Bacteriophage-Resistant Mutant of Enterococcus faecalis Is Impaired in Biofilm Formation

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Enterococcus faecalis is a common gram-positive non-spore-forming bacterium in nature and is found in the upper respiratory tract, intestine, and mouth of healthy people. E. faecalis is also one of the common pathogens causing nosocomial infections and is resistant to several antibiotics commonly used in practice. Thus, treating drug-resistant E. faecalis with antibiotics is challenging, and new approaches are needed. In this study, we isolated a bacteriophage named EFap02 that targets E. faecalis strain EFa02 from sewage at Southwest Hospital. Phage EFap02 belongs to the Siphoviridae family with a long tail of approximately 210 nm, and EFap02 can tolerate a strong acid and alkali environment and high temperature. Its receptor was identified as the capsular polysaccharide. Phage-resistant mutants had loss-of-function mutations in glycosyltransferase (gtr2), which is responsible for capsular polysaccharide biosynthesis, and this caused the loss of capsular polysaccharide and interruption of phage adsorption. Although phage-resistant mutants against EFap02 can be selected, such mutants are impaired in biofilm formation due to the loss of capsular polysaccharide, which compromises its virulence. Therefore, this study provided a detailed description of the E. faecalis EFap02 phage with the potential for treating E. faecalis infection.

Keywords: bacteriophage, Enterococcus faecalis, phage resistance, phage receptor, capsular polysaccharide, biofilm

INTRODUCTION

Enterococcus faecalis is a common opportunistic pathogen that causes blood and urinary tract infections. It is one of the primary pathogens that infect root canals (Khalifa et al., 2016). E. faecalis is intrinsically resistant to several commonly used antibiotics, such as cephalosporin and aminoglycoside (García-Solache and Rice, 2019). Vancomycin-resistant Enterococcus (VRE) is resistant to vancomycin (Miller et al., 2016). With the emergence of multidrug-resistant strains of E. faecalis and its ability to form biofilms (Ch’ng et al., 2019), E. faecalis infection has caused great concern in practice (Fiore et al., 2019), and there is an urgent need to find new treatments.
In recent years, phage therapy has renewed interest as a promising alternative to antibiotics (Khalifa et al., 2016). Phage therapy has successfully treated patients with multidrug-resistant bacterial infections. In Belgium and France, a randomized, controlled, double-blind phase I/II trial (PhagoBurn) used a cocktail of 12 natural lytic phages to treat burn wounds infected with *Pseudomonas aeruginosa*. Compared to standard care, the bacterial load was reduced in the phage cocktail treatment group, indicating that phage therapy is promising, although the quality of the phage product needs improvement (Jault et al., 2019). Furthermore, in China, a 63-year-old woman who developed a recurrent urinary tract infection with extensively drug-resistant *Klebsiella pneumoniae* was cured by antibiotic and phage synergism (Bao et al., 2020). Only one human study described the treatment of chronic prostatitis associated with *E. faecalis* by phage therapy in 2007. Three patients who suffered chronic *E. faecalis* infection were not cured with antibiotics, autovaccines, and laser biostimulation. They were then treated with phage therapy twice daily for 1 month. The pathogen was eradicated, and clinical symptoms were relieved without recurrence (Lekkiewicz et al., 2009).

In this study, we successfully isolated a lytic phage, EFap02, from the sewage of Southwest Hospital in China. Phage EFap02 belongs to the *Siphoviridae* family and has a potent lytic effect against *E. faecalis*. The capsular polysaccharide was identified as the EFap02 receptor. The phage-resistant mutant had loss-of-function mutations in glycosyltransferase Group 2 (*gtr2*), which is responsible for capsular polysaccharide biosynthesis. The mutations caused the loss of capsular polysaccharide and interruption of phage adsorption. The loss of capsular polysaccharide significantly decreased the biofilm formation capability compromising the EFap02 virulence. Overall, this study suggested that EFap02 could be a promising candidate for *E. faecalis* phage therapy.

