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The lytic bacteriophage vB_EfaH_EF1TV, a new member of the *Herelleviridae* family, disrupts biofilm produced by *Enterococcus faecalis* clinical strains.

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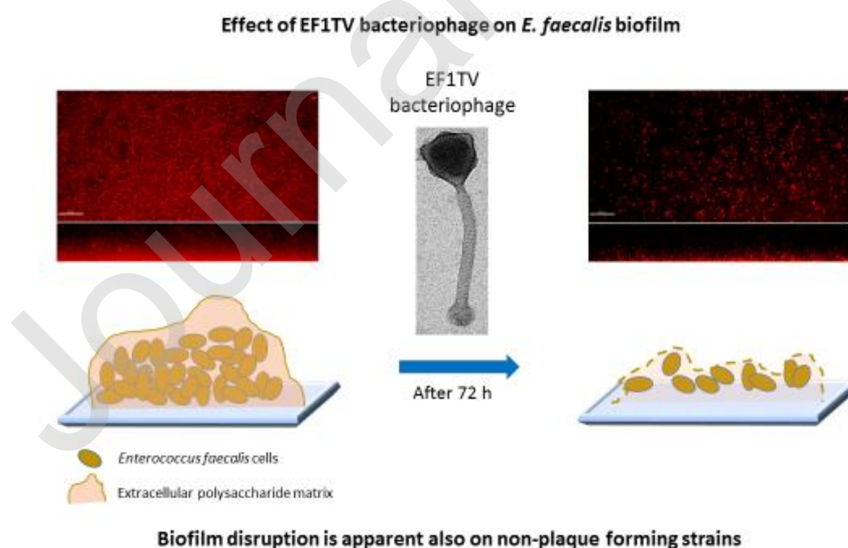
Graphical abstract



Highlights

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- EF1TV is a newly discovered lytic bacteriophage specific for *Enterococcus faecalis*.
- Phage EF1TV is the fourth characterized member of the *Kochikohdavirus* genus within the *Herelleviridae* family.
- EF1TV disrupts biofilm produced by strains of *Enterococcus faecalis*, even if they are not able to produce productive infection.
- Integrity of the enterococcal polysaccharide antigen gene cluster is necessary for the infectivity of EF1TV.

Abstract

Objectives: The aim of this study is to characterize a new bacteriophage able to infect *Enterococcus faecalis*, and to evaluate its ability to disrupt biofilm.

Methods: The vB_EfaH_EF1TV (EF1TV) host-range was determined by spot test and efficiency of plating using a collection of 15 *E. faecalis* clinical strains. The phage genome was sequenced with a next generation sequencing approach. Anti-biofilm activity was tested by crystal violet method and confocal laser scanning microscopy. Phage-resistant mutants were selected and sequenced to investigate receptors exploited by phage for infection.

Results: EF1TV is a newly discovered *E. faecalis* phage which belongs to the *Herelleviridae* family. EF1TV, whose genome is 98% identical to φEF24C, is characterized by a linear dsDNA genome of 143,507 bp with direct terminal repeats of 1,911 bp. The phage is able to infect *E. faecalis* and shows also the ability to degrade biofilm produced by strains of this species. The results were confirmed by confocal laser scanning microscopy analyzing the biofilm reduction in the same optical field before and after phage infection.

Conclusions: The EF1TV phage shows promising features such as an obligatory lytic nature, an anti-biofilm activity and the absence of integration-related proteins, antibiotic resistance determinants and virulence factors, and therefore could be a promising tool for therapeutic applications.

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Keywords: Phage therapy; biofilm degradation; *Herelleviridae* family; SPO1-like phage; bacteriophage resistance.

