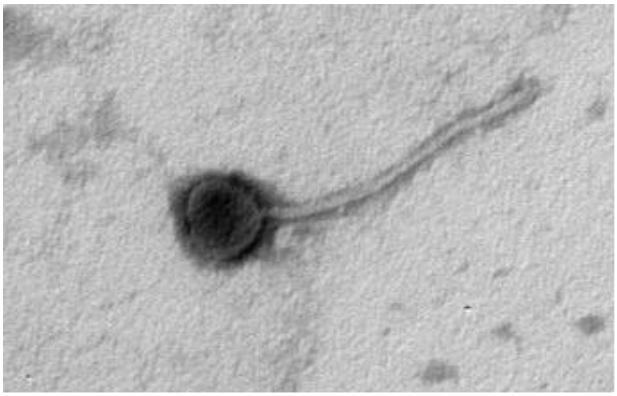

Fig. 1 Transmission electron micrographs of negatively stained phage φEc-ZZ2 (*Bar* corresponds to 100 nm)

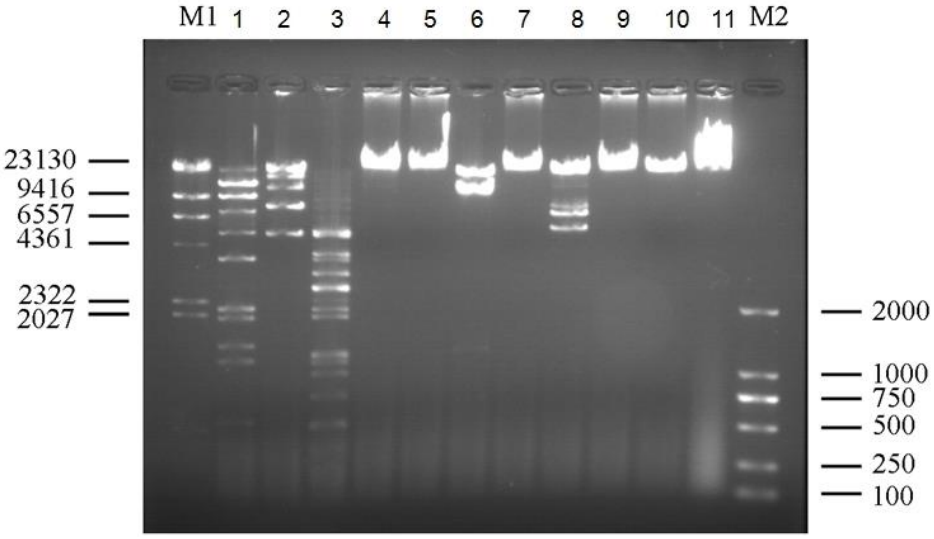

Fig. 2 Restriction analysis of phage φEc-ZZ2 genome (M1 and M2: DNA marker, Lane 1: *Hind* III, Lane 2: *EcoR* V, Lane 3: *Nde* I, Lane 4: *Pst* I, Lane 5: *Bgl* II, Lane 6: *Xba* I, Lane 7: *Not* I, Lane 8: *EcoR* I; Lane 9: *Xho* I; Lane 10: *Sac* II; Lane 11: phage genome).