### MATERIALS AND METHODS

#### Strains and Cultural Conditions

The bacterial strains and phages are listed in Table 1. The EFa02 strain was isolated from the Daping Hospital Department of Clinical Laboratory Medicine. *E. faecalis* was cultured in brain heart infusion (BHI) medium at 37°C. When necessary, erythromycin (20 μg/ml) was added to the medium. The stock cultures were stored in medium supplemented with 20% glycerol at −80°C.

| Strain or phage | Description | Source |
|-----------------|-------------|--------|
| EFa02           | Wild type *Enterococcus faecalis* strain | This study |
| EFa02R          | Phage-resistant mutant                  | This study |
| EFa02R:gtr2     | EFa02R complemented with *gtr2*        | This study |
| EFap02          | Phage targets EFa02                    | This study |

#### Isolation of the Phage That Infects *Enterococcus faecalis*

Isolation of bacteriophages was performed as previously described (Shen et al., 2018). Sewage (10 ml) from the Southwest Hospital was centrifuged at 10,000 × g for 10 min. The supernatant was filtered with a 0.45 μm aseptic filter, and 2 ml of the filtered supernatant was added to a 50 ml clean centrifuge tube. The logarithmic phase host bacteria EFa02 was added and then cultured in a 37°C shaking incubator overnight. The mixture was centrifuged at 21,000 × g for 1 min, and the supernatant was filtered using a 0.22 μm aseptic filter. Then, 10 μl supernatant was mixed with 100 μl host bacteria EFa02 in a 15 ml centrifuge tube. BHI soft agar (5 ml) was added, and the content was poured onto the surface of agar plates. The plates were incubated at 37°C overnight, and the plaques were observed on the top agar.

#### Electronic Microscope

Phage morphology was observed by transmission electron microscopy (TEM; HT7700, Hitachi, Japan; Lee et al., 2019). The filtered phage lysate was dropped onto the prepared Formvar/carbon-coated copper grid and incubated for 1 min. The grid was negatively stained with 2% uranium acetate for 10 min and then observed using TEM at an acceleration voltage of 80 kV. Phages are classified according to the guidelines of the International Committee on the Taxonomy of Viruses (ICTV; Lefkowitz et al., 2018).

#### Determination of the Optimal Multiplicity of Infection

The optimal multiplicity of infection (MOI) is the ratio of bacteriophages to bacteria at the time of infection. The optimal MOI is the number of infections when the phage can achieve the best growth state (Lee et al., 2019). According to different MOIs, the phage (2 ml) and host bacteria (2 ml) were mixed. BHI medium was added to the mixture to a final volume of 10 ml. The mixture was incubated at 37°C with shaking at 220 rpm for 6 h. The 1 ml mixture was centrifuged at 12,000 × g for 1 min and filtered with a 0.45 μm filter. The phage titer was determined by the double-layer agar (DLA) method (Lee et al., 2019). The MOI with the highest phage titer was the optimal MOI. Three biological repeats were performed.

#### One-Step Growth Curve

As previously described, one-step phage growth was performed (Lu et al., 2013). EFa02 was cultivated to the early logarithmic stage (OD₆₀₀ = 0.5). The phage was mixed with EFa02 (10⁴ CFU/ml) at an MOI of 0.01 and incubated at 37°C with shaking at 220 rpm. Samples were taken at time points 0, 10, 20, 30, 40, 50, 60, and 90 min. The phage titer was measured using the DLA method. Three biological replicates were performed.

#### pH and Thermal Stability of EFap02

The BHI medium was adjusted with HCl or NaOH to pH 2.0–14.0. Then, 990 μl of BHI medium was mixed with 10 μl...
phage stock solution (3 × 10⁶ PFU/ml). After incubation at 37°C for 60 min, the phage titer was determined by the DLA method.

BHI medium (900 μl) was mixed with 100 μl phage stock solution (9 × 10⁹ PFU/ml) and treated at 4, 25, 37, 50, 60, and 70°C. After 60 min, the samples were removed and cooled to room temperature. The phage titer was determined by the DLA method (Ding et al., 2020).

**Sensitivity of EFap02 to Chloroform**
Phage EFap02 (10¹⁰ PFU/ml) was mixed with chloroform at ratios of 0, 10, 25, 50, 75, and 95%. The mixtures were incubated at 37°C with shaking at 220 rpm for 60 min. And the phage titer was determined using the DLA method (Oduor et al., 2020).