1. Introduction

Enterococci are aerotolerant anaerobic Gram-positive bacteria that are part of the human and animal microbiota. In recent years, members of this genus have emerged as important causes of nosocomial and community-acquired infections [1]. Of the over 20 *Enterococcus* species [2], *Enterococcus faecalis* is particularly pathogenic to man and causes 85–90% of all enterococcal infections [3], including urinary tract, wound and endodontic infections, endocarditis and bacteremia. The ability to produce biofilm is a very common feature among isolates from infected patients, and many data suggest that biofilm formation is an important virulence factor in *E. faecalis* pathogenesis, especially in several chronic infections including cystic fibrosis, urinary infections, otitis and periodontitis [4], [5], [6]. Bacterial biofilms are communities composed of one or more bacterial species adhering to a surface and embedded in a hydrated matrix mostly constituted by extracellular polymeric substances (EPS) of bacterial origin [7]. The EPS matrix gives to bacterial cells an extra-protection from the immune response and limits also the penetration and diffusion of antibiotics and disinfectants [4]. Indeed, it was demonstrated that the antibiotic tolerance of bacterial cells embedded in biofilm could increase up to thousand folds compared to that of planktonic cells [8]. Altogether, these observations emphasize the need to develop new therapeutic approaches for the control of pathogenic bacteria that should not be only limited to planktonic cells. Two main different approaches are currently being developed: i) limit the adhesion and formation of biofilm and ii) target biofilm tolerance by killing also persister cells [9]. A third promising approach involves bacteriophages [10], [11] by exploiting their ability to produce polysaccharide depolymerases that can degrade the biofilm-associated EPS matrix [12]. These

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enzymes, that usually are part of phage structural proteins such as tail fibers and base plates, could promote both phages and antibiotics diffusion through the biofilm matrix, thus facilitating their access to bacterial cells.

In this study, vB_EfaH_EF1TV (abbreviated hereinafter EF1TV), a newly discovered *E. faecalis* phage, was isolated and characterized. In addition, we demonstrated that this phage has the ability to target biofilm produced *in vitro* by *E. faecalis* clinical strains.

2. Material and methods

2.1. Bacterial strains and growth conditions

The *E. faecalis* clinical strain 1/1112 was used as host for phage isolation and propagation. This strain was isolated during 2014 from a blood sample processed at the Careggi University Hospital (Florence, Italy), showed a multi-drug resistant (MDR) phenotype (resistance to levofloxacin, vancomycin and teicoplanin) and belonged to Sequence Type (ST) 6. A collection of 15 *E. faecalis* and 5 *E. faecium* clinical strains obtained from the same center was employed to determine the EF1TV host-range. Additional reference strains obtained from American Type Culture Collection (ATCC) or Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), and used for the host-range determination are reported in Table 1.

Tryptic Soy Broth (TSB) or Tryptic Soy Agar (TSA) (Liofilchem srl, Italy) were used to grow bacterial strains. Soft agar for double-layer plating was composed by TSB plates solidified with 0.7% agar. SM buffer (10 mM Tris-HCl, pH 7.5; 100 mM NaCl; 10 mM $MgSO_4$) was used for suspending and titrating bacteria and phages.

2.2. Phage isolation and propagation

A single colony of *E. faecalis* 1/1112 was cultured overnight (O/N) in 20 ml of TSB medium at 37°C with shaking. After centrifugation, the bacterial pellet was suspended in half of the

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initial volume in SM buffer. To select phages, an aliquot of 50 ml of wastewater samples collected at the “Tor Vergata” University Hospital (Rome, Italy) was centrifuged at 4,700 x *g* for 10 min at 25 °C and then filtered through 0.2 µm syringe filter. A 500 µl volume of this suspension was then mixed with 0.2 ml of indicator bacteria and plated by standard double-layer-agar method. To obtain pure bacteriophages preparations, three rounds of infection and picking of isolated plaques were performed. According to the systematic naming scheme proposed by Kropinski et al.[13], the obtained phage was named vB_EfaH_EF1TV, abbreviated EF1TV. Phage propagation was performed as previously described [14]. The phage lysate was centrifuged 100 min at 18,000 rpm in Beckman JA-20 rotor, the pellet suspended in SM buffer and stored at 4°C.

2.3. Electron microscopy

Phage morphology was examined by transmission electron microscopy (TEM) of negatively stained preparations as previously described [15].

2.4. Bacteriophage host range

The EF1TV host range was determined by both spot test and efficiency of plating (EOP) analyses on the bacterial strains listed in Table 1. In the spot tests, 10^8 phage particles were spotted on the bacterial lawn of each tested strain. Lytic activity was recorded after 18 h of incubation at 37°C using a scale ranging from absence of lysis (-), turbid spot (+) and clear spot (++). To determine the EOP, EF1TV was tested at different dilutions ranging from 10^8 to 10^3 PFU/ml against all the strains displaying turbid or clear zones in the spot test. For this purpose, bacterial strains were grown O/N in TSB at 37°C and, following incubation, 200 µl of bacterial suspension were infected with 100 µl of diluted phage and plated. Plates were incubated O/N at 37°C and PFUs were enumerated. Spot test and EOP were repeated three

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times for each bacterial strain. The EOP was finally computed as the ratio between average PFU on target bacteria and average PFU on the *E. faecalis* 1/1112 strain.