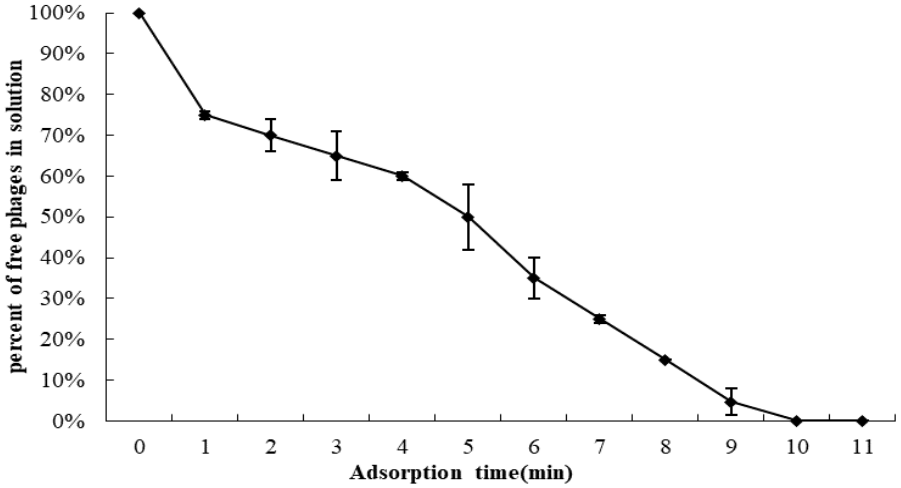

Fig. 3 Adsorption of phage φEc-ZZ2 to *E. faecium*

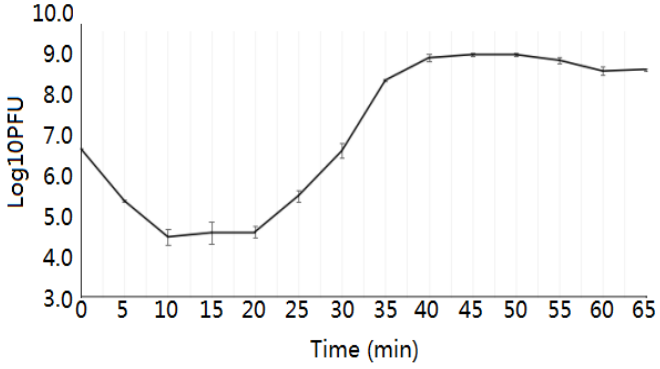

Fig. 4 One-step growth curve of phage φEc-ZZ2 on *E. faecium*

Analysis of phage proteins by SDS-PAGE

SDS-PAGE analysis of the phage φEc-ZZ2 revealed that two major proteins (with molecular weight of 34 kDa and 20 kDa, respectively) and three minor proteins (with molecular weight ranging from 41-110 kDa) were observed on the gel (Fig. 5). A major protein, with a molecular weight of 34 kDa, might be coincident with the major capsid protein of *E. faecium* bacteriophage φEc-ZZ2, another one, with an approximate molecular weight of 20 kDa, probably constituted tail protein of phage φEc-ZZ2.

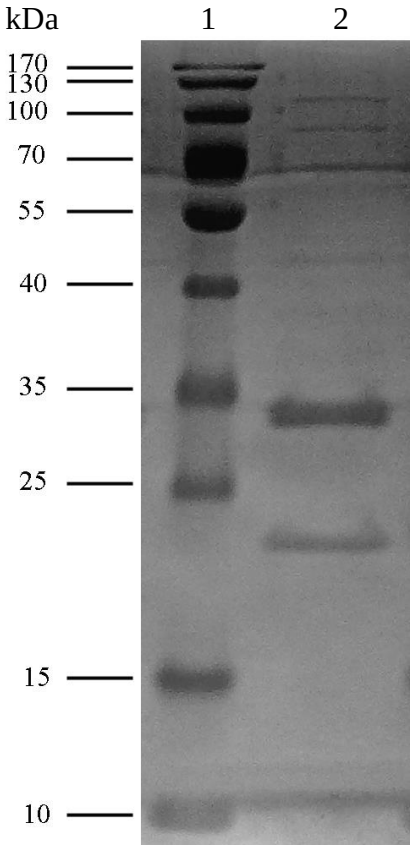

Fig. 5 SDS-PAGE analysis of phage structural proteins:
1. Protein ladder; 2. Phage φEc-ZZ2

Stability investigation

As shown in Fig. 6, no obvious effect on phage φEc-ZZ2 activity was observed after 1 h of incubation at pH levels ranging from 5 to 10. However, the phage φEc-ZZ2 completely lost its activity at pH 11 or higher and pH 3 or lower. When incubated at pH 4 for 1 h, a titer of 7.5×10^4 PFU/ml of active phage φEc-ZZ2 was detected at the end of the incubation. The moderate stability of the phage was observed at pH 5.0, 6.0, 7.0, 8.0, and 9.0.

Discussion

Phage therapy has been discussed in recent reviews [7, 15] due to the emergence of drug-resistant pathogens. In the search for bacteriophages to control infectious diseases caused by vancomycin-resistant *E. facecium*, Biswas et al. [3] demonstrated that ENB6, an *Enterococcus* phage, can protect mice against infection with 10^9 bacteria using 3×10^8 PFU/ml, however, the information on the biology of *E. faecium* phages is relatively minimal.