**Selection of Phage-Resistant Mutants**
Isolating phage-resistant mutants was performed as described previously (Yang et al., 2020). Briefly, 10 μl of E. faecalis culture (OD₆₀₀=0.2) was added to EFap02 (10⁹ PFU), and the mixture was placed on BHI agar and incubated at 37°C for 24 h. Then, the phage resistance of the colonies was validated by the DLA assay.

**Phage Genome Extraction, Sequencing, and Analysis**
The extraction of the phage genome was performed as previously described with a slight modification (Yuan et al., 2020). To remove contaminated DNA and RNA from the phage stocks, DNase I and RNase A were added to a final concentration of 1 μg/ml, and the phage stocks were incubated at 37°C for 60 min. EDTA (0.5 mol/l) was added to a final concentration of 0.02 mol/l. Proteinase K (20 g/l) and 10% SDS were added (final concentration 0.5%). The mixture was treated at 56°C for 60 min. An equal volume of balanced phenol (pH = 8.0) was added to extract nucleic acids, and then, the mixture was centrifuged at 10,000 × g for 5 min. The upper aqueous phase was transferred to a new 1.5 ml centrifuge tube. An equal volume of chloroform was added. The tube was gently mixed and centrifuged at 10,000 × g for 10 min. The supernatant was transferred to a new centrifuge tube, mixed with 0.6 volume of isooamyl alcohol, and placed at −20°C for 1 h. The mixture was centrifuged at 12,000 × g for 20 min to collect the DNA pellet. The DNA pellet was washed with 75% ice-cold ethanol and centrifuged at 12,000 × g for 20 min. Finally, the pellet was dried at room temperature, dissolved in 50 μl of water, and stored at −20°C for future use.

The Bacterial Genomic DNA Kit (Tiangen-DP302) was used for DNA extraction. The phage genome was sequenced using the Illumina HiSeq 2,500 platform (~1 Gbp/sample) and assembled with Newbler (Version 2.9) under default parameters. The characteristics of the phage genes were predicted by RAST² (Aziz et al., 2008) and FgeneSV. The homologous DNA sequences and proteins were searched and analyzed using BlastN and BlastP in the BLAST program. Visualization of the phage circular genome was performed using the CGView server database. Phage virulence factors were analyzed by searching a virulence database (Underwood et al., 2005). A comparative analysis of the EFa02 genomes and phage-resistant mutants was performed by Bresseq (Barrick et al., 2014). Analysis of phage DNA termini was performed using PhageTerm (Garneau et al., 2017), and the phylogenetic tree was constructed and displayed by MEGA 7.0 with the neighbor-joining method (Tamura et al., 2013).

**Bacterial Growth Curve and Construction of Enterococcus faecalis Strains**
EFa02 and EFap02 were cocultured for 72 h to explore the effect of phage on bacterial growth. The OD₆₀₀ of the culture medium was measured at given time points. Three biological replicates were performed. The gtr2 gene was amplified by PCR using the primers listed in Table 2 to complement gtr2 in EFa02R. The PCR product was ligated into the plasmid pGM23 by Gibson assembly to generate pGM-gtr2, and pGM-gtr2 was electroporated into EFa02R, and the strain was named EFa02R::gtr2.

**Phage Adsorption Assay and Efficiency of Plating Assay**
Phage adsorption tests were performed on different E. faecalis strains according to a previously reported protocol (Keski-Szeloch et al., 2013; Yang et al., 2020). The following steps were performed to verify the difference in the adsorption capacity of phage EFap02 to E. faecalis EFa02, EFa02R, and EFa02R::gtr2. The phage filtrate was diluted to 10⁹ PFU/ml in sterile water to form the initial titer. The host bacteria (1 ml) and phage (10⁴ PFU) were mixed and left in the EP tube for 5 min and centrifuged at 21,000 × g for 2 min. The phage titer was calculated by the DPA method (residual titer). The phage adsorption rate was calculated as [(initial titer − residual titer)/initial titer] × 100%. Three biological replicates were performed.

