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*Article*

# Characterization and Anti-Biofilm Activity of Lytic Enterococcus Phage vB_Efs8_KEN04 against Clinical Isolates of Multidrug-Resistant *Enterococcus faecalis* in Kenya

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**Abstract:** *Enterococcus faecalis* is a growing cause of nosocomial and antibiotic-resistant infections. Treating drug-resistant *E. faecalis* requires novel approaches. The use of bacteriophages (phages) against multidrug-resistant (MDR) bacteria has recently garnered global attention. Biofilms play a vital role in *E. faecalis* pathogenesis as they enhance antibiotic resistance. Phages eliminate biofilms by producing lytic enzymes, including depolymerases. In this study, Enterococcus phage vB_Efs8_KEN04 (ΦKEN04), isolated from a sewage treatment plant in Nairobi, Kenya, was tested against clinical strains of MDR *Enterococcus faecalis*. This phage had a broad host range against 100% (26/26) of MDR *E. faecalis* clinical isolates and cross-species activity against *Enterococcus faecium*. It was able to withstand acidic and alkaline conditions, from pH 3 to 11, as well as temperatures between -80°C and 37°C. It could inhibit and disrupt the biofilms of MDR *E. faecalis*. Its linear double-stranded DNA genome of 142,402 bp contains 238 coding sequences with a G+C content and coding gene density of 36.01% and 91.46%, respectively. Genomic analyses showed that ΦKEN04 belongs to the genus *Kochikohdavirus* in the family *Herelleviridae.* It lacked antimicrobial resistance, virulence, and lysogeny genes, and its stability, broad host range, and cross-species lysis indicate strong potential for the treatment of Enterococcus infections.

**Keywords:** bacteriophage; biofilm; *Enterococcus faecalis*; genome; multidrug resistance; phage therapy

## 1. Introduction

*Enterococcus faecalis* is a gram-positive, facultative anaerobic cocci that causes difficult-to-treat infections in the nosocomial setting [1]. It is commonly found in nature and is a part of the human intestinal microbiota, comprising less than 1% of the microbiome [2,3]. Early in its evolution, *Enterococcus faecalis* acquired traits that enabled it to become an effective nosocomial pathogen, resistant to several drugs and causing severe infections in humans. It causes many human infections, including bacteremia, soft tissue and wound infections, pneumonia, endocarditis, and urinary tract infections [4–6]. It can persist for extended periods on medical equipment, and because of its high tolerance and genetic adaptability, *E. faecalis* is a significant contaminant in the hospital environment [7]. The ability of *Enterococcus faecalis* to form biofilms is particularly concerning in clinical settings, as its biofilms form on medical equipment such as catheters and prosthetic heart valves, leading to persistent infections that exhibit increased resistance to antibiotics within the biofilm structure [8]. Biofilms are organized communities of microorganisms that attach to surfaces and are embedded in

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self-produced extracellular polymeric substances (EPS) consisting of proteins, extracellular DNA, and polysaccharides [9]. Bacterial biofilms enhance pathogenicity; for example, they contribute significantly to persistent chronic urinary tract infections (UTIs), including recurrences and relapses [10]. Existing antibiotics have limited efficacy in eliminating biofilms and are less effective in treating the growing number of MDR infections [11], prompting the exploration of phage-based therapies as promising alternatives for eradicating biofilms and treating MDR pathogens. The dramatic increase in the frequency of antibiotic therapy failures due to resistance has prompted scientists to search for novel solutions.

Bacteriophages, viruses that infect bacteria, have been investigated for the development of highly effective antimicrobials with low toxicity and minor environmental impact. Bacteriophages, known for their narrow host range, are the most represented biological entities on Earth, and their number in ecosystems is estimated to exceed $10^{31}$ [12]. Phages can eliminate biofilms by producing enzymes that prevent biofilm formation and disrupt existing biofilms [13]. Depolymerases and lysins are bacteriophage enzymes that selectively degrade biofilms' extracellular polymeric substance matrix components, enhancing the phages' access to bacterial biofilm [14].

Phages with narrow host ranges are highly specific for specific bacterial strains or species. This specificity can be advantageous when precise targeting is needed, such as treating specific bacterial infections [15]. However, this makes them less valuable when targeting a wide range of bacteria, such as when treating polymicrobial infections or during the emergence of phage resistance [16]. In this regard, a phage with a broad host range is particularly advantageous because it can target more than one bacterial strain, presumably leading to fewer treatment failures [15].

Many *Enterococcus faecalis* phages have been identified to date [17–20] and have been shown to inhibit and disrupt the biofilms of their host bacteria [21–23]. For instance, studies have shown the ability of phage EFDG1 to reduce two-week-old biofilms of *E. faecalis* V583 [24]. Additionally, a genetically engineered orthocluster VIII phage phiEf11 reduced the established biofilm of *E. faecalis* strains JH2-2 and V583, which had formed on coverslips [25]. After 24 and 48 h of incubation, a significant decrease of 10–100-fold in viable cells was observed [26]. Despite the promising nature of phage therapy in the fight against antimicrobial-resistant bacteria, a few infrequent case studies have identified certain limitations. As an illustration, a patient suffering from a *Pseudomonas aeruginosa* multidrug-resistant prosthetic vascular graft infection was treated using a cocktail of phages (PT07, 14/01, and PNM) in combination with ceftazidime-avibactam. The outcome, nonetheless, did not meet expectations. After phage treatment and without antimicrobial therapy, a new bloodstream infection, increased biofilm production, and the emergence of phage-resistant mutants in the bacterial isolate occurred, highlighting the challenges and potential risks associated with phage therapy in complex infections [27].

This study presents the genomic characterization and antibiofilm activity of *Enterococcus faecalis* phage vB_Efs8_KEN04 (ΦKEN04), isolated from community wastewater in Nairobi, Kenya. This phage exhibits a relatively broad host range against clinical MDR *E. faecalis* isolates and a potent capacity to disrupt (eliminate already formed) and inhibit (prevent biofilm initiation) *E. faecalis* biofilms under laboratory conditions. It also evaluates the stability of lytic ΦKEN04 in vitro under different temperatures and pH ranges. The discovery of ΦKEN04 offers a promising phage-based therapy to effectively combat multidrug-resistant enterococcal infections and their biofilms.

## 2. Materials and Methods

### *2.1. Strains and Cultural Conditions*

Archived clinical isolates of multidrug-resistant *Enterococcus faecalis* and *Enterococcus faecium* (37 in total, 26 MDR *E. faecalis* and 11 MDR *E. faecium)* from patients in different hospitals around Kenya were obtained from an ongoing surveillance study (protocol WRAIR 209/KEMRI 2767) in the Department of Emerging Infectious Diseases, Walter Reed Army Institute of Research-Africa (WRAIR-A) in Kenya. Bacterial identity and antimicrobial susceptibility testing profiles were first confirmed using the Vitek 2 version 9.02 automated platform (bioMérieux, Marcy-l'Étoile, France),

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and they were cultured in tryptic soy broth (Oxoid Ltd., Basingstoke, Hampshire, England) under aerobic conditions with agitation at 37°C and a speed of 200 rpm for phage isolation.

