Isolation of a lytic bacteriophage vB_EfaS_PHB08 and its endolysin lys08 against Enterococcus faecalis biofilm

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Research

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Abstract

Background: *Enterococcus faecalis* is an opportunistic pathogen can cause a variety of diseases, such as urinary tract infections and wound infections in human and animals. Recently, bacteriophages and their derivatives represent a good effect on fighting against bacterial infections.

Methods: We isolated virulent bacteriophages of *E. faecalis* using the double-layer plate method. The bioactivities of the phage isolated were determined via one-step growth curve testing and bacterial killing assays. Illumina HiSeq sequencing was performed to determine the genetic characteristics and the lysins of the phage. Protein expression and antibiofilm assays were also performed to highlight the bioactivities of the phage lysins.

Results: We isolated a virulent bacteriophage vB_EfaS_PHB08 (thereafter PHB08) from the sewage nearby hospital. PHB08 possessed a linear double-stranded DNA genome with 55,244 bp in length, which encoded 91 putative coding sequences (CDS). We found that PHB08 could inhibit the growth of host bacteria for 12 h. In vegetable models, PHB08 can reduce $1 \times 10^5$ Colony Forming Units (CFU) of *E. faecalis* per square centimeter at room temperature (25 °C) for 24 h. In addition, PHB08 and its endolysin can remove the biofilm formed by *E. faecalis*.

Conclusions: A virulent phage and endolysin displayed a good effect on reducing and/or eradicating *E. faecalis* infection and biofilm.

Background

The Gram-positive pathogenic bacteria *Enterococcus faecalis* is the causative agent of endocarditis, sepsis and meningitis in both animals and humans [1–3]. It has been reported that *E. faecalis* is the third most common pathogen that is associated with hospital-acquired infections and it accounts for 15% of catheter-associated urinary tract infections (CAUTI) and 5–15% of infective endocarditis (IE) cases [4–6]. *E. faecalis* strains are also closely associated with many hard-to-treat persistent interradicular infections, suggesting that *E. faecalis* plays a role in the pathogenesis of dental disease [7, 8]. In most cases, treatment of *E. faecalis* infections relies on the use of antimicrobials. However, due to the frequency of use of antibacterial drugs, the resistance of *E. faecalis* clinical isolates is increasing, suggesting a worrisome condition of using antimicrobials continuously [9–11]. Moreover, different genotypes of *E. faecalis* play a role in spreading drug resistance genes, which further promotes the clinically difficult treatment of *E. faecalis* infections [12].

During the infection, *E. faecalis* strains always form single and mixed-species biofilms on both tissue and medical devices in the host, often under exposure to fluid flow, giving rise to infections that are recalcitrant to treatment [13]. Research showed that produce biofilm of *E. faecalis* is related with clinical disease [14]. Formation of biofilms confers the bacteria capacity to escape the killing of antibiotics and the elimination of the host immune system [15]. Therefore, eradication of biofilms formed by *E. faecalis* during the infection is beneficial for clinical treatment.
Bacteriophages (thereafter phages) are the natural predator of bacteria and they are probably the most abundant biological entities in nature [16]. Regarding their ability to kill pathogens with high specificity, phages have been proposed as promising therapeutic tools since their discovery in 1915 [17]. Recently, *E. faecalis* phages have been found to have the potential to specifically inhibit (or kill) the reproduction and survival of *E. faecalis* as a biological control agent [18–20]. Phages and their derivates such as the lysins have also displayed a good effect on reducing and/or eradicating bacterial biofilms in addition to their effective bactericidal activity [21–23]. In this study, we isolated a virulent vB_EfaS_PHB08 (thereafter PHB08) specific infect for *E. faecalis* and constructed a recombinant plasmid expressing endolysin lys08. Further studies revealed that PHB08 as a potential biological agent in lettuce model. In addition, PHB08 and its derivative exhibited effectively for removing *E. faecalis* biofilms.

**Methods**

**Bacterial strains and cultural conditions**

*E. faecalis* strain EF3964 was recovered from a patient with urinary tract infections. It grows well on tryptic soy agars (TSA; Becton, Dickinson and company, MD, USA) and/or in tryptic soy broth (TSB; Becton, Dickinson and company, MD, USA) at 37° C for 16 h.

**Phage isolation and purification**

Phages against *E. faecalis* were isolated from sewages using *E. faecalis* strain EF3964 as an indicator by a double-layer plate methodology, as described previously [24]. In briefly, sewage samples from the hospital sewage were centrifuged at 4,000 × g for 10 min. The supernatants were harvested and were filtered using a 0.22 µm membrane to remove bacteria. After that, 300 µl of the filtrates were mixed with 300 µl of the bacterial culture of EF3964 at mid-log phase, and the mixture incubated using a double-layer TSA plate 37 °C for 12 h to form the phage plaques. Presumptive single plaque was picked and was resuspended in 6 ml of sterile SM buffer (5.8 g of NaCl, 2.0 g of MgSO₄·7H2O, 50 ml of Tris-HCl [pH7.4], 5.0 ml of 2% gelatin). The phage-containing SM buffer was then centrifuged at 12,000 × g for 30 s and the supernatant was filtered through a 0.22 µm pore size membrane. In the next, the phage preparations were given serial 10-fold dilutions with sterile SM buffer. Finally, the phage preparations were inoculated into the indicator bacteria at mid-log phase, which was then incubated using a double-layer TSA plate 37 °C to the next cycle. Phage isolation by the double-layer agar method was repeated four more times. The phages were purified by CsCl gradient ultra-centrifugation and then stored at 4 °C [25]. The morphology of the phages was observed under transmission electron microscope (HITACHI H-7650, Japan).