The phage was diluted in tenfold increments. Then, 100 μl of E. faecalis and 4 ml of BHI soft agar were mixed and poured onto a BHI agar plate. Then, 1 μl of the diluted phage was pipetted onto agar plates and incubated at 37°C overnight (Forti et al., 2018).

**Capsule Staining**
Bacterial was stained with crystal violet solution for 5 min and then stained with 20% copper sulfate solution. Then, the bacteria were observed under the microscope immediately.

**Biofilm Experiment**
Crystal violet staining was used to monitor biofilms (Forti et al., 2018). EFa02, EFa02R, and EFa02R::gtr2 were inoculated
in 1 ml of BHI medium and cultured at 37°C overnight. The OD₆₀₀ was measured, and the culture was diluted to an OD₆₀₀ of 0.02. A diluted bacterial solution (1 ml) was added to a 24-well plate and incubated at 37°C for 24 h, 48 h, or 72 h. The medium was then gently removed, and the wells were washed three times with 1 ml of phosphate-buffered saline (PBS). Finally, the plate was stained with 1 ml of 0.1% crystal violet at room temperature for 20 min. The excess crystal violet was carefully removed, washed with sterile water three times, and air-dried for 3 h. Then, 1 ml of 95% ethanol was added for 10 min, and the OD₅₇₀ of each well was measured.

**Statistical Analysis**

Statistical analysis was performed using one-way ANOVA or Student’s t-test. A p < 0.05 was considered statistically significant.

**RESULTS**

**Isolation and Identification of Bacteriophage Against Enterococcus faecalis**

Phage plaques were first discovered on the top agar lawn that contained a clinical strain of *E. faecalis* EFa02. Clear plaques, approximately 2 mm in diameter, were formed on the DLA plate (Figure 1A). The plaque was purified three times, and the isolated phage was named EFap02. Electron microscopy showed that phage EFap02 had an icosahedron head and a long tail of approximately 210 nm (Figure 1B). Phage EFap02 belonged to the Siphoviridae family according to the ICTV guidelines and was named vB_EFaS_EFap02.

**The Bactericidal Effect of Phage EFap02**

The highest phage EFap02 amount was 2.3 × 10⁶ PFU/mL when the MOI was 0.0001. Therefore, the optimal MOI of phage EFap02 is 0.0001 (Figure 1C). The one-step growth curve was measured to determine the latent period of phage EFap02 (Figure 1D). The phage titer increased 10 min after infection and peaked at 30 min, indicating a phage lysis time of approximately 30 min. These data suggest that EFap02 is effective at infecting EFa02. However, the EFap02 titer dropped to approximately 5 × 10⁴ PFU/mL after chloroform treatment (Figure 2C). These data indicate that EFap02 can tolerate a strong acid and alkali environment and high temperature but is partially sensitive to chloroform.

**Genomic Characterization of Phage EFap02**

The phage EFap02 genome structure is shown in Figure 3. The whole-genome sequencing results and phageTerm analysis results revealed that EFap02 had a linear double-stranded DNA, with a length of 39,776 bps and a (G + C) content of 35%. The complete genome sequence is available at GenBank (accession no. OL505084). The EFap02 genome sequence was searched as a query in the nucleotide database of the National Center for Biotechnology Information (NCBI). The results showed that the genome sequences of *Enterococcus* phage phiSHEF4 (GenBank accession no. NC_042022.1) and *Enterococcus* phage phiSHEF11 (GenBank accession no. OL799257.1) shared similar query coverage above 80% and identity above 91% with the EFap02 genome. The phylogenetic tree of EFap02 was constructed based on the nucleotide sequence terminase large subunit (ORF58), as shown in Figure 4. The result showed that EFap02 is closely related to the *Enterococcus* phage LY0323 (GenBank accession no. MH375074) genome, but was distantly related to the other phages included in the analyses. Based on these results, phage EFap02 could be considered as a novel phage. The EFap02 genome sequence encodes 60 predicted open reading frames (ORFs), 20 were functional genes, and 40 were hypothetical proteins (Table 3). No homologs of bacteriophage excisionases, repressors, integrases, or transposases were predicted in the EFap02 genome, supporting phage EFap02 as a lytic phage.