*2.2. Bacteriophage Isolation, Purification, and Propagation*

Raw sewage water samples were collected from a sewage treatment plant in Nairobi East. It treats domestic and industrial wastewater, handling approximately 80% of the wastewater generated in Nairobi city daily. It is, therefore, a significant source of bacteria in the environment [28]. Enterococcus phage vB_Efs8_KEN04 was isolated through an enrichment method using *E. faecalis* isolate EFS8 as a host according to the method described by D'Souza et al. with slight modifications (D'Souza et al., 2020). Briefly, 50 mL of environmental wastewater was centrifuged at 12,000 × g for 10 min (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Eight milliliters of water sterilized by filtration through a 0.22 µm membrane was mixed with 2 ml of 5x Tryptic Soy Broth (TSB; Oxoid Ltd., Basingstoke, Hampshire, England) and 50 µL of bacterial culture grown in 1x TSB for 16-24 h at 37°C with agitation at 200 rpm. The mixture was incubated for 24 h at 37°C with agitation at 200 rpm. Bacterial debris was eliminated by centrifugation, and the supernatant was filter-sterilized. Serial 10-fold dilutions of ΦKEN04 in sodium chloride-magnesium sulfate (SM) buffer were spotted onto double-layer (0.7% top/1.5% bottom) Tryptic Soy Agar (TSA) agar overlaid with 100 µL of a culture of *E. faecalis* isolate EFS8 in the semisolid top layer. The next day, a well-isolated phage plaque was suspended in SM buffer and filter sterilized. Phage KEN04 was purified by three rounds of single-plaque isolation through plaque assays and propagated to reach a high titer. Briefly, the following components were mixed in a 50 ml falcon tube to amplify the phage: 20 ml of TSB, 10 µL of 1 M CaCl2, 40 µL of 1M MgCl2, 200 µL of 10% glucose, and 400 µL of the overnight host bacteria and incubated at 37°C with shaking at 200 rpm for 1−2 h to reach the mid-log phase. When the host bacterium reached the exponential growth phase, 250 µL of a single pure phage suspension was added, followed by incubation at 37°C, 200 rpm until lysis occurred. Bacterial debris was removed by a 10-minute centrifugation at 12,000 x g, after which the supernatant was filtered through a sterile 0.22 µm filter. The filtrate was centrifuged for 16−18h at 10,000 × g to pellet the phages. The supernatant was discarded after ultracentrifugation, leaving approximately 2 ml of supernatant to resuspend the pellet. The phage titers were determined using a spot assay [29].

*2.3. Phage Stability*

2.3.1. Thermal Stability

Thermal stability was determined by dispensing 20 µL of the propagated phage suspension with a titer of $2 \times 10^9$ PFU/ml into 0.2 ml PCR tubes and incubating at different temperatures (−80°C, −20°C, +4°C, 20°C, 22−30°C, 37°C, 40°C, 45°C, 50°C, and 60°C) for 1 h. After incubation, the phage lysate was diluted in SM buffer using a 10-fold serial dilution technique in 96-well round-bottom (U) microplates (Thermo Scientific, Danmark), and the phage titer was then evaluated using a spot assay as described elsewhere [29]. The experiment was performed in triplicate, and the phage lysate stored at +4°C was used as the reference titer.

2.3.2. pH Stability

The effect of pH 1, 3, 5, 7, 9, 11, and 13 on phage titer and viability was studied for 1 h in TSA plates using the spot test method, as described elsewhere [29]. The pH of the SM buffer was adjusted to the desired value using 1M NaOH and 1M HCl. The pH of SM was determined using a pH meter (Thermo Scientific, Danmark). After incubation, the phage titer was evaluated. The experiment was performed in triplicate, and the phage lysate stored at pH 7.5 was used as the reference titer.

*2.4. Host Range Analysis*

To investigate the activity of ΦKEN04 against other endemic bacterial strains, its host range was determined using a spot test [29] against a panel of 27 clinical isolates of MDR enterococci, and the

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efficiency of plating (EOP) was determined using a double-layer agar plate method, following a previously described protocol [30,31]. The bacterial strains used for this study were associated with skin and soft tissue infection, urinary tract infection, surgical site infection, and blood infection and were widely spread across Kenyan regions. All the tested strains were cultured in broth overnight at 37°C. Briefly, 2 µL of an individual phage stock was spotted on a TSA plate with a lawn of 100 µL of host bacteria cultured overnight in soft agar, which was examined for bacterial lysis after 18–24 h. Host range tests were performed in duplicates. A phage was termed 'potent' upon infecting and lysing bacterial strain in the host range panel [32]. The EOP was calculated by dividing the average plaque-forming units (PFU) of the test bacteria by the average PFU of the host bacteria. Phages were categorized as high production (EOP ≥ 0.5) when the productive infection on the test bacteria resulted in at least 50% of the PFU found for the primary host; medium production (0.1 ≤ EOP < 0.5); low production efficiency (0.001 <EOP< 0.1); inefficient (EOP ≤ 0.001), and reference (EOP = 1) [33,34].

*2.5. Biofilm Formation Assay*

The ability of enterococci to form biofilms was assessed using a crystal violet biomass assay [35]. Briefly, the bacterial isolates were grown overnight at 37°C, 200 rpm in tryptic soy broth. The enterococcal cultures were diluted 1:100 in fresh TSB containing 2% glucose monohydrate (Oxoid Ltd., Basingstoke, Hampshire, England), and 100 µL of the diluted solution was dispensed into the wells of 96-well round-bottom (U) microplates (Thermo Scientific, Danmark) and incubated under static conditions at 37°C, 5% $CO_2$ for 72 h without changing the medium. Wells with sterile TSB containing 2% glucose were used as controls for contamination. *Enterococcus faecalis* strain ATCC 29212 was used as a positive biofilm control, whereas *Enterococcus faecalis* isolate EFS4 (ST947), an in-house isolate, was used as the negative control. The experiments were performed in triplicate. After incubation, planktonic bacteria were pipetted off, the wells were washed three times with distilled water, and the plates were allowed to air-dry for 15 min. Adhesive bacteria were fixed at 60°C for 1 h and stained with 100 µL of 1% crystal violet for 20 min. This was followed by three washes with 100 µL of sterile distilled water to remove the excess dye. The microplates were air-dried for 15 min. Then, 100 µL of 33% glacial acetic acid was added to each well, followed by pipetting to release the bound crystal violet dye from the biofilm [36]. The stained adherent cells' optical density (OD) was quantified at 630 nm using a microtiter plate reader (BioTek Instruments, Gen5TM version 3.10, USA). The strains were divided into groups based on the OD values of the bacterial biofilms. Bacterial strains were classified as follows: OD values ≤0.0551 as non-biofilm formers, weak biofilm-producing isolates (0.0551<OD<0.102), moderate biofilm formers (0.102<OD<0.204), and those with OD > 0.204 were classified as strong biofilm-producing bacterial strains [37,38].

*2.6. Biofilm Inhibition Assay by Phage*

The anti-biofilm effect of the phage was evaluated as described by Goodarzi et al., with some modifications [39]. To investigate the inhibitory effect of phages on biofilm formation, a 3-day-old biofilm was formed in the presence of phages. Briefly, single colonies of *E. faecalis* strains were cultured in TSB at 37°C, 200 rpm for 24 h. After incubation, the bacterial culture was diluted 1:100 in fresh TSB, supplemented with 2% glucose. Diluted bacterial culture (100 µL) was dispensed into the wells, and 2 µL of phage lysate (titer 9 × $10^9$ PFU/ml) was added. The plates were incubated under static conditions at 37°C, 5% $CO_2$ for 72 h without changing the medium. Wells with sterile TSB containing 2% glucose were used as controls for contamination. *Enterococcus faecalis* strain ATCC 29212 was used as a positive biofilm control, whereas *Enterococcus faecalis* isolate EFS4 (ST947) was used as the negative control. Biofilm formation was performed in triplicate for treated and untreated samples. After incubation for 72 h, the suspension was drained from the wells and rinsed with sterile distilled water three times, and biofilm fixation, staining, and OD measurements were performed as described in Section 2.5.