**Physical parameter of phage PHB08**

For temperature sensitivity assay, 100 µl (10⁹ Plaque Forming Units, PFU) of the purified phages were treated at different temperature (4, 20, 40, 50, 60, 70, and 80 °C) for 1 h. For acid-base sensitivity assay, 100 µl of phage (10⁹ PFU) with 900 µl SM buffer of pH (3.0, 5.0, 7.0, 9.0, and 11.0), were treated at 37 °C
for 1 h. Phage titer was determined using the double-layer plate method. This experiment was repeated three times.

**One-step growth curve**

The one-step growth curve of PHB08 is determined as described previously [24]. In briefly, PHB08 with multiplicity of infection (MOI) of 0.01 was inoculated into the indicator bacteria at mid-log phase and the mixture was incubated at 37 °C for 5 min. After incubation, the mixture was centrifuged at 12,000 rotation per minute (rpm) for 30 s. The supernatant was discarded with equal volume TSB. The titer of phage was determined by double-layer plate method. This experiment was repeated three times.

**Lytic Activity of PHB08**

The lytic activity of phage PHB08 was analyzed in a 96-well microtiter plate by examining the optical density measurement method [26, 27]. Briefly, 100 µl of the mid-log phase *E. faecalis* EF3964 (6.6 × 10^7 Colony Forming Units, CFU) mixed with 100 µl of phage PHB08 of different MOI (0.001, 0.01, 0.1, 1.0, 10, 100, and 1000) was incubated at 37 °C (160 rpm). Wells with equal volume of TSB medium or Phosphate buffer saline (PBS) buffer added were used as controls. The absorbance value of resulting supernatant was measured at 590 nm using a multimode microplate reader (Tecan Spark 10M). This experiment was performed in triplicate.

**Killing assay in vegetable module**

The effect of phage PHB08 on host strain EF3964 in vegetable module was evaluate as previously described [28]. Briefly, the vegetable was sterilized with sodium hypochlorite (100 µg/ml) for 5 min. After washing with sterile water, the vegetable sample was covered evenly host strain EF3964 (10^5 CFU/cm^2) until sample dried naturally. Subsequently, phage PHB08 with different MOI values (1000, 100, 10, and 1.0) was sprayed on the vegetable leaves at 25 °C for 6, 12, and 24 h, respectively. The control group was added equal volume phosphate buffered saline (PBS; pH = 7.4). The survival of EF3964 was counted by 10-fold dilutions method. This experiment was repeated three times.

**DNA extraction and analysis of genome sequence**

The phages’ genomic DNA extracted using the phenol-chloroform protocol was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH = 8.0]) and was sequenced on an Illumina HiSeq 2,500 sequencer with 2 × 100 bp read length. The short reads were assembled into the genome by means of SOAPdenovo [29]. Open reading frames (ORFs) were predicted using Glimmer [30, 31]. The final assembled sequence was searched against the current protein and nucleotide databases (http://www.ncbi.nlm.nih.gov/) by means of the basic local alignment search tool (BLAST). Protein BLAST (BLASTP) was used to identify putative homologies and proteins sharing similarities with the predicted phage proteins. The genomes were scanned for tRNAs using tRNA scan-SE [32] and ARAGORN [33]. Phylogenetic tree was analyzed using the ClustalW program in MEGA 6.0 [34]. The complete genome sequence of PHB08 was deposited in GenBank under the accession number MK570225.

**Cloning, expression and activity identification of lys08**
The putative lysin gene lys08 of phage PHB08 was amplified by polymerase chain reaction (PCR) with specific primers (5’-CGTGTGTCACATACCTGAATTG-3’; 5’-GCAGTAACAGCCATTCTCATCTATG-3’), and then was cloned into the expression vector pET-28a, generating pET-lys08, which was finally transformed into the expressed strain BL21 (DE3). Single colonies of BL21 were picked and inoculated into TSB medium containing 50 µg/ml of kanamycin, incubated overnight at 37 °C. After that, bacterial culture was transferred into fresh TSB containing 50 µg/ml of kanamycin and the mixture was cultured at 37 °C until Optical Density (OD_{600}) value reaches 0.6 ~ 0.8. The expression of protein Lys08 was induced by the addition of 0.6 mM/L IPTG in the bacterial culture and further incubation at 25 °C for 16 h. Lys08 was purified by Ni-nitrilotriacetic acid column as previously described [35]. The purified protein lys08 was dialyzed in a protein preservation solution (50 mmoL/l Tris, 0.3 mol/l NaCl, pH 8.0) in 0.45 µm membrane concentrated. Purified protein was quantified by the Bradford Protein Assay Kit (Thermo Fisher Scientific) and stored at -80 °C until uses.