The EFap02 phage genome includes DNA replication and modification, transcription regulation, phage packaging, structural protein, and host lysis protein modules. ORF53 encodes phage DNA packaging protein in the packaging module. ORF58 encodes a large terminase subunit, an enzyme that inserts a single viral genome into the viral procapsid. ORF56, a portal protein, injects DNA into the host cell through a pathway formed by portal protein. Among the structural proteins, ORF49 encodes the phage major tail protein. ORF44 encodes the phage tail fiber. ORF45 encodes the phage tail assembly protein, and ORF47 encodes the phage tail tape measure protein. These structural proteins are involved in binding to the host bacterium. For the lysis module, ORF42 is annotated as N-acetylmuramoyl-L-alanine amidase, and ORF43 is annotated as holin, which can form micron-scale holes in the inner membrane. The phage releases

| Table 2 | Primers used in this study. |
|---|---|
| Primer use or name | Sequence (5′→3′) |
| Complementing with gtr2 pMG44-F | TTCAAAATTCTTGCGATTA |
| pMG44-R | COGCGGCTACGGATATT |
| pMG44-02R-F | AAAAACTCCGAGGGAATTTT |
| pMG44-02R-R | ATATCGTAGGGCGGTATT |

**Stability of Bacteriophage**

The stability of EFap02 was tested under different pH, temperature, and chloroform treatments. EFap02 was viable when the pH was 4–11 (Figure 2A). Moreover, EFap02 was viable at 60°C and was rapidly inactivated at 70°C (Figure 2B).
active endolysin (N-acetylmuramoyl-L-alanine amidase) into the periplasm to digest the peptidoglycan of \textit{E. faecalis}. The Virulence Search Database shows that none of the ORFs encode virulence factors or antibiotic resistance genes, indicating that EFap02 can be safely used to treat \textit{E. faecalis}-associated diseases.

**Isolation and Sequencing of the Phage-Resistant Strain EFa02R**

In the bacterial growth curve, the OD$_{600}$ of the phage–bacteria coculture increased 30h after the phage was added (Figure 5A). The culture medium became turbid due to the growth of
FIGURE 3 | Genome characterization of phage EFap02. Genome map of phage EFap02 and genetic characteristics. Circular genome visualization of EFap02 was performed using the CGView server database. Annotation of the specific function of ORFs was conducted using RAST and the BLASTP database. The first inner circle with the red histogram indicates the GC content, while the second inner circle with the purple and green histograms indicates the GC skew. The outer black circle indicates the predicted ORFs of phage EFap02.

FIGURE 4 | Phylogenetic relationships of phages infecting Enterococcus faecalis. Terminase large subunit and the neighbor-joining method were used to construct the phylogenetic tree.
phage-resistant bacteria. We then isolated a phage-resistant mutant named EFa02R. Since most resistant bacteria exhibit phage resistance mediated by receptor mutation (Labrie et al., 2010), an adsorption assay was conducted. The results showed that the adsorption rate of EFa02R decreased to approximately 20% (Figure 5B). After sequencing, it was found that resistant bacteria contain a mutation