2.5. Selection of phage-resistant mutants

Two independent cultures of *E. faecalis* ATCC 19433 were prepared in TSB starting from single colonies. Bacterial suspensions were incubated O/N at 37°C, diluted 1:100 in 20 ml of fresh TSB and further incubated until an OD_{600} value of 0.4 was reached. Cultures were then infected with 2 x 10^{10} PFU, incubated for an additional 48 h and finally plated on TSA. Two randomly selected colonies, one for each infected culture, were picked-up and analyzed by spot assay to confirm the phage resistance phenotype. The phage-resistant mutants were named 19433-FR1 and 19433-FR2.

2.6. DNA extraction

Bacteriophage DNA was extracted from phage lysate (4 x 10^{10} PFU/mL) by using the Phage DNA Isolation Kit (Norgen Biotek Corp., Canada) following manufacturer instructions. At the end of the extraction procedure, DNA samples were dissolved in 100 µL of sterile ddH_2O. Whole bacterial DNA was extracted from O/N cultures of phage resistant mutants by using the phenol-chloroform method [16]. Aliquots of all DNA preparations were resolved by agarose gel electrophoresis (0.75% w/v) followed by staining with ethidium bromide (0.05 µg/ml). All DNA samples were quantified by using a Qubit fluorometer (Thermo Scientific) and stored at 4°C.

2.7. Genome sequencing and bioinformatics analysis

Bacteriophage genome was sequenced with the MiSeq instrument (Illumina Inc., San Diego, CA, USA) and a paired-end approach (2 x 300 bp) by using the kit Illumina Nextera XT™. Raw reads were assembled by using the SPAdes software [17]. Topology of the phage

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genome has been inferred with PhageTerm [18]. Direct Sanger sequencing was performed on PstI restriction fragments containing the estimated 5' and 3' genome ends to validate software predictions (data not shown). Phage genome was annotated by using the Rapid Annotation using Subsystem Technologies (RAST) web-service [19]. Automatic annotation was manually reviewed by BLASTP analysis against RefSeq proteins deposited in the INSDC databases. The on-line instance of tRNAScan-SE (http://lowelab.ucsc.edu/tRNAscan-SE/) was used to identify phage tRNA genes, while host tRNA genes were searched using ARAGORN [20]. Phage lifestyle was predicted using Phage Classification Tool Set (PHACTS) [21]. Comparison of phage genomes was performed by using EasyFig [22]. Genomes of phage-resistant mutants 19433-FR1 and 19433-FR2 were sequenced with the Illumina HiSeq 4000-PE150 platform and a 2x150 paired ends approach at an external facility (Beijing Novogene Bioinformatics Technology Co., Ltd). Raw reads were assembled by using the SPAdes software [17]. Nodes from assemblies of phage resistant mutants were then compared with the genome of ATCC 19433 (assembly accession number: **GCA_000392875.1**) by BLASTN analysis to find out genome variants. Genes that following this analysis were found to be different between a given mutant and its parental strain were further filtered out to remove results likely due to alignment or sequencing errors, using criteria as previously described [23].

2.8. Influence of pH and temperature on phage viability

To assess the stability of EF1TV to pH variations, phages were suspended in 1 ml of SM buffer (final concentration 1 x 10^7 PFU/ml), previously adjusted with 1 M NaOH or 1 M HCl to yield a pH range from 2.0 to 12.0. After 60 min of incubation at 25°C, samples were serially diluted and tested against *E. faecalis* 1/1112 by using the double-layer-agar method. The effect of temperature on phage viability was assessed by incubating 1 ml of phage

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suspension at 1 x 10^7 PFU/ml at 40, 50, 60 and 65°C for 60 min. Phage suspensions were then titrated. Both assays were carried out in triplicate and results are reported as the mean of phage counts (PFU/ml) ± standard deviation.