**Antibiofilm by phage PHB08 and its endolysins lys08**

Biofilm formation was detected using a 96-well microtiter plate as previously described [36]. In briefly, 100 µl of the overnight cultured bacterial strain EF3964 was added to each well and was incubated at 37 °C for 24 h, 48 h, or 72 h, respectively. Equal volumes of PBS (pH = 7.4) was included as controls. Each well was washed three times with sterile PBS buffer. Subsequently, 100 µl phage PHB08 (2.0·10^9 PFU/ml, 2.0·10^8 PFU/ml, 2.0·10^7 PFU/ml, and 2.0·10^6 PFU/ml) with TSB medium or 100 µl lys08 (50 µg and 100 µg) was added to every well at 37 °C for 4 h. After incubation, 1% crystal violet solution was added 100 µl to each well at 37 °C for 15 min, then washed three times with sterile PBS buffer until the liquid has no more crystal violet color. Finally, 150 µl of 33% acetic acid added to each well. The absorbance value of resulting supernatant was measured at 600 nm as mentioned above. This experiment was repeated three times.

**Statistical Analysis**

Student’s unpaired t-test was used for statistical significance when comparing results for two groups; while ordinary one-way analysis of variance (ANOVA) with post-hoc analysis by Dunnett’s test was used when comparing the results of more than two groups. Data are present as “Mean ± SD”. Differences were considered statistically significant if $P < 0.05$ (∗). All statistical analyses were performed using GraphPad Prism software.

**Results**

**Microbiological characteristics of phage PHB08**

The isolated phage PHB08 had a clear, translucent, uniform size plaque on a double-layer agar plate (Fig. 1a). Under the observation of electron microscope, phage PHB08 had a rectangular head (length 124 mm ± 5, width 61 mm ± 5) and a long tail (158 mm ± 5) (Fig. 1b). Based on these morphological...
characteristics and according to the latest International Committee on Taxonomy of Viruses (ICTV) classification, PHB08 was determined as a member of the family *Siphoviridae*.

**Phenotypic parameters of phage PHB08**

Phage PHB08 was treated in different pH (3.0–11.0) at 37 °C for 1 h and treated in different temperature (4–80 °C for 1 h. The Acid-base tolerance results exhibited that the activity of PHB08 is relatively stable between 5.0–11.0 (Fig. 2a). Temperature tolerance results showed that the titer of phage PHB08 is quite stable between 4 °C and 60 °C (Fig. 2b). PHB08 was treated at 70 °C for 40 min or at 80 °C for 20 min, no titer was observed (Fig. 2b). The one-step growth curve of PHB08 showed that PHB08 has a latency of 20 min and a high-speed growth period of 40 min with an average burst size of 64 phage particles per infected (Fig. 2c). The host range assays indicated that phage PHB08 specifically infected 15 out of 19 *E. faecalis* clinical isolated, but not infecting other species including *Enterococcus faecium* (Table 1).
Table 1  
The host range of PHB08.

| Strains                  | Isolated locations | Plaque formation |
|--------------------------|--------------------|------------------|
| *Enterococcus faecalis* EF3964 (Host Strain) | Hubei, China       | +                |
| *Enterococcus faecalis* EF1833 | Hubei, China       | +                |
| *Enterococcus faecalis* EFHB01 | Hubei, China       | +                |
| *Enterococcus faecalis* EFHB02 | Hubei, China       | +                |
| *Enterococcus faecalis* EFHB03 | Hubei, China       | +                |
| *Enterococcus faecalis* EFHB04 | Hubei, China       | -                |
| *Enterococcus faecalis* EF1067 | Anhui, China       | +                |
| *Enterococcus faecalis* EF7011 | Anhui, China       | +                |
| *Enterococcus faecalis* EFhn15 | Hunan, China       | +                |
| *Enterococcus faecalis* EFhn20 | Hunan, China       | -                |
| *Enterococcus faecalis* FJ1801 | Fujian, China      | +                |
| *Enterococcus faecalis* Hu6 | Unknown            | +                |
| *Enterococcus faecalis* Hu7 | Unknown            | +                |
| *Enterococcus faecalis* Hu8 | Unknown            | -                |
| *Enterococcus faecalis* XN01 | Shanxi, China      | +                |
| *Enterococcus faecalis* XN02 | Shanxi, China      | +                |
| *Enterococcus faecalis* 20170714-1 | Unknown            | +                |
| *Enterococcus faecalis* 20170714-2 | Unknown            | +                |
| *Enterococcus faecalis* 20170714-3 | Unknown            | -                |
| *Enterococcus faecium* HBM101 | Hubei, China       | -                |
| *Enterococcus faecium* HBM1-2 | Hubei, China       | -                |
| *Enterococcus faecium* 14409 | Beijing, China     | -                |
| *Enterococcus faecium* STT-03 | Hainan, China      | -                |

NOTE: (+) indicates that plaques were observed; (-) indicates no plaques were observed.
Enterococcus faecium STT-04 Hainan, China -
Enterococcus faecium 21905 Hubei, China -
Enterococcus faecium 180724 Unknow -
Enterococcus faecium 180801 Unknow -
Enterococcus faecium SX11 Shanxi, China -
Enterococcus faecium HN12 Hainan, China -
Escherichia coli 18701 Hubei, China -
Escherichia coli 14997 Hubei, China -
Escherichia coli 0157:H7 Hubei, China -
Escherichia coli DH5α Hubei, China -
Salmonella 268 Hubei, China -
Salmonella 140411 Hubei, China -
Salmonella 1003 Hubei, China -

NOTE: (+) indicates that plaques were observed; (-) indicates no plaques were observed.