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*2.7. Biofilm Disruption Assay by Phage*

A 2-day-old biofilm was formed in the absence of phages and then treated. Briefly, single colonies of *E. faecalis* strains were cultured in TSB at 37°C, 200 rpm for 24 h. After incubation, the bacterial culture was diluted 1:100 in fresh TSB, supplemented with 2% glucose. The diluted bacterial culture (100 µL) was dispensed into the wells, followed by incubation under static conditions at 37°C, 5% $CO_2$ for 48 h without changing the media. After 48 h of incubation, the plates were removed from the incubator, the planktonic bacteria were pipetted off, the wells were washed twice to remove all planktonic cells, and 100 µL of the phage lysate was added. For untreated wells, the medium was replaced with TSB supplemented with 2% glucose. The plates were then placed back in the incubator for 24 h. Biofilm formation was performed in triplicate for treated and untreated samples. After 24 h of treatment, the suspension was drained from the wells and rinsed three times with sterile distilled water. Biofilm fixation, staining, and OD measurements were performed as described in Section 2.5.

*2.8. Genomic DNA Extraction*

Before DNA extraction, the pure phage suspension ($2 \times 10^9$ PFU/ml) was propagated to reach a titer of $3.5 \times 10^{11}$ PFU/ml. The phage was concentrated by centrifugation for 18 h at 10,000 x g. The supernatant was then discarded. One milliliter of the propagated phage suspension was treated with RNase A (Thermo Fisher Scientific, USA) and DNase I (ThermoFisher Scientific, USA) to remove host RNA and DNA, respectively. Deproteinization was achieved by adding Proteinase K and incubating at 56°C for 1 h 30 min [40]. Phage DNA was isolated and purified using the Norgen phage DNA isolation kit (Norgen Biotek Corporation, Thorold, ON, Canada), following the manufacturer's instructions. The quality and quantity of the extracted DNA were determined using a Nanodrop One spectrophotometer and a Qubit4 fluorometer (Fisher Scientific, Waltham, MA, USA), respectively.

*2.9. Genome Sequencing and Bioinformatic Analysis of Sequencing Data*

The genome was sequenced on the Illumina MiSeq platform (Illumina, Inc., USA). The quality of the raw reads was assessed using FastQC v0.12.1 [41], trimmed with fastp v0.23.4 [42], and assembled using shovill v1.1.0 (https://github.com/tseemann/shovill). Genome annotation was performed using pharokka 1.5.1 [43]. The complete phage genome was further queried against CRISPR-Cas Finder (https://proksee.ca/, accessed on December 11, 2023), PhageLead [44] (https://phageleads.dk/, accessed on 11 December 2023), PhageTerm platforms [45] (https://cpt.tamu.edu/galaxy-pub, accessed on 27 November 2023) to determine CRISPR-like systems, lysogeny genes, and termini in the phage genome, respectively. ARAGORN v1.2.41 [46] and tRNAscan-SE v2.0.12 [47] were used to predict the tRNA and tmRNA genes. Nucleic acid sequence similarity searches were performed using default parameters in BLASTn [48]. The identification of antimicrobial resistance and virulence genes was conducted by scanning the assembled nucleotide sequence using Abricate version 1.0.1 [49] at https://github.com/tseemann/abricate, accessed on November 27, 2023, with the following datasets: NCBI AMRFinderPlus [50], Comprehensive Antibiotic Resistance Database (CARD) [51], Virulence FactorDatabase (VFDB) [52] and ResFinder [53].

*2.10. Phylogenetic Tree and Comparative Genomics of Phage Genomes*

To examine the genetic relationships between ΦKEN04 and other Enterococcus phages, a phylogenetic tree was generated using the entire genome sequences of 38 phages retrieved from the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 23 December 2023), including ΦKEN04. The phages included in the phylogenetic tree were selected according to the following criteria: i) should be a complete genome sequence [54]; ii) should exhibit a high similarity of >70% to ΦKEN04 [55]; and iii) should have a genome size similar to that of ΦKEN04 [56]. The analysis was conducted using the Virus Classification and Tree Building Online Resource (VICTOR), a method for the genome-based phylogeny and classification of prokaryotic viruses [54] (https://victor.dsmz.de, accessed on 23 December 2023), enabling the evaluation of similarities and differences in genetic

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characteristics. The nucleotide sequences were compared using the genome explosion distance phylogeny (GBDP) method in the settings recommended for prokaryotic viruses [57], and the branch length was magnified using the distance formula d0, according to GBDP. In addition, the intergenomic similarities between ΦKEN04 and the 20 closest related Enterococcus phages were determined using a virus intergenomic distance calculator (VIRIDIC) [58] to further our understanding of their interactions. The nucleotide identity of the complete genome length cut-off for genera (>70%) and species (>95%) was used [55].

### *2.11. Statistical Analysis*

Statistical analysis of the biofilm results was conducted using GraphPad Prism 8.4.0 (GraphPad Software, Inc., San Diego, CA, USA), and a Student's t-test was employed to determine significance. Statistical significance was set at $p < 0.05$.

## 3. Results

### *3.1. Bacteriophage Isolation and Purification*

Phage vB_Efs8_KEN04 isolated from a wastewater plant using the Enterococcus faecalis isolate EFS8 as the host strain. ΦKEN04 forms clear plaques on a double-layered agar plate, as shown in Figure 1.

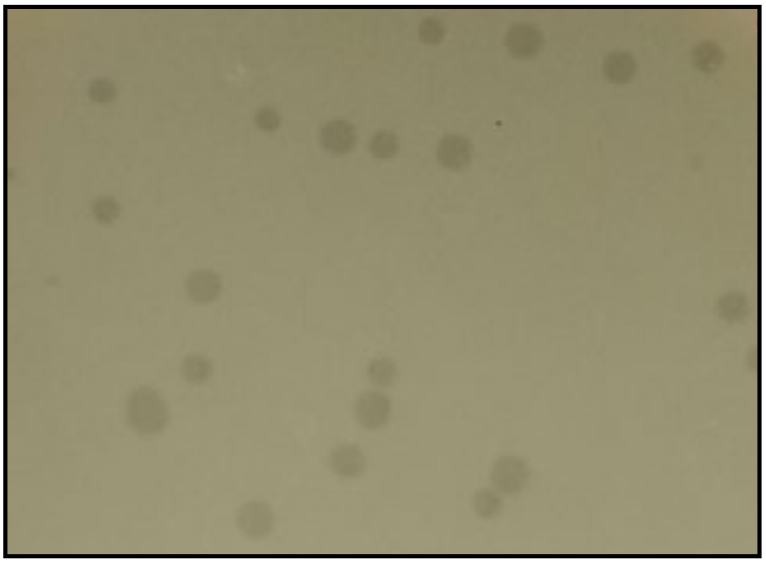

**Figure 1.** Plaque morphology of phage vB_Efs8_KEN04.

### *3.2. Host Range Analysis*

The host range and efficiency of plating studies were conducted on 37 multidrug-resistant (MDR) Enterococci isolates, with 26 being E. faecalis isolates and the remaining eleven being E. faecium isolates. These bacteria were isolated from urinary tract infections, skin and soft tissue infections, surgical site infections, and blood infections in humans. Enterococcus faecalis phage vB_Efs8_KEN04 was active against all 26 MDR E. faecalis and 1/11 of the E. faecium in the spot assay (Table 1). The EOP was greater than 0.5 for 13/27 (48.15%) isolates, indicating high production of the phage and an EOP≥0.5 is considered good for therapy [34].