| ORF  | Start | Stop  | Strand | Function                                              |
|------|-------|-------|--------|-------------------------------------------------------|
| ORF01| 481   | 110   | −      | Phage-associated homing endonuclease                 |
| ORF02| 681   | 481   | −      | hypothetical protein                                  |
| ORF03| 967   | 764   | −      | hypothetical protein                                  |
| ORF04| 1,524 | 1,679 | +      | hypothetical protein                                  |
| ORF05| 1,676 | 2,095 | +      | hypothetical protein                                  |
| ORF06| 2,061 | 2,648 | +      | hypothetical protein                                  |
| ORF07| 2,673 | 2,792 | +      | hypothetical protein                                  |
| ORF08| 2,805 | 2,984 | +      | hypothetical protein                                  |
| ORF09| 2,996 | 3,220 | +      | hypothetical protein                                  |
| ORF10| 3,217 | 3,429 | +      | hypothetical protein                                  |
| ORF11| 3,426 | 3,644 | +      | hypothetical protein                                  |
| ORF12| 3,641 | 3,856 | +      | hypothetical protein                                  |
| ORF13| 3,853 | 4,050 | +      | hypothetical protein                                  |
| ORF14| 4,145 | 5,725 | +      | hypothetical protein                                  |
| ORF15| 5,817 | 6,011 | +      | hypothetical protein                                  |
| ORF16| 6,018 | 6,338 | +      | hypothetical protein                                  |
| ORF17| 6,376 | 6,564 | +      | hypothetical protein                                  |
| ORF18| 6,637 | 7,060 | +      | hypothetical protein                                  |
| ORF19| 7,213 | 7,398 | +      | hypothetical protein                                  |
| ORF20| 7,382 | 7,552 | +      | hypothetical protein                                  |
| ORF21| 7,566 | 7,838 | +      | hypothetical protein                                  |
| ORF22| 7,840 | 8,046 | +      | hypothetical protein                                  |
| ORF23| 8,030 | 8,404 | +      | hypothetical protein                                  |
| ORF24| 8,397 | 9,689 | +      | hypothetical protein                                  |
| ORF25| 9,730 | 10,269| +      | HNH homing endonuclease                               |
| ORF26| 10,306| 10,500| +      | hypothetical protein                                  |
| ORF27| 10,514| 11,254| +      | hypothetical protein                                  |
| ORF28| 11,254| 11,483| +      | hypothetical protein                                  |
| ORF29| 11,630| 12,154| +      | hypothetical protein                                  |
| ORF30| 12,211| 12,842| +      | hypothetical protein                                  |
| ORF31| 12,853| 13,632| +      | hypothetical protein                                  |
| ORF32| 13,653| 14,130| +      | hypothetical protein                                  |
| ORF33| 14,277| 15,096| +      | hypothetical protein                                  |
| ORF34| 15,096| 15,386| +      | hypothetical protein                                  |
| ORF35| 15,387| 15,632| +      | hypothetical protein                                  |
| ORF36| 15,713| 16,420| +      | hypothetical protein                                  |
| ORF37| 16,490| 16,714| +      | hypothetical protein                                  |
| ORF38| 16,749| 19,040| +      | hypothetical protein                                  |
| ORF39| 19,107| 19,400| +      | hypothetical protein                                  |
| ORF40| 19,412| 19,618| -      | hypothetical protein                                  |
| ORF41| 20,805| 19,708| -      | Phage lysis, N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28) |
| ORF42| 21,041| 20,808| -      | Phage holin                                           |
| ORF43| 21,041| 20,808| -      | Phage tail fiber                                      |
| ORF44| 21,301| 21,056| -      | Phage tail assembly protein                           |
| ORF45| 23,711| 21,483| -      | Phage protein                                         |
| ORF46| 25,830| 23,755| -      | Phage protein                                         |
| ORF47| 30,144| 25,912| -      | Phage major tail protein                              |
| ORF48| 30,712| 30,401| -      | Phage major tail protein                              |
| ORF49| 31,451| 30,888| -      | Phage major tail protein                              |
| ORF50| 31,895| 31,530| -      | Phage major tail protein                              |
| ORF51| 32,299| 31,692| -      | Phage protein                                         |
| ORF52| 32,631| 32,296| -      | Phage protein                                         |
| ORF53| 32,902| 32,603| -      | Phage DNA packaging protein                           |
| ORF54| 34,453| 33,257| -      | Phage major capsid protein of Caudovirales           |
| ORF55| 35,091| 34,528| -      | Phage maturation protease                             |
| ORF56| 36,229| 35,078| -      | Phage portal (connector) protein                      |
| ORF57| 36,398| 36,234| -      | hypothetical protein                                  |
| ORF58| 38,189| 36,468| -      | Phage terminase, large subunit                        |
| ORF59| 39,076| 38,603| -      | hypothetical protein                                  |
| ORF60| 39,277| 39,077| -      | hypothetical protein                                  |
in the gtr2 gene. The mutant site changed from AGC to ATC, and the amino acid changed from serine to isoleucine (Figure 5C).