Features of phage PHB08 genome

The complete genome of phage PHB08 was linear double-stranded DNA genome of 55,244 bp, with a G + C content of 40% (Fig. 3). The genome of PHB08 was predicted 91 putative CDS (gene annotation for each CDS of PHB08 shown in Table 2), and one tRNA (Trp-CCA) prediction. A BLASTn search revealed that the PHB08 was closely related to phage vB_EfaS_IME198 (GenBank accession no. KT932699.1; 96.18% identity, 89% coverage) and vB_EfaS_HEf13 (GenBank accession no. MH618488.1; 95.95% identity, 84% coverage). The phage PHB08 genome contains genes that encode the structure and assembly protein of phage PHB08, including tail fibers (CDS61), tail length tape-measure protein (CDS62), major capsid protein (CDS71), portal protein (CDS74), and terminase large subunit (CDS75). Encoding DNA replication and regulation modules such as DNA binding protein (CDS6), DNA polymerase I (CDS22), adenylate kinase and related kinases (CDS35), HNH homing endonuclease (CDS39, CDS58, and CDS87), replicative DNA helicase (CDS41), DNA replication protein (CDS42), and DNA primase (CDS44). Genes encoding endolysin protein were found in the PHB08, such as phage lysin (CDS59), with 96% amino acid sequence identity to the CHAP domain protein of Enterococcus phage Entf1. No lysogeny associated gene and encoding known antibiotic resistance were predicted [37]. Phylogenetic tree analysis the amino acid sequence of the major capsid protein (CDS71) and terminase large subunit (CDS75) indicated that PHB08 belongs to Saphexavirus, Siphoviridae family (Fig. 4).
Table 2
Gene annotation for each CDS of PHB08.

| CDS | Start | Stop | Length (bp) | Size (aa) | Function                  | Accession numbers | % identity | E value |
|-----|-------|------|-------------|-----------|---------------------------|-------------------|------------|---------|
| 1   | 151   | 17   | 135         | 44        | hypothetical protein      | -                 | -          | -       |
| 2   | 603   | 229  | 375         | 124       | hypothetical protein      | -                 | -          | -       |
| 3   | 982   | 596  | 387         | 128       | hypothetical protein      | -                 | -          | -       |
| 4   | 1406  | 108  | 324         | 107       | hypothetical protein      | -                 | -          | -       |
| 5   | 2683  | 217  | 513         | 170       | hypothetical protein      | -                 | -          | -       |
| 6   | 2991  | 268  | 306         | 101       | Phage DNA binding protein | YP_009603915.1    | 99.01%     | 2e-71   |
| 7   | 3259  | 299  | 267         | 88        | hypothetical protein      | -                 | -          | -       |
| 8   | 3479  | 325  | 228         | 75        | hypothetical protein      | -                 | -          | -       |

NOTE: (−) indicates that no analysis or no results.
| CDS | Start | Stop | Length (bp) | Size (aa) | Function | Accession numbers | % identity | E value |
|-----|-------|------|-------------|-----------|----------|------------------|------------|---------|
| 9   | 3722  | 351 3| 210         | 69        | hypothetical protein | --         | --       | --      |
| 10  | 3947  | 373 5| 213         | 70        | hypothetical protein | --         | --       | --      |
| 11  | 4207  | 394 7| 261         | 86        | hypothetical protein | --         | --       | --      |
| 12  | 4398  | 420 4| 195         | 64        | hypothetical protein | --         | --       | --      |
| 13  | 4895  | 447 6| 420         | 139       | hypothetical protein | --         | --       | --      |
| 14  | 5109  | 490 9| 201         | 66        | hypothetical protein | --         | --       | --      |
| 15  | 5707  | 512 0| 588         | 195       | hypothetical protein | --         | --       | --      |
| 16  | 6053  | 573 0| 324         | 107       | hypothetical protein | --         | --       | --      |

NOTE: (--) indicates that no analysis or no results.
| CDS | Start | Stop   | Length (bp) | Size (aa) | Function               | Accession numbers | % identity | E value |
|-----|-------|--------|-------------|-----------|------------------------|-------------------|------------|---------|
| 17  | 6259  | 6023   | 237         | 78        | hypothetical protein    | --                | --         | --      |
| 18  | 6521  | 6243   | 279         | 92        | hypothetical protein    | --                | --         | --      |
| 19  | 7275  | 6580   | 696         | 231       | hypothetical protein    | --                | --         | --      |
| 20  | 7663  | 7268   | 396         | 131       | hypothetical protein    | --                | --         | --      |
| 21  | 8120  | 7665   | 456         | 151       | hypothetical protein    | --                | --         | --      |
| 22  | 10726 | 8195   | 2532        | 843       | DNA polymerase I (EC 2.7.7) | YP_009218931.1    | 98.3%    | 0.0     |
| 23  | 10991 | 10806  | 186         | 61        | hypothetical protein    | --                | --         | --      |
| 24  | 11232 | 11005  | 228         | 75        | hypothetical protein    | --                | --         | --      |