**Table 1.** Host range analysis of Enterococcus phage vB_Efs8_KEN04.

| No. | Bacterial isolates | Sequence Types (ST) | Origin | Spot assay | Efficiency of Plating (EOP) |
|---|---|---|---|---|---|
| 1 | *E. faecalis* EFS8 * | 1904 | Urinary tract infection | ++ | 1 |
| 2 | *E. faecalis* EFS1 | 6 | Skin and soft tissue infection | ++ | 0.15 |
| 3 | *E. faecalis* EFS4 | 947 | Skin and soft tissue infection | + | <0.001 |

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| | | | | | |
|---|---|---|---|---|---|
| 4 | *E. faecalis* EFS5 | 6 | Skin and soft tissue infection | ++ | 0.076 |
| 5 | *E. faecalis* EFS6 | 6 | Skin and soft tissue infection | ++ | 0.05 |
| 6 | *E. faecalis* EFS9 | 6 | Urinary tract infection | ++ | 0.01 |
| 7 | *E. faecalis* EFS10 | 6 | Urinary tract infection | ++ | 1.1 |
| 8 | *E. faecalis* EFS11 | 368 | Urinary tract infection | ++ | 0.0004 |
| 9 | *E. faecalis* EFS13 | 59 | Skin and soft tissue infection | ++ | 0.5 |
| 10 | *E. faecalis* EFS14 | 6 | Skin and soft tissue infection | ++ | 1.7 |
| 11 | *E. faecalis* EFS15 | | Urinary tract infection | + | <0.001 |
| 12 | *E. faecalis* EFS17 | 6 | Skin and soft tissue infection | + | <0.001 |
| 13 | *E. faecalis* EFS18 | 368 | Urinary tract infection | ++ | 0.0011 |
| 14 | *E. faecalis* EFS19 | | Urinary tract infection | ++ | 0.12 |
| 15 | *E. faecalis* EFS21 | 44 | Skin and soft tissue infection | + | <0.001 |
| 16 | *E. faecalis* EFS22 | | Skin and soft tissue infection | + | <0.001 |
| 17 | *E. faecalis* EFS23 | 6 | Urinary tract infection | ++ | 3 |
| 18 | *E. faecalis* EFS25 | 6 | Surgical site infection | ++ | 1.5 |
| 19 | *E. faecalis* EFS26 | 6 | Skin and soft tissue infection | ++ | 1.2 |
| 20 | *E. faecalis* EFS27 | 1903 | Urinary tract infection | ++ | 0.14 |
| 21 | *E. faecalis* EFS28 | 28 | Skin and soft tissue infection | ++ | 0.6 |
| 22 | *E. faecalis* EFS29 | 6 | Blood infection | ++ | 0.8 |
| 23 | *E. faecalis* EFS30 | 28 | Skin and soft tissue infection | ++ | 0.8 |
| 24 | *E. faecalis* EFS31 | 6 | Urinary tract infection | ++ | 1.2 |
| 25 | *E. faecalis* EFS32 | 1903 | Urinary tract infection | ++ | 0.9 |
| 26 | *E. faecalis* EFS33 | 1903 | Skin and soft tissue infection | ++ | 6 |
| 27 | *E. faecium* EFM5 | 80 | Urinary tract infection | + | <0.001 |
| 28 | *E. faecium* EFM1 | | Skin and soft tissue infection | - | N/A |
| 29 | *E. faecium* EFM2 | 80 | Skin and soft tissue infection | - | N/A |
| 30 | *E. faecium* EFM3 | | Skin and soft tissue infection | - | N/A |
| 31 | *E. faecium* EFM4 | | Skin and soft tissue infection | - | N/A |
| 32 | *E. faecium* EFM6 | 612 | Skin and soft tissue infection | - | N/A |
| 33 | *E. faecium* EFM7 | | Skin and soft tissue infection | - | N/A |
| 34 | *E. faecium* EFM8 | 80 | Urinary tract infection | - | N/A |
| 35 | *E. faecium* EFM9 | 80 | Skin and soft tissue infection | - | N/A |
| 36 | *E. faecium* EFM10 | | Urinary tract infection | - | N/A |
| 37 | *E. faecium* EFM11 | 761 | Surgical site infection | - | N/A |

EFS, *Enterococcus faecalis*; EFM, *Enterococcus faecium*; EOP, Efficiency of plating. The EOP was determined by dividing the mean Plaque Forming Units (PFU) of the target bacteria by the mean PFU of the host bacteria (EFS8). *Host bacteria; ++, very clear plaques; +, turbid plaques; −, no plaques; N/A, not applicable.

### *3.3. Phage Stability*

The stability of vB_Efs8_KEN04 was evaluated at different temperatures and pH values. The results revealed that ΦKEN04 was stable from − 80°C to 37°C (Figure 2A, Table S1). Phage titer declined at temperatures of 40°C and above. Similarly, the stability rate of ΦKEN04 was high at pH 5–11 (slightly acidic to strongly basic) but low at pH 3 (strongly acidic). No phage activity was observed at pH 1 or 13 (Figure 2B, Table S2). These findings suggest that phage vB_Efs8_KEN04 can withstand moderate acidic and alkaline conditions and a wide range of temperature conditions between − 80°C and 37°C.

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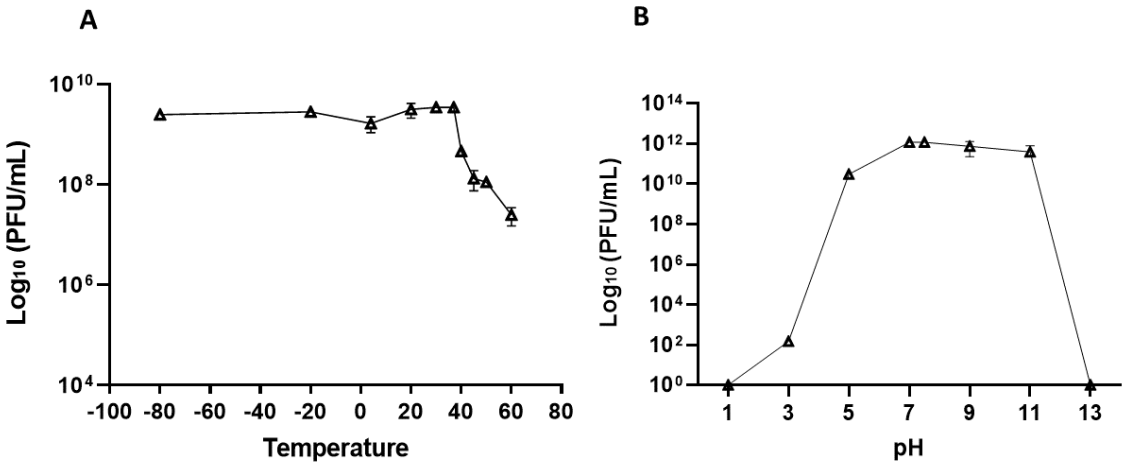


**Figure 2.** Phage stability test of Enterococcus phage vB_Efs8_KEN04. (A) Thermal stability test. (B) pH stability test.

*3.4. Biofilm Formation of Enterococcus faecalis*

Of the 26 clinical MDR E. faecalis isolates that were examined for biofilm formation, 22 isolates (84.62%) were strong biofilm formers, one isolate (3.85%) was identified as a weak biofilm former, one (3.85%) isolate as a moderate biofilm former, and two isolates (7.69%) as non-biofilm formers (Table S3). A total of 24 MDR E. faecalis isolates (92.31%) showed the ability to produce biofilms. Enterococcus faecalis isolate EFS8, the phage host, was a strong biofilm former (Figure 3).