2.9. One-step growth curve

For the one-step growth experiment, *E. faecalis* 1/1112 was incubated at 37°C to the mid-exponential phase. An aliquot of 0.9 ml was infected with 0.1 ml of 1 x 10^7 PFU/ml phage stock (MOI 0.01). After 5 min at 37°C, the mixture was centrifuged twice at 12,000 x *g* for 1 min to remove the non-adsorbed phages. The pellet was finally suspended in 1 ml of SM buffer, diluted 1 x 10^{-3} in 10 ml of TSB and incubated at 37°C with shaking. Aliquots of 0.1 ml were then taken at 5 or 10 min intervals and phage titre was determined. The experiment was carried out in triplicate. The latent period was computed excluding the 5 min of phage adsorption and the 5 min centrifugation interval needed to remove non-absorbed phages. The burst size was computed as the ratio of the average phage titre after the rise period to the average of infected bacterial cells during the latent period.

2.10. Biofilm degradation assay of EF1TV

Biofilm degradation assay was performed as previously described [24]. Each *E. faecalis* strain (*n*=16) was grown in 5 ml of TSB O/N at 37°C. Biofilm production assay was performed on 96-well microtiters plates. O/N cultures were diluted in TSB (25 µl in 2.5 ml), vortexed and aliquots of 250 µl were dispensed in two columns of the microtiter plate (test and control columns), covered and incubated 3 days at 37°C in the dark. After incubation, 10 µl containing 10^9 EF1TV phages were added to the test wells (MOI=1) and then plates were incubated for two additional days at 37°C. An aliquot of 15 µl of crystal violet 0.1% was then added to the test and control wells and, after 30 minutes of staining at room temperature in the dark, wells were rinsed 3 times with 250 µl of SM and dried for 1 h. Subsequently, 300 µl of

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95% ethyl alcohol were added to each well and plates were incubated at 4°C for 1 h. The absorbance of wells at 595 nm was finally measured by using a microplate reader (Benchmark Microplate Reader, Bio-Rad, California). The biofilm degradation due to phage activity was evaluated as the difference of the average absorbance between the control and test wells. Assays included seven technical replicates for each strain, and a total of three biological replicates were performed . Statistical significance was assessed by using the One-Way ANOVA test (https://www.socscistatistics.com/tests/anova/default2.aspx).

2.11. Confocal microscope analysis

E. faecalis strains 4/4692 and 11/8181 were cultured in 2.0 ml of TSB for three days at 37°C in uncoated 8-wells µ-slides (glass bottom 170 µm thick; IBIDI, GMBH company, Germany). After three days, cells were infected with EF1TV at MOI 1, incubated for three additional days at room temperature and then analyzed. Images were obtained using a confocal laser scanning microscope Olympus FV 1000 (Olympus, Shinjuku, Tokyo, Japan) equipped with 60x oil immersion objective (numerical aperture: 1.35). In order to preserve the viability of the microbial biofilm for the phage treatment, images were acquired at 635 nm laser line (550-650 filter emission) exploiting light naturally reflected by bacterial cells without any staining [25], [26]. The native biofilms were approximately 30-40 µm thick, and Z-slices were obtained every 0.80 µm, for 80 optical sections in total. The same field was acquired before and after 72h of phage treatment. Images elaboration with orthogonal projection and 3D rendering with isosurface for volume and intensity measurement was carried out by Imaris software (version 6.2.1, Bitplane, Zurich, Switzerland). To determine thickness size, isosurfaces of volume adhering to the substrate were considered in both samples.

2.12. Nucleotide sequence accession numbers

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The nucleotide sequence of vB_EfaH_EF1TV was deposited in the DDBJ/EMBL/GenBank databases under the accession no. **MK268686.1**.

3. Results

3.1. Phage isolation and host range determination

EF1TV was isolated from wastewaters of "Tor Vergata" University hospital in Rome by using the agar overlay method and *E. faecalis* 1/1112 as indicator strain. The EF1TV host range was determined by using a recent collection of non replicate, non consecutive *E. faecalis* clinical isolates. A spot test analysis was used for the determination of the phage bactericidal activity. As reported in Table 1, all the *E. faecalis* strains were sensitive to the phage infection at the concentration of 10^8 PFU/spot, even if with differences in the turbidity of lytic zone. Productive infection and plaquing ability of EF1TV was assessed by efficiency of plating analysis (EOP) [27], which demonstrated that only strains exhibiting a clear spot in the spot assay (8/17) were able to form evident plaques (EOP values between 0.44 and 1).