NOTE: (−) indicates that no analysis or no results.
| CDS | Start | Stop  | Length (bp) | Size (aa) | Function | Accession numbers | % identity | E value |
|-----|-------|-------|-------------|----------|----------|-------------------|------------|---------|
| 25  | 115   | 11232 | 354         | 117      | hypothetical protein | -          | -        |
|     | 85    |       |             |          |          |                   |            |         |
| 26  | 117   | 11586 | 213         | 70       | hypothetical protein | -          | -        |
|     | 98    |       |             |          |          |                   |            |         |
| 27  | 120   | 11801 | 210         | 69       | hypothetical protein | -          | -        |
|     | 10    |       |             |          |          |                   |            |         |
| 28  | 122   | 12007 | 225         | 74       | hypothetical protein | -          | -        |
|     | 31    |       |             |          |          |                   |            |         |
| 29  | 124   | 12245 | 195         | 64       | hypothetical protein | -          | -        |
|     | 39    |       |             |          |          |                   |            |         |
| 30  | 126   | 12441 | 180         | 59       | hypothetical protein | -          | -        |
|     | 20    |       |             |          |          |                   |            |         |
| 31  | 130   | 12820 | 207         | 68       | hypothetical protein | -          | -        |
|     | 26    |       |             |          |          |                   |            |         |
| 32  | 131   | 13038 | 117         | 38       | hypothetical protein | -          | -        |
|     | 54    |       |             |          |          |                   |            |         |

**NOTE:** (−) indicates that no analysis or no results.
| CDS | Start | Stop | Length (bp) | Size (aa) | Function | Accession numbers | % identity | E value |
|-----|-------|------|-------------|-----------|----------|-------------------|------------|---------|
| 33  | 137   | 13157| 564         | 187       | hypothetical protein | --         | --        | --      |
|     | 20    |      |             |           |          |                   |            |         |
| 34  | 144   | 13812| 633         | 210       | hypothetical protein | --         | --        | --      |
|     | 44    |      |             |           |          |                   |            |         |
| 35  | 150   | 14437| 570         | 189       | adenylate kinase and related kinases | --         | --        | --      |
|     | 06    |      |             |           |          |                   |            |         |
| 36  | 154   | 15003| 435         | 144       | hypothetical protein | --         | --        | --      |
|     | 37    |      |             |           |          |                   |            |         |
| 37  | 159   | 15574| 330         | 109       | hypothetical protein | --         | --        | --      |
|     | 03    |      |             |           |          |                   |            |         |
| 38  | 169   | 15903| 102         | 342       | hypothetical protein | --         | --        | --      |
|     | 31    |      |             |           |          |                   |            |         |
| 39  | 173   | 16924| 441         | 146       | HNH homing endonuclease | NP_389885.1 | 28.4%     | 1e-05   |
|     | 64    |      |             |           |          |                   |            |         |

NOTE: (−) indicates that no analysis or no results.
| CDS | Start | Stop  | Length (bp) | Size (aa) | Function | Accession numbers | % identity | E value |
|-----|-------|-------|-------------|----------|----------|-------------------|------------|---------|
| 40  | 176   | 17437 | 225         | 74       | hypothetical protein |                   |           |         |
| 41  | 190   | 17676 | 136         | 454      | Replicative DNA helicase (Dn aB) | NP_719448.1 | 24.3     | 8e-04   |
| 42  | 198   | 19052 | 777         | 258      | DNA replication protein | YP_009036407.1 | 99.6     | 0.0      |
| 43  | 202   | 19877 | 354         | 117      | hypothetical protein | --              | --        | --      |
| 44  | 212   | 20305 | 948         | 315      | DNA primase | --              | --        | --      |
| 45  | 214   | 21264 | 189         | 62       | hypothetical protein | --              | --        | --      |
| 46  | 216   | 21452 | 159         | 52       | hypothetical protein | --              | --        | --      |
| 47  | 218   | 21607 | 255         | 84       | hypothetical protein | --              | --        | --      |

NOTE: (−) indicates that no analysis or no results.
| CDS | Start | Stop | Length (bp) | Size (aa) | Function | Accession numbers | % identity | E value |
|-----|-------|------|-------------|----------|----------|-------------------|------------|---------|
| 48  | 222   | 21861| 393         | 130      | hypothe cial prot ein | -          | -        | -       |
|     | 53    |      |             |          |          |                   |            |         |
| 49  | 224   | 22255| 147         | 48       | hypothe cial prot ein | -          | -        | -       |
|     | 01    |      |             |          |          |                   |            |         |
| 50  | 225   | 22373| 213         | 70       | hypothe cial prot ein | -          | -        | -       |
|     | 85    |      |             |          |          |                   |            |         |
| 51  | 234   | 23346| 126         | 41       | hypothe cial prot ein | -          | -        | -       |
|     | 71    |      |             |          |          |                   |            |         |
| 52  | 238   | 23656| 201         | 66       | hypothe cial prot ein | -          | -        | -       |
|     | 56    |      |             |          |          |                   |            |         |
| 53  | 242   | 23856| 387         | 128      | hypothe cial prot ein | -          | -        | -       |
|     | 42    |      |             |          |          |                   |            |         |
| 54  | 246   | 24245| 435         | 144      | hypothe cial prot ein | -          | -        | -       |
|     | 79    |      |             |          |          |                   |            |         |
| 55  | 255   | 24732| 825         | 274      | hypothe cial prot ein | -          | -        | -       |
|     | 56    |      |             |          |          |                   |            |         |