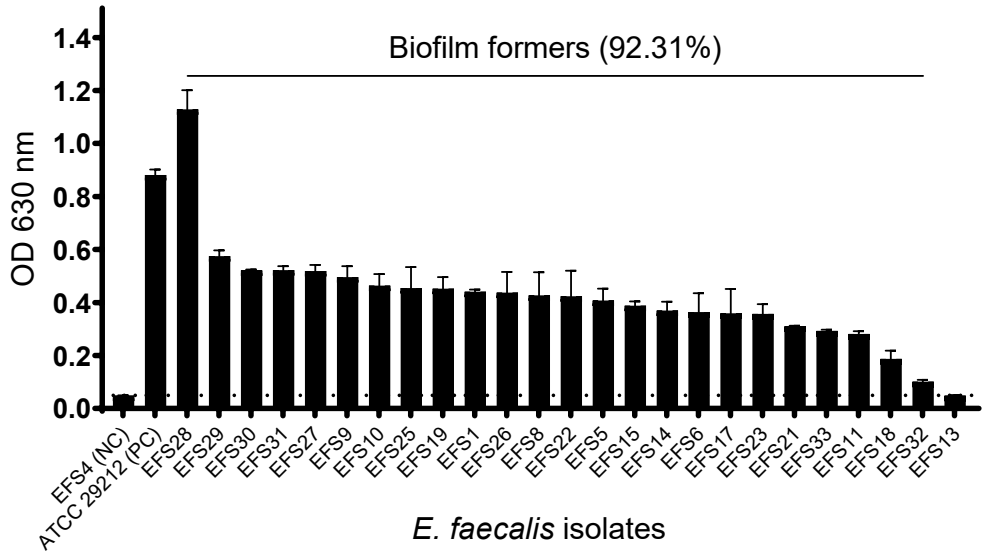


**Figure 3.** Biofilm formation profile of *Enterococcus faecalis* isolates. The biofilm formation experiment was performed in triplicate, and the error bars represent the standard deviation. The horizontal grid line represents the OD threshold of 0.551 for biofilm-forming isolates.

*3.5. Biofilm Inhibition and Disruption by Phage vB_Efs8_KEN04.*

The effects of phage vB_Efs8_KEN04 treatment on the inhibition and disruption of biofilms of multidrug-resistant *E. faecalis* isolates are shown in Figure 4. For the inhibition of biofilm formation (Figure 4A, Table S4), phage vB_Efs8_KEN04 treatment for 72 h at 37°C reduced the bacterial population significantly (**, $p < 0.05$) for some susceptible bacteria. However, it could not prevent

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other bacteria (EFS5, EFS6, EFS15, EFS17, EFS21, EFS22, EFS26, EFS27, EFS30, EFS31, EFS31, EFS32, and EFS33) from forming biofilms ($p>0.05$, highlighted by ns). For the disruption of already formed biofilms (Figure 4B, Table S5), phage vB_Efs8_KEN04 treatment for 24 h significantly reduced the bacterial population (**, $p <0.05$) for all the bacteria, including its host bacteria EFS8, except for the isolate EFS18 (ns, $p>0.05$).

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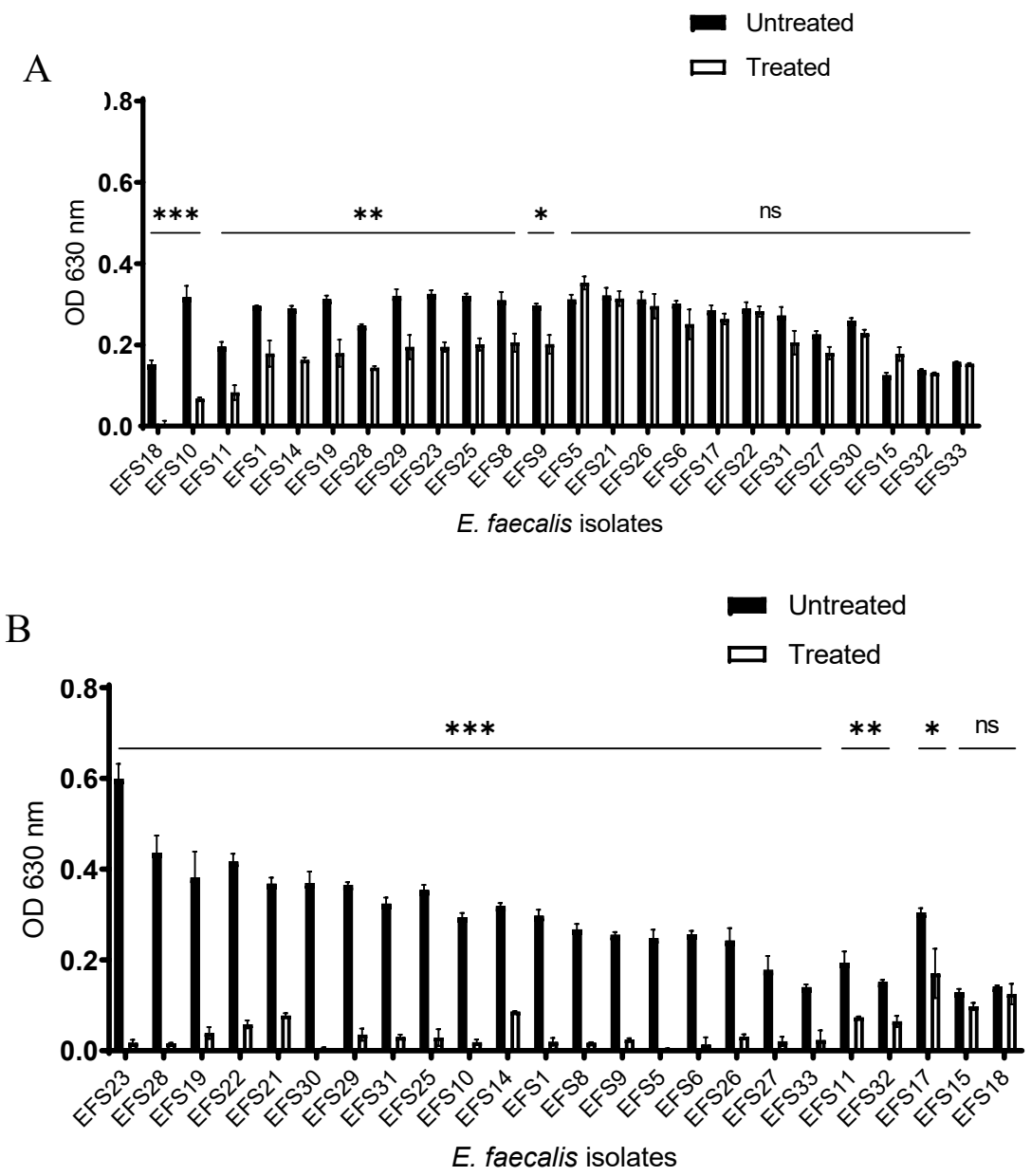


**Figure 4.** (A) Inhibition of biofilm by Enterococcus phage vB_Efs8_KEN04; (B) Biofilm disruption by phage vB_Efs8_KEN04. Biofilm inhibition and disruption experiments were performed in triplicate, and the error bars represent the standard deviations. Significance level: *, $p<0.05$ significant; **, $p<0.01$ Very significant; ***, $p<0.001$ highly significant; ns, non-statistically significant.