3.2. Electron microscopy

EF1TV morphology was studied by transmission electron microscopy (Fig. 1). Results from this experiment demonstrated that EF1TV showed a typical morphology of myovirids (Ackermann's viral morphological group A1). The phage is characterized by an isometric head of 87±4 nm with clearly visible capsomers. The long contractile tail is 191±12 nm in length, 19.5±0.7 nm in width and ends with a complex baseplate structure exhibiting the double disk morphology upon contraction seen in many SPO1-related phages (Fig. 1B). The contracted tail is 26.3±1.7 nm wide. Altogether these features suggested that EF1TV is a member of the newly proposed *Herelleviridae* family [28].

3.3. Bioinformatics analysis of phage genome

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Reads obtained from phage genome sequencing were assembled using the SPAdes software into 3 nodes. Analysis by BLASTN software using the nr database (http://blast.ncbi.nlm.nih.gov/) revealed that the longest node (141,324 bp; 133X coverage) represented a draft of the phage genome. Analysis with the PhageTerm software suggested that the very 5' and 3' ends of the phage genome were characterized by two long direct terminal repeats (DTRs) of 1,911 bp. This prediction was confirmed by direct Sanger sequencing of terminal fragments of the phage genome obtained by digestion with the PstI restriction enzyme (data not shown). The phage genome therefore consisted of a linear dsDNA of 143,507 bp (% GC= 35.8), characterized by two long DTRs, in which at least 7 tRNA organized in a modular fashion and distributed between ORF67 and ORF68 can be detected. Comparison of the EF1TV genome with sequences deposited in nucleotide databases revealed that the closest homologs were φEF24C (98.0% overall nucleotide identity) and φECP3 (97.5% overall nucleotide identity) (Fig. 2), demonstrating that EF1TV was a SPO1-like virus member of the recently described *Herelleviridae* family [28].

The presence of domains possibly involved in the degradation of bacterial cell-wall was detected in four proteins (ORF78, ORF80, ORF98 and ORF99) which were homologous of the φEF24C ORF9 and ORF10 endolysins (98% and 98% similarity) and of the ORF29 and ORF30 lysins (99% and 99% similarity), respectively. Conversely, no significant similarity with known antibiotic resistance, virulence or toxin genes, or with elements commonly associated with lysogeny (*i. e.*, integrases, repressors and anti-repressors) was revealed.

3.4. One-step of EF1TV

The one-step growth curve of EF1TV on *E. faecalis* 1/1112 was determined (Fig. 3). The triphasic curve obtained shows a latent period (excluding 10 min of pre-treatment) of 15 min, followed by a rise period of about 15 min. The average burst size, computed as the ratio of

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released phages at plateau to the infected bacterial cells at the latent period, was about 27 PFU/infected cell.

3.5. Influence of pH and temperature on phage viability

The effect of different pH values on the infectivity of EF1TV was assessed. The maximal infectivity has been observed at pH 7.0 (Fig. 4A), even if the phage retains almost 100% activity also after one hour incubation at pH 6.0, while a 60% activity was observed at pH 8.0. At pH 4.0 and pH 11.0 EF1TV still retains a good infectious activity of 36% and 31% respectively. Extreme pH values have negative effects on the phage stability as no plaques were observed after incubation at pH 2.0, and a residual activity of 0.02% was retained after incubation at pH 12.0. In order to evaluate heat-resistance EF1TV was incubated at 40, 50, 60 and 65°C for 60 min (Fig. 4B). The phage is highly stable up to 50°C without significant reduction of infectivity, but there was almost 2 log reduction in titre after exposure to 60°C, and a more drastic reduction of 5 log after exposure at 65°C.

3.6. Biofilm susceptibility to EF1TV

The ability of EF1TV to degrade biofilm of *E. faecalis* strains was assessed as described above. Tested strains produced different amounts of biofilm biomass, as demonstrated by OD_{595} values with the crystal violet assay that ranged from 0.32 to 1.36. Treatment with EF1TV was always able to reduce biofilm, even if with different extents (9%-68%). Interestingly, degradation of biofilm by EF1TV was observed with either plaque-forming and non-plaque-forming strains (Fig. 5).