**NOTE:** (−) indicates that no analysis or no results.
| CDS | Start   | Stop   | Length (bp) | Size (aa) | Function                  | Accession numbers     | % identity | E value   |
|-----|---------|--------|-------------|-----------|---------------------------|------------------------|------------|-----------|
| 56  | 26060   | 26284  | 225         | 74        | hypothetical protein       | --                     | --         | --        |
| 57  | 26286   | 26714  | 429         | 142       | hypothetical protein       | --                     | --         | --        |
| 58  | 26732   | 27292  | 561         | 186       | HNH homing endonuclease   | QDB70581.1             | 97.8%      | 2e-138    |
| 59  | 28035   | 27319  | 717         | 238       | Phage lysin               | --                     | --         | --        |
| 60  | 31337   | 28110  | 322         | 107       | Phage minor structural protein | QBZ69423.1             | 76.4%      | 0.0       |
| 61  | 35341   | 31349  | 399         | 133       | Phage tail fibers         | YP_009004020.1         | 87.1%      | 0.0       |
| 62  | 38240   | 35355  | 288         | 961       | Phage tail length tape measure protein | YP_009218894.1         | 98.9%      | 0.0       |

NOTE: (--) indicates that no analysis or no results.
| CDS | Start | Stop | Length (bp) | Size (aa) | Function       | Accession numbers | % identity | E value |
|-----|-------|------|-------------|-----------|----------------|-------------------|------------|---------|
| 63  | 384   | 38253| 225         | 74        | hypothetical   | --                | --         | --      |
| 64  | 389   | 38488| 441         | 146       | hypothetical   | --                | --         | --      |
| 65  | 397   | 39072| 690         | 229       | hypothetical   | --                | --         | --      |
| 66  | 402   | 39782| 435         | 144       | hypothetical   | --                | --         | --      |
| 67  | 406   | 40229| 381         | 126       | hypothetical   | --                | --         | --      |
| 68  | 409   | 40594| 378         | 125       | hypothetical   | --                | --         | --      |
| 69  | 413   | 40987| 405         | 134       | hypothetical   | --                | --         | --      |
| 70  | 418   | 41451| 441         | 146       | Chitinase (EC 3.2.1.14) | AYH927 97.9 5% 2e-100 | --         | --      |

NOTE: (−) indicates that no analysis or no results.
| CDS | Start | Stop  | Length (bp) | Size (aa) | Function                          | Accession numbers | % identity | E value |
|-----|-------|-------|-------------|-----------|-----------------------------------|-------------------|------------|---------|
| 71  | 428   | 42046 | 807         | 268       | Phage major capsid protein         | YP_006488741.1    | 99.6       | 0.0     |
| 72  | 435   | 42901 | 675         | 224       | hypothetical protein               | -                 | -          | -       |
| 73  | 444   | 43686 | 756         | 251       | hypothetical protein               | -                 | -          | -       |
| 74  | 459   | 44453 | 153         | 511       | Phage portal protein               | AYH92724.1        | 99.8       | 0.0     |
| 75  | 473   | 46045 | 127         | 423       | Phage terminase, large subunit     | YP_009603889.1    | 99.5       | 0.0     |
| 76  | 476   | 47379 | 249         | 82        | hypothetical protein               | -                 | -          | -       |
| 77  | 479   | 47646 | 345         | 114       | hypothetical protein               | -                 | -          | -       |

NOTE: (−) indicates that no analysis or no results.
| CDS | Start      | Stop      | Length (bp) | Size  | Function               | Accession numbers | % identity | E value |
|-----|------------|-----------|-------------|-------|------------------------|-------------------|------------|---------|
| 78  | 48603      | 48004     | 600         | 199   | hypothetical protein    |                   | --         | --      |
| 79  | 48884      | 49198     | 315         | 104   | hypothetical protein    |                   | --         | --      |
| 80  | 49198      | 49455     | 258         | 85    | hypothetical protein    |                   | --         | --      |
| 81  | 49455      | 49847     | 393         | 131   | hypothetical protein    |                   | --         | --      |
| 82  | 49849      | 50235     | 387         | 128   | hypothetical protein    |                   | --         | --      |
| 83  | 50232      | 50492     | 261         | 86    | hypothetical protein    |                   | --         | --      |
| 84  | 50494      | 50700     | 207         | 68    | hypothetical protein    |                   | --         | --      |
| 85  | 50690      | 50992     | 303         | 100   | hypothetical protein    |                   | --         | --      |

NOTE: (−) indicates that no analysis or no results.
| CDS | Start   | Stop    | Length (bp) | Size  | Function                        | Accession numbers | % identity | E value |
|-----|---------|---------|-------------|-------|---------------------------------|-------------------|------------|---------|
| 86  | 510     | 51380   | 369         | 121   | hypothetical protein            | --                | --         | --      |
| 87  | 518     | 52582   | 759         | 252   | HNH homing endonuclease         | APU002            | 98.8%      | 0.0     |
| 88  | 526     | 53511   | 855         | 284   | hypothetical protein            | --                | --         | --      |
| 89  | 535     | 53730   | 219         | 72    | hypothetical protein            | --                | --         | --      |
| 90  | 537     | 53944   | 213         | 70    | hypothetical protein            | --                | --         | --      |
| 91  | 541     | 54262   | 141         | 46    | hypothetical protein            | --                | --         | --      |

NOTE: (--) indicates that no analysis or no results.