*3.6. Genome Characteristics of Enterococcus faecalis Phage vB_Efs8_KEN04*

The genome structure of phage vB_Efs8_KEN04, a newly isolated *E. faecalis* phage in Kenya, was investigated in this study. The phage genome contained 8 tRNA genes and was shown to be a linear double-stranded DNA with a length of 142,402 base pairs and a G+C content of 36.01% (Figure 5). It belongs to the genus *Kochikohdavirus* of the family *Herelleviridae*. The genome contained 238 coding sequences (CDS) with a coding gene density of 91.46%. Seventy CDSs (29.41%) were predicted to encode functional proteins, and the remaining 168 (70.59%) were annotated as hypothetical proteins (Table S6). The functional proteins were divided into the following categories:

i) DNA replication, transcription, translation, and nucleotide metabolism: A total of 25 CDSs were predicted to encode for DNA replication, transcription regulation, translation, and metabolism-

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related proteins, such as HNH homing endonuclease, DNA helicase, exonucleases, transcriptional repressor, DNA helicase, DNA primase, and a transcriptional regulator, RNA polymerase beta subunit, and thymidylate synthase.

ii) Structural and packaging proteins: 27 CDS were predicted to encode for tail, head, and packaging proteins such as portal proteins, head proteins, tail fiber proteins, head maturation proteases, virion structural proteins, tail proteins, tail assembly chaperones, minor and major head proteins, and terminase large and small subunits.

iii) Host lysis and adhesion proteins: Two CDS were predicted to encode holin and endolysin proteins. BLASTP analysis of the Enterococcus phage vB_Efs8_KEN04 genome revealed no similarities to the genes encoding integrase or excisionase. The genome of phage vB_Efs8_KEN04 lacks genes encoding toxins, virulence factors, antibiotic resistance genes, and CRISPR. These data indicate that phage vB_Efs8_KEN04 is a strictly lytic phage that can be used to treat *E. faecalis* infection.

iv) Sixteen CDS encode for moron, auxiliary metabolic genes, and host takeover.

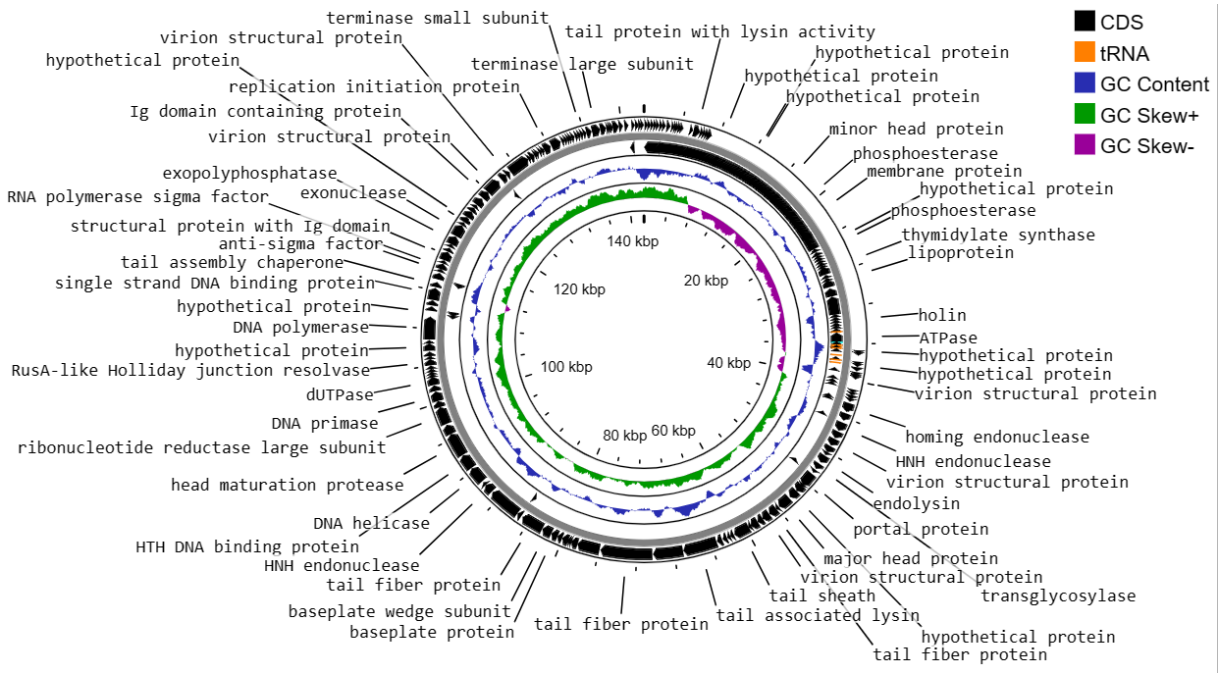


**Figure 5.** Circular genome map of Enterococcus phage vB_Efs8_KEN04 constructed using CGView.

Analysis of phage vB_Efs8_KEN04 DNA termini and phage packaging mechanisms revealed long Direct Terminal Repeats (DTR) of 2,849 bp with a specific packaging site called the cos site, which serves as a recognition signal for the packaging machinery. This DTR is comparable to that of bacteriophage T5 [59].

### *3.7. Phylogenetic Analysis*

To gain a deeper understanding of the evolution and relationship between phage vB_Efs8_KEN04 and other Enterococcus phages, the genome of phage vB_Efs8_KEN04 was compared with that of 37 Enterococcus phages. These phage sequences were obtained from the National Center for Biotechnology Information (NCBI) database, and all had homology ranging from 78.06 to 99.29% with phage vB_Efs8_KEN04 (Table S7).

The phylogenetic tree, generated with the whole genome sequences, indicated that phage vB_Efs8_KEN04 had the highest similarity to Enterococcus phage PBEF129 (GenBank accession