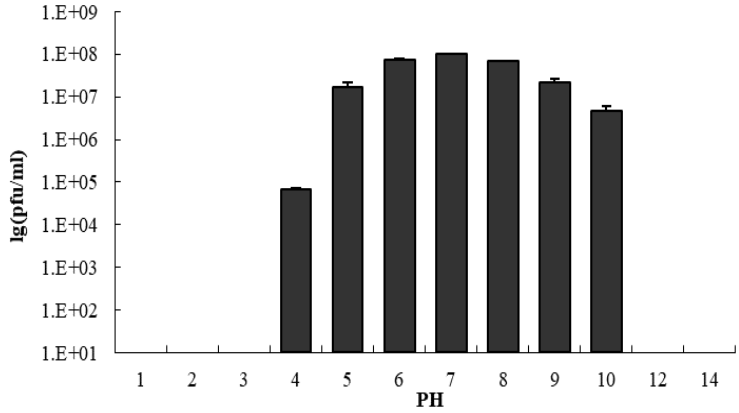

Fig. 6 pH stability test of phage φEc-ZZ2

In this study, we focused our efforts on the isolation and characterization of *E. faecium* phages with potential for therapeutic application. Recently, two phages (IME-EFm1 and IME-EFm5) were obtained from the environment using clinical isolates of *E. faecium* as indicator hosts [12, 24]. Examination by transmission electron microscopy revealed that phage IME-EFm1 and IME-EFm5 belonged to the family *Siphoviridae*. Similarly, the phage φEc-ZZ2 exhibits the same morphological features as those of phages IME-EFm1 and IME-EFm5, which are tailed bacteriophages with icosahedral heads and long flexible tails.

With respect to host ranger determination, we found that phage φEc-ZZ2 could infect 12 of 22 *E. faecium* isolates and did not infect *E. faecalis*, suggesting the phage φEc-ZZ2 exhibits a broader host range and its specificity to *E. faecium*. Adsorption rate and one-step growth curve are the parameters used to measure phage infectivity. The latent period and burst size for tailed phages are usually 40-60 min and 50-100 min, respectively [19]. The adsorption rate for φEc-ZZ2 showed that more than 90% of the phage particles were adsorbed in 9 min. A one-step growth curve for φEc-ZZ2 demonstrated a latent period of 20 min, and an average burst size of 180 min. Furthermore, optimal pH was determined by examining the stability of phage φEc-ZZ2 under different pH.

Our experiment showed that almost no reduction of active phage φEc-ZZ2 was observed after one hour of incubation at pH 6.0, 7.0 and 8.0, and the moderate stability of the phage was observed at pH 5.0, 6.0, 7.0, 8.0, and 9.0. Considering these, phage φEc-ZZ2 is promising for possible applications in the eradication of *E. faecium* contamination or treatment of *E. faecalis* infections. However, the use of single phages is usually ineffective because many phages have a narrow host range when used alone. To overcome this limitation, the phage cocktail [17] and phage-derived lysins [10, 13, 20] are considered as promisingly effective agents for biocontrol purposes. Therefore, further study about *E. faecium* phages will be focused on developing the phage cocktail and phage-derived lysins to control vancomycin-resistant *Enterococcus* infections.

Conclusion

E. faecium is inherently resistant to several antibiotics due to its ability to acquire resistance to antibiotics, either by mutation or by receipt of foreign DNA via the transfer of plasmids and transposons. Therapeutic difficulties presented by *E. faecium* have become a threat in both community and nosocomial settings. Phage therapy utilizes a live bacterial virus as a bioagent to kill pathogenic bacteria and is predicted to be a promising alternative therapy against drug-resistant bacterial infections, including drug-resistant *E. faecium*.

In this study, we successfully isolated a lytic phage, designated φEc-ZZ2, specifically infecting *E. faecium* isolates. Biological analysis revealed that φEc-ZZ2 presented a relatively broader host range among *E. faecium* stains, a latent time of 20 min a large burst size of 180 PFU/infected cell, and pH stability. These characteristics mean that φEc-ZZ2 has significant potential for use in the prophylaxis of *E. faecium* infections. Moreover, the therapeutic effectiveness of φEc-ZZ2 must also be examined *in vivo* because bactericidal effectiveness differs *in vitro* and *in vivo.*

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