**Killing of PHB08 in vitro**

The effect of the PHB08 on host strain EF3964 was evaluated in liquid medium at 37 °C for 12 h. As shown in Fig. 5a, phage PHB08 showed a strong antibacterial ability at different MOI value (from 0.0001 to 100) for 12 h. After two hours, the curve of the OD\textsubscript{600nm} value showed a downward trend, implying that the host bacteria were killed by phage PHB08. The effect of phage PHB08 on host strain was evaluated in lettuce as a vegetable model at room temperature (25 °C). With higher (MOI = 1000) phage-treated, the number of living bacteria EF3964 have a significantly reduction (\(P< 0.001\)) compared to control group. With phage-treated at MOI = 1, no significantly reduction of living bacteria EF3964 was observed within 24 h (Fig. 5b).
**Antibiofilm activity**

The recombinant protein lys08 was predicted to be 26.4 kDa, which was consistent with Polyacrylamide Gel Electrophoresis (PAGE-Gel) test results (Fig. 6a). The purified lys08 was detected by the spot method. The results showed 5 µl lys08 (5 µg) can form translucent halo on the plate, suggesting bactericidal activity of lys08 (Fig. 6b). The effect of the lys08 and phage on the biofilm was evaluated using 96-well microtiter plate method. In phage challenge groups, PHB08 was able to remove the formation of biofilm in co-culture 24 h at 37 °C ($P < 0.001$) (Fig. 6c). In lys08-treated groups, obviously reduction was observed ($P < 0.001$) (Fig. 6d).

**Discussion**

In recent years, the use of bacteriophages and their derivates fighting against bacterial infections and antimicrobial resistance have received more attentions [17]. In the present study, we isolated a virulent phage PHB08 from sewages using *E. faecalis* EF3964 as the indicator bacterium. The morphology and phylogenetic tree analysis further revealed that phage PHB08 belonged to the *Siphoviridae* family. The phenotypic parameters assays showed that PHB08 had relatively good stability at temperatures between 4 °C and 60 °C, and pH between 5.0 and 9.0, the average survival rate of phages during incubation is maintained at 40–50%. Our results were consistent with those of phages including *Enterococcus* phage vB_EfaS_HEf13 [38] and *Enterococcus* phage EF-P10 [39] when they were exposed to the same temperature (4–60 °C) and acid-base (5.0–9.0). It is worthy note that PHB08 can lyse 15 of the 19 *E. faecalis* strains, with a host range of 78.9%, which has a wider lytic range than the other *E. faecalis* bacteriophages (7%-70.5%) [38, 40–42]. The average burst of PHB08 was 64 phage particles per infected, in line with the generally reported estimate of about 30–122 phage particles per infected [43, 44]. The genome sequence of phage PHB08 has the highest similarity (89–98%) with those phages (IME198, HEf13, Ef7.1, EF-P29, EF-P10, UD13, IME-EF1, SAP6, EF1c55, BC-611, Entf1, and Ef2.2) with ~ 40% G + C (Table 3). Phylogenetic analysis of the complete genome sequences of PHB08 and other representative the complete *Saphexavirus* genome. Our data showed that PHB08 was closely related to phage SAP6 (Figure S1). The results indicated those phages, which were isolated from different countries (China, USA, Japan, South Korea, Poland, Russia) may have the complex evolutionary relationship.
Table 3
Sequence information for the *E.faecalis* phages belonging to Saphexavirus subfamily used in this study.

| Name                        | GenBank     | Length   | Similarity | G + C% | ORF | tRNA | Countries     |
|-----------------------------|-------------|----------|------------|--------|-----|------|---------------|
| *Enterococcus* phage vB_EfaS_PHB08 | MK570225.1  | 55,244 b p | 100%       | 40.00% | 91  | 1    | China         |
| *Enterococcus* phage vB_EfaS_IME198 | KT932699.1  | 58,000 b p | 96.18%     | 40.02% | 95  | 0    | China         |
| *Enterococcus* phage vB_EfaS_HEf13 | MH618488.1  | 57,811 b p | 95.95%     | 40.03% | 95  | 1    | South Korea   |
| *Enterococcus* phage vB_EfaS_Ef7.1 | MK721194.1  | 58,018 b p | 94.09%     | 40.03% | 102 | 3    | The United States |
| *Enterococcus* phage EF-P29 | KY303907.1  | 58,984 b p | 96.07%     | 39.77% | 101 | 0    | China         |
| *Enterococcus* phage EF-P10 | KY472224.1  | 57,408 b p | 96.05%     | 39.82% | 127 | 0    | China         |
| *Enterococcus* phage VD13 | KJ127303.1  | 55,726 b p | 94.89%     | 40.01% | 88  | 1    | The United States |
| *Enterococcus* phage IME-EF1 | NC_041959   | 57,081 b p | 96.40%     | 40.05% | 98  | 0    | The United States |
| *Enterococcus* phage SAP6 | NC_041960   | 58,619 b p | 97.53%     | 40.00% | 44  | 0    | South Korea   |
| Name                          | GenBank    | Length     | Similarity | G + C% | ORF | tRNA | Countries |
|-------------------------------|------------|------------|------------|--------|-----|------|-----------|
| *Enterococcus* phage vB_EfaS_EF1c55 | MN103542.1 | 55,876 bp  | 89.20%     | 39.79% | 94  | 0    | Poland    |
| *Enterococcus* phage BC-611   | AB712291.1 | 53,996 bp  | 95.40%     | 40.45% | 88  | 1    | Japan     |
| *Enterococcus* phage Entf1    | MK800154.1 | 58,938 bp  | 95.24%     | 39.93% | 105 | 1    | Russia    |
| *Enterococcus* phage vB_EfaS_Ef2.2 | MK721189.1 | 58,400 bp  | 89.52%     | 39.92% | 103 | 2    | The United States |