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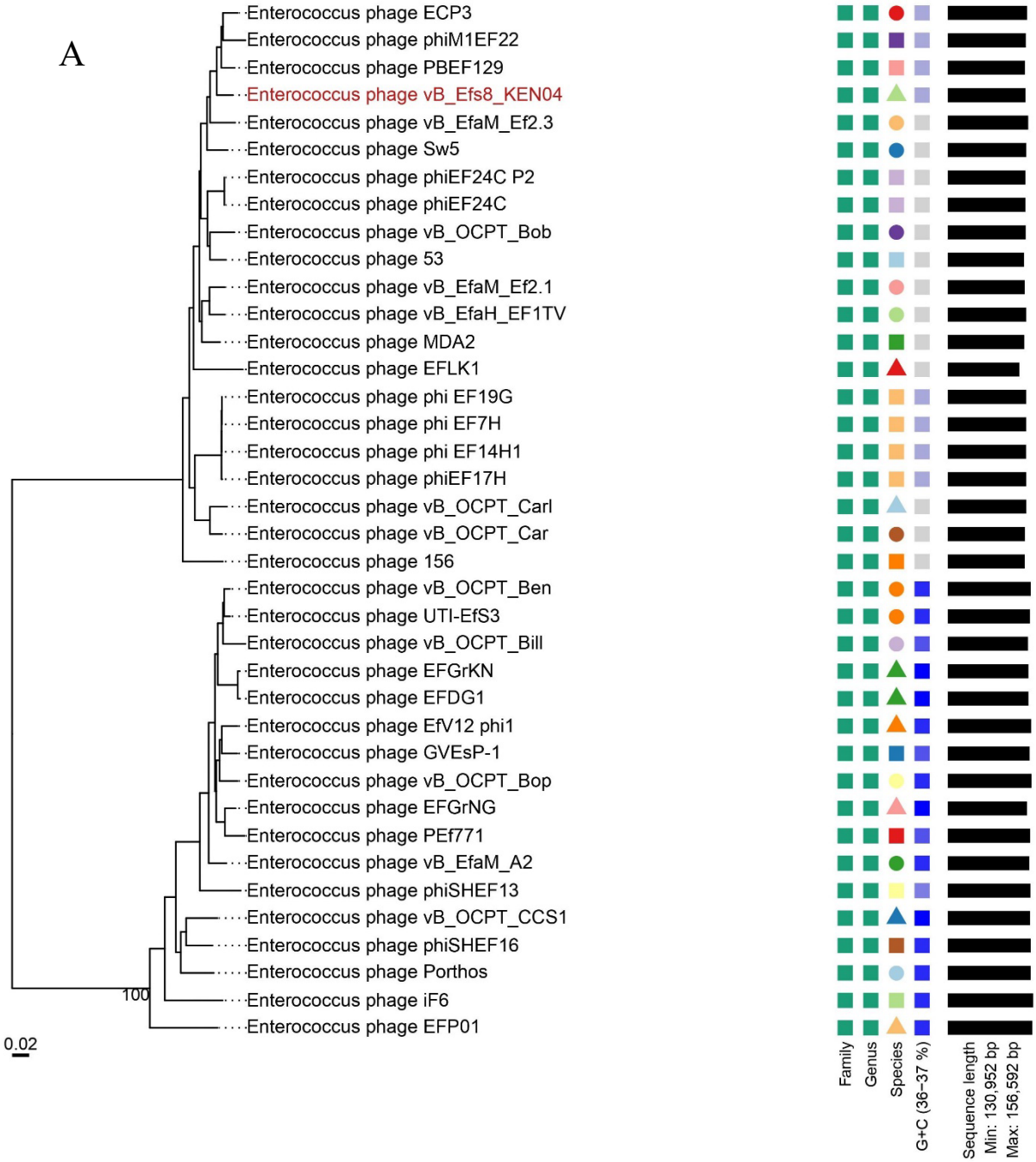
number MN854830.2), Enterococcus phage phiM1EF22 (GenBank accession number AP018715.1), Enterococcus phage ECP3 (GenBank accession number NC_027335.2), and Enterococcus phage vB_EfaM_Ef2.3 (GenBank accession number MK721192.1) (Figure 6A, Table S7). Subsequently, we employed VIRIDIC to compute the inter-genomic similarities, revealing the degree of similarity between phage vB_Efs8_KEN04 and the top 20 phages most closely linked to it (Figure 6B). This indicated that the similarity of phage vB_Efs8_KEN04 and the other Enterococcus phages' complete genome was significantly greater than the genus threshold of 70% and lower than the species threshold of 95% [55], suggesting that they belong to the same genus but distinct species. The intergenomic similarities between phage vB_Efs8_KEN04 and the four most closely related Enterococcus phages were as follows: Enterococcus phage PBEF129 (94.6%), Enterococcus phage phiM1EF22 (93.3%), Enterococcus phage ECP3 (94.5%), and Enterococcus phage vB_EfaM_Ef2.3 (93.5%) (Figure 6B).



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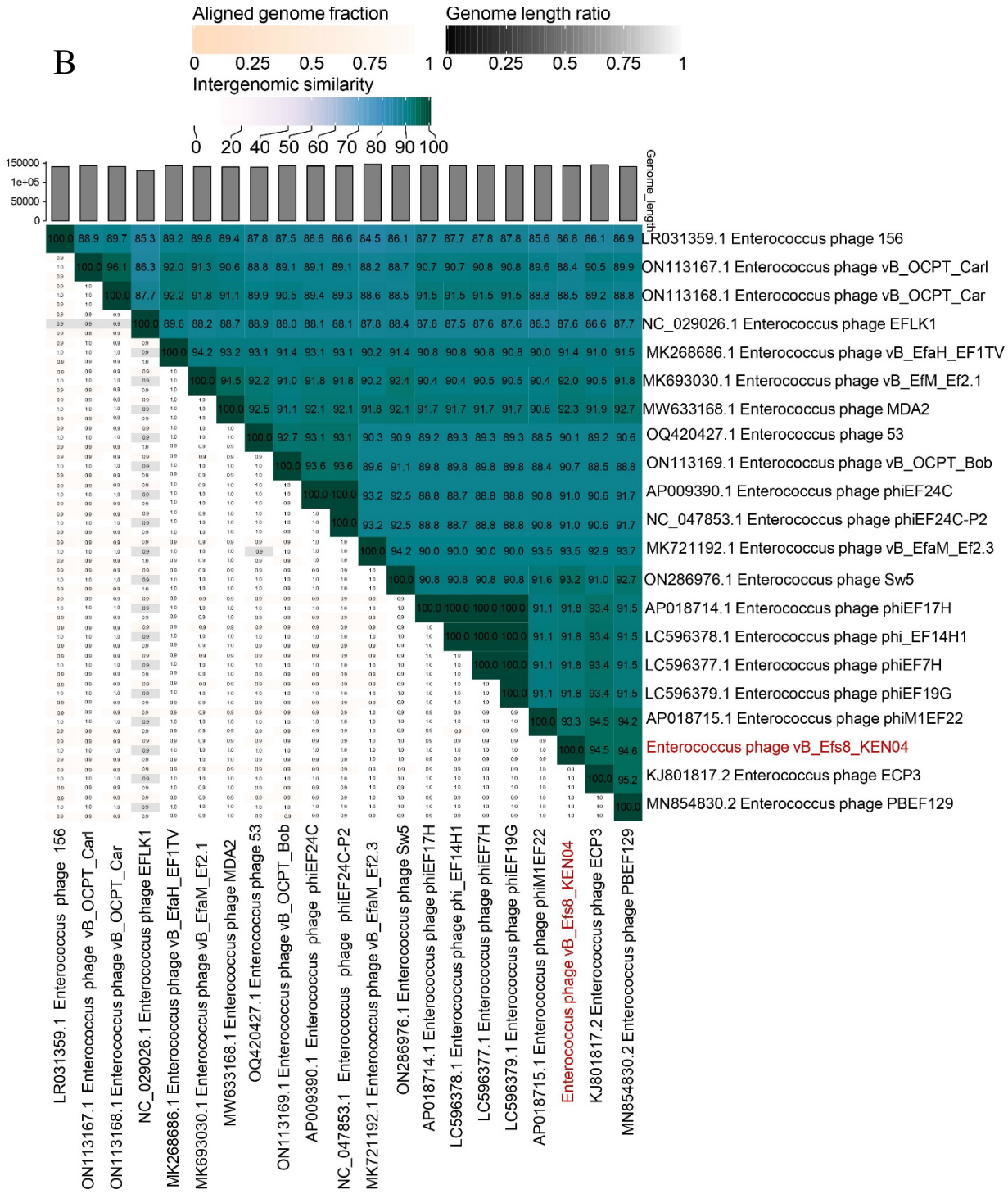


**Figure 6.** (A) Phylogenetic analysis of Enterococcus phage vB_Efs8_KEN04 and other related Enterococcus bacteriophages based on the similarity of whole genome sequences. The phylogenetic tree was generated using the online VICTOR platform with the formula d0. (B) Heatmap of the average nucleotide identity values between phage vB_Efs8_KEN04 and the top 20 most similar Enterococcus bacteriophages.

## 4. Discussion

The use of bacteriophages, also known as phages, in treating bacterial infections, including *E. faecalis*, has gained particular attention. This is due to the increase in antibiotic resistance and the phages' ability to infect and eliminate bacteria. Phages have the potential to serve as a natural, safe, and efficient method for preventing and controlling multidrug-resistant (MDR) organisms [60].

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Studies have also shown that phages can be used to control biofilms formed by *Enterococcus faecalis* [23,61].