### Identification of the Phage EFap02 Receptor

The gtr2 gene is related to the synthesis of capsular polysaccharide on the surface of *Acinetobacter baumannii* (Kenyon and Hall, 2013; Singh et al., 2018). We also performed capsule staining of EFa02, EFa02R, and EFa02R::gtr2, which showed that EFa02 and EFa02R::gtr2 have the capsule, but EFa02R lost the capsule (Figure 6A). Furthermore, we performed a phage adsorption experiment to test whether capsular polysaccharide is the receptor for EFap02. We found that the adsorption capacity of EFa02R dropped dramatically to 23.9%, and the adsorption capacity recovered to 89% after complementing the gtr2 gene (Figure 5B). Additionally, EOP testing showed that EFa02R::gtr2 was resensitive to EFap02 (Figure 5D). These experiments suggest that the mutation of gtr2 leads to the loss of capsular polysaccharides and prevents EFap02 from adsorbing to EFa02R.

**EFa02R Is Impaired in Biofilm Formation**

We measured the biofilm formation of these strains on the polystyrene surface after 24, 48, and 72 h of incubation. At each time point, the biofilm of the resistant mutant EFa02R was much less than that of the wild-type strain EFa02 and the complemented strain EFa02R::gtr2 (Figure 6B), indicating that the phage-resistant mutant had impaired biofilm formation.

### DISCUSSION

Phage therapy has been used to treat *E. faecalis* infectious diseases (Letkiewicz et al., 2009; Bolocan et al., 2019), as well as many other diseases. For example, phages can treat alcoholic hepatitis by killing the *E. faecalis* that produces cytolysin which leads to hepatocyte lysis (Duan et al., 2019). Thus, the characterization of *E. faecalis* phages is important for treating *E. faecalis*-associated diseases.

Identification of the phage receptors is essential for the rational selection of phages for therapy. The *E. faecalis* phage receptors had been described previously. Duerkop et al. found that phage infection of *E. faecalis* requires a predicted integral membrane protein named PIP_{EF} (phage infection protein of *E. faecalis*). PIP_{EF} is an integral membrane protein that spans the membrane six times (Duerkop et al., 2016). In 2018, Ho et al. showed that mutation of the glycosyltransferase epaR leads to the resistance to phage NPV1 by preventing phage adsorption (Ho et al., 2018). And these phage receptors are cell wall-associated structures that are important for cell viability under external stresses (Canfield and Duerkop, 2020).

In this study, the isolated EFap02 phage is stable under high temperatures and other conditions, making it convenient to store
and transport for future clinical use. Although EFap02 has strong lytic activity, we observed the rapid emergence of phage-resistant mutants. Resistance is an issue in phage therapy, and its mechanisms are diverse. The receptor mutation is the most likely to occur, and it prevents phage adsorption (Labrie et al., 2010). In this study, the phage-resistant strain EFa02R had loss-of-function mutations in the glycosyltransferase gene Group 2 family responsible for the biosynthesis of capsular polysaccharides (Kenyon and Hall, 2013; Singh et al., 2018). We confirmed that the gtr2 mutation in EFa02 causes phage resistance and that the capsular polysaccharide is the phage receptor. Identifying phage receptors is essential for the rational development of phage cocktails (Gordillo Altamirano and Barr, 2021).

The loss of receptors prevents phage adsorption; however, there is always a trade-off for phage resistance. We observed that the capsular polysaccharide-loss phage-resistant mutant reduced the formation of biofilms. Previous studies found that the formation of biofilms increased the resistance to antibiotics (Ch’ng et al., 2019). Capsular polysaccharide loss may make bacteria resensitive to some antibiotics, which may allow for clearance of the bacteria by the body’s immune system or antibiotics (Gordillo Altamirano and Barr, 2021).

In summary, this study revealed the biological and genomic characteristics of a phage EFap02 and identified its receptor as the capsular polysaccharide. This study suggests EFap02 as a potential phage therapy agent.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/genbank/, OL505084.

AUTHOR CONTRIBUTIONS

JL, QZ, KX, and JW conceived and designed the experiments. JL performed the experiments. JL, YZ, YLi, and YLu analyzed the data. JL, KX, QZ, and JW wrote the paper. All authors contributed to the article and approved the submitted version.

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