The harm caused by foodborne pathogens has become a serious challenge to humans [45]. Researchers began to use phages as natural antimicrobials in food to kill or inhibit foodborne pathogens, thus ensuring food safety [46, 47]. A series of studies have shown that phages can be used in food safety. For example, application phages on *Salmonella* in cantaloupes resulted *Salmonella* significantly reduced [48]; With phage treated, 95% reduction the number of *Campylobacter jejuni* was observed, and also founded that *Salmonella* can be killed or inhibited by phage [49]. In our study, killing assay exhibited the host strain EF3964 can be inhibited or killed at different MOI for 12 h in medium. Potential bactericidal ability at low MOI was similar to those of the single phage 13076 and phage 14028 [26]. In vegetable model, PHB08 can kill $1 \cdot 10^5$ CFU/cm² *E. faecalis* at 25 °C for 24 h. It can be inferred that phage-based biological control methods have great potential in improving the safety of food microorganisms. *E. faecalis* with biofilm formation can provide resistance to antibiotics [50], explore the new methods of against cell biofilms is currently one of the major problems in medicine [51]. Although some research have reported of phages fighting bacterial biofilms [52, 53], only a few studies on the effects of *E. faecalis* phage endolysin on host bacteria biofilms. In our study, endolysin lys08 and phage PHB08 can effectively remove the host strain biofilm at 37 °C for 72 h ($P<0.001$), suggesting that phage and endolysin have the development potential to against the biofilm formation of *E. faecalis*.

**Conclusion**

A virulent phage of *E. faecalis* was isolated and characterized in this study. This phage displayed a high survival stability and capacity to lyse cells. *In vitro* tests revealed that PHB08 and its endolysin lys08 could remove the biofilm formed by *E. faecalis*. Overall, our study offers an option for treating *E. faecalis* infections through phage and its derivates.
Abbreviations

CDS
Coding Sequences
CFU
Colony Forming Units
CAUTI
Catheter-associated Urinary Tract Infections
TSA
tryptic soy agars
TSB
tryptic soy broth
PFU
Plaque Forming Units
MOI
Multiplicity of infection
rpm
Rotation per minute
PBS
Phosphate buffer saline
ORFs
Open Reading Frames
BLAST
Basic Local Alignment Search Tool
BLASTP
Protein BLAST
PCR
Polymerase chain reaction
OD
Optical Density
PAGE-Gel
Polyacrylamide Gel Electrophoresis

Declarations

Ethics approval and consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and materials
The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

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Author Contributions

DY and YC performed the data and drafted the main manuscript; DY, YC, ES, and LH planned and performed experiments; BW, HC, and ZP were responsible for experimental design, project management, and manuscript revision. All authors reviewed and agreed the publication of this manuscript.

Conflicts of interest

The authors declare that they have no competing interests.

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Figures

Figure 1

Morphological characteristics of phage PHB08. a. Appearance of phage PHB08 plaque on host strain EF3964. b. Electron microscopy of phage PHB08. Phage PHB08 has a rectangular head (length 124 mm ± 5, width 61 mm ± 5) and a long tail (158 mm ± 5). The scale in the right corner is 200 nm.
Figure 2

Biological characteristics of phage PHB08. a. Stability of phage PHB08 at different temperatures. b. Stability of phage PHB08 at different pH values. c. Curves for one-step growth of phage PHB08.

Figure 3

Circular genetic map of PHB08. The red part represents the distribution of the CDS region. The black part represents the total content of GC (40%). The green part represents the GC skew +, which means the GC shift on the leading chain is positive and the purple is the GC skew −.

Figure 4

Phylogenetic tree analysis of phage PHB08. a. The amino acid of terminase large subunit large terminal subunit and b. the major capsid proteins of phage PHB08 was analyzed by MEGA6.0. The neighbor-joining method was used to construct phylogenetic with a bootstrap re-sampling analysis of 1,000 replications. The numbers next to the branches are bootstrap values.
Figure 5

Lytic Activity of PHB08 in vitro. a. Killing assay in TSB medium. b. Killing assay in vegetable module. Data are expressed as the mean ± SD. Validation of the killing by CFU count of host bacteria EF3964 after 6 h, 12 h, and 24 h with or without treatment by PHB08 at MOIs of 101-104, respectively. Data are expressed as the mean ± SD. Significance was determined by ANOVA (**P < 0.001).
Antibiofilm activity. a. SDS polyacrylamide gel electrophoresis analysis of purified protein lys08. Lane 1: Unpurified protein, lane 2: Purified protein. b. The activity of PHB08 and purified lys08 was determined by spot tests. 5 μl endolysin lys08 (5 μg) spotted on the plated containing host strain EF3964 at 37°C for 12 h. c. The effect of the phage PHB08 on the biofilm and (d.) lys08 on the biofilm was evaluated using 96-well microtiter plate method. Three independent experiments were performed and data are expressed as means ± SD (n = 3). Significance was determined by ANOVA (***, P < 0.001)

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