This study characterized a highly lytic and broad-spectrum *E. faecalis* phage vB_Efs8_KEN04 isolated from environmental wastewater in Nairobi, Kenya. It is a dsDNA phage belonging to group I of Baltimore's classification of viruses. The genome of this phage did not encode any lysogenic, antibiotic resistance, or virulence and CRISPR-cas genes. Thus, it is an effective and safe candidate for phage therapy. Based on genome annotation, phage vB_Efs8_KEN04 was classified as a member of the genus *Kochikohdavirus* in the family *Herelleviridae*. Its genome contains eight genes encoding for transfer RNAs (tRNAs). The presence of tRNAs in bacteriophage genomes is widespread, especially among virulent phages [62]. However, their precise role has remained ambiguous for almost five decades as phages utilize the host's transcriptional machinery to control the expression of their own genes after initial infection [63]. Several hypotheses have been proposed for the role of these phage-encoded tRNAs. The most established is codon compensation, where codons rarely used by the host but necessary to the phage are supplemented by the tRNAs encoded by the phage [62]. Recently, a study proposed a new hypothesis that phage-encoded tRNAs counteract the tRNA-depleting strategies of the host using enzymes such as VapC, PrrC, Colicin D, and Colicin E5 to defend from viral infection, and they have evolved to be insensitive to host anticodon nucleases [64].

Phage vB_Efs8_KEN04 exhibited a wide host range by displaying lysis activity against all 26 clinical MDR *E. faecalis* isolates tested. These isolates belonged to various sequence types (ST6, ST44, ST28, ST59, ST368, ST947, ST1903, and ST1904) and were associated with different infection types such as urinary tract infection, skin and soft tissue infection, surgical site infection, and blood infection. It also has an EOP≥0.5 for 13 isolates, indicating high progeny production. In addition, it showed a cross-species activity against a clinical isolate of multidrug-resistant *Enterococcus faecium*. The phage's ability to target its host bacteria is due to its host receptors involved in recognition, interaction and adsorption during phage attachment [65]. Additionally, the receptors are recognized by the ends of the virion's long tail fibers of the phage towards the host bacteria [66]. When subjected to different temperatures and pH conditions, phage vB_Efs8_KEN04 showed the ability to withstand moderate acidic and alkaline conditions from pH 3-11 and a wide temperature range from – 80°C to 37°C. Many external physical and chemical factors, including but not limited to temperature, acidity, salinity, and ions, determine bacteriophages occurrence, viability, and storage. These factors can inactivate the phage by damaging its structural components (head, tail, envelope), lipid depletion, and/or DNA structural changes [67]. The studied phage exhibited remarkable stability throughout a broad range of temperatures and pH levels, making it advantageous for formulation into a suitable pharmaceutical form and therapeutic applications. Furthermore, the phage's stability across acidic and alkaline environments (pH 3–11) enables it to be administered orally without compromising its viability in the gastrointestinal tract [23].

In this study, we also investigated the effect of phage vB_Efs8_KEN04 on the biomass reduction of *E. faecalis* biofilm by inhibition and disruption experiments. Biofilms are communities of bacteria that can be highly resistant to antibiotics and contribute to persistent infections [24,68]. Several factors contribute to the enhanced antimicrobial resistance of microorganisms in a biofilm. These include the physical barrier created by the extracellular matrix, which hinders the diffusion of antimicrobial agents [69]. Additionally, nutrient and oxygen depletion within the biofilm can cause certain bacteria to enter a stationary state, making them less susceptible to microbial killing [70].

Furthermore, a subpopulation of bacteria might differentiate into a phenotypically resistant state, and some bacteria within the biofilm have been found to express specific antimicrobial resistance genes unique to biofilms [71]. Recent studies have demonstrated that extracellular DNA (eDNA) in the biofilm matrix protects microbial cells against various antimicrobial agents [72]. The biofilm formation phenotype of multidrug-resistant *Enterococcus faecalis* was investigated, and the results revealed that out of 26 MDR tested for this purpose, 92.31% showed the ability to form biofilm. To date, several investigations have been performed to test bacteriophages' ability to inhibit and destroy *Enterococcus faecalis* biofilms [17,22,73,74], but the mechanisms of phage-biofilm interaction are not well understood [75]. As indicated by the crystal violet biomass assay, the inhibition assay

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indicated that the isolated phage vB_Efs8_KEN04 significantly reduced biofilm biomass (p-value <0.05) compared to the control for most of the biofilm-forming *E. faecalis* isolates. Based on genome and structural proteome analysis, this can be explained by the endolysins encoded by phage vB_Efs8_KEN04, which may destroy the EPS component. Furthermore, phage endolysin lyses some bacteria at the edge of the EPSs [76]. Phage vB_Efs8_KEN04 then induces the production of depolymerase, such as endolysins [77] (CDS121) (Table S6) since they can penetrate the inner layers of the biofilm by degrading structural components of the established biofilm exopolymeric matrix, allowing them to break it down or disrupt its integrity [78]. The reduction of bacteria on the biofilm causes the reduction of EPS material; thus, the biofilm is completely eliminated [79]. Similarly, phage vB_Efs8_KEN04 showed the ability to significantly decrease biofilm biomass when compared with an untreated biofilm of an already formed biofilm. Therefore, phage vB_Efs8_KEN04 has the potential to be successfully used as a biofilm eradication agent. In future research, the phage-derived enzymes may be studied as biological antibacterial agents to control Enterococcus and its biofilm.

## 5. Conclusions

Phage vB_Efs8_KEN04 is a lytic phage belonging to the genus *Kochikohdavirus* in the family *Herelleviridae*. It was isolated from a municipal sewage treatment plant located in Nairobi East. Phage vB_Efs8_KEN04 exhibits efficacy against all the clinical multidrug-resistant strains of *Enterococcus faecalis* tested and one *E. faecium* isolates, including the ability to destroy bacterial biofilms. The genome analysis revealed that the phage lacks genes of concern, including virulence, antibiotic resistance, lysogeny genes, and the CRISPR or CRISPR-like system. The phage vB_Efs8_KEN04 has great potential as a candidate for phage therapy against enterococci infections and for controlling biofilms.

## 6. Limitations

A limitation of this study is the lack of animal models as representative models of enterococci infection in humans, as the experimental models can mimic the pathogenesis of natural disease.

**Supplementary Materials:** The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Table S1: Temperature stability test (Mean ± SD); Table S2: pH stability test (Mean ± SD); Table S3: Biofilm formation profile of MDR *Enterococcus faecalis* isolates; Table S4: Biofilm inhibition of phage vB_Efs8_KEN04; Table S5: Biofilm disruption of phage vB_Efs8_KEN04; Table S6: Predicted molecular function for gene products of phage vB_Efs8_KEN04; Table S7: Summary of similar genomic sequence with phage vB_Efs8_KEN04.

**Author Contributions:** Conceptualization, O.S., A.N. and L.M.; methodology, O.S.; software, O.S. and C.K.; validation, C.K., A.N. and L.M.; formal analysis, O.S.; investigation, O.S.; resources, O.S., C.K., A.N. and L.M.; data curation, O.S.; writing—original draft preparation, O.S.; writing—review and editing, C.K., A.N. and L.M.; visualization, O.S.; supervision, A.N. and L.M.; project administration, O.S.; funding acquisition, O.S., A.N. and L.M. All authors have read and agreed to the published version of the manuscript.

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**Institutional Review Board Statement:** Not applicable.

**Data Availability Statement:** Data is contained within the article or supplementary material. The raw data are available in Sequence Read Archive under the bioSample accession number SAMN40604471 and the complete genome of Enterococcus phage vB_Efs8_KEN04 is available in GenBank under the accession number PP582180.

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