3.7. Confocal microscopy analysis of biofilm digestion

The crystal violet assay showed that strains 4/4692 and 11/8181 produced the highest levels of biofilm. At the same time, these strains showed also the highest reduction of biofilm

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biomass after EF1TV infection. For these reasons, both strains were chosen for further characterization by confocal laser scanning microscopy analysis of the biofilm *in vivo*. Results from these experiments, reported in Figure 6, revealed a strong reduction of reflected light from bacteria after phage treatment for both the plaque-forming 4/4692 and the non-plaque-forming 11/8181 strain. The signal intensity of non-infected control strains did not change after three and six days of incubation.

3.8. Genomic analysis of phage resistant mutants

The whole-genome sequence of two randomly selected phage resistant derivatives of *E. faecalis* ATCC 19433, namely 19433-FR1 and 19433-FR2, was obtained. Comparison of the draft genomes of these two mutants with ATCC 19433 revealed that the only significant differences observed affected the same chromosomal locus. In particular, 19433-FR1 displayed a 1 bp deletion (A638del) in the gene encoding for the EpaR sugar transferase, while 19433-FR2 was characterized by a large deletion that included part of *epaR* and extends for 16,214 bp downstream of this gene. Taken together these results suggest that the integrity of the enterococcal polysaccharide antigen (*epa*) gene cluster is necessary for the infectivity of EF1TV, as recently demonstrated by Ho et al. for the ΦNPV1 phage [29].

4. Discussion

SPO1-related phages infect *Firmicutes*, are characterized by an obligatory virulent lifestyle, a broad host range and are non-transducing, with the exception of the *Bacillus* phage CP-51 [30]. Given these features such phages are promising candidates for therapy against pathogenic *Firmicutes* [31]. In this work we describe EF1TV, a lytic phage that infects clinical isolates of *E. faecalis*. According to its morphology and genome sequence, EF1TV represents a newly discovered member of the recently proposed *Herelleviridae* family

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(previously known as *Spounavirinae* subfamily within the family of *Myoviridae*). Indeed, EF1TV shares a high identity with other lytic phages against *E. faecalis* such as φEF24C [32], EFLK1 [33] and φECP3, and an analysis with the VICTOR web service clearly demonstrated that EF1TV is the fourth characterized member of the *Kochikohdavirus* genus (data not shown). EF1TV has a linear non-permuted genome of 143,507 bp characterized by DTR of 1,911 bp. To our knowledge, these DTRs are shorter than those of any other SPO1-related phages. EF1TV has been selected by using the *E. faecalis* 1/1112 strain, a representative of the ST6 genetic lineage. *Enterococcus faecalis* of ST6, which are part of the hospital adapted clonal complex (CC) 2, are one of the leading lineages frequently associated to nosocomial infections in several European countries including Spain, Portugal and The Netherlands, and are also characterized by a high prevalence of major antibiotic resistance mechanisms and a prominent ability to acquire exogenous genes via recombination [34]. These observations make this bacteriophage a promising tool for targeted clinical applications against serious infections caused by *E. faecalis*. The phage host range was evaluated on *E. faecalis, E. faecium* and *S. aureus* strains, and similarly to φEF24C, EF1TV is able to infect only *E. faecalis*. All *E. faecalis* strains were sensitive to the phage infection by spot testing but with a different turbidity of the lytic zones. Since it is well known that the host range determined by spot testing is potentially broader than that determined by other techniques [35], we determined also the phage plaquing ability and, according to EOP, only 8 out of 17 strains were sensitive. As previously observed, these differences could depend by mechanisms such as abortive infection or lysis from without [35].

Bacterial biofilms play a key role in the pathogenesis of many human infections and represent a relevant challenge for their treatment [36]. Antibiotics have not been specifically developed to target microbial biofilm infections, and their activity is often limited in such cases. The development of novel anti-biofilm strategies is therefore essential, and phages could represent

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an interesting option, giving their ability to eradicate biofilms and clear infections by working in synergy with antibiotics [37]. The biofilm dispersing ability can depend either by the expression of depolymerizing enzymes that degrade the EPS or by the capacity to infect metabolically inactive persister cells [38]. EF1TV, similar to other SPO1-like phages of *E. faecalis* [33], [39], has an outstanding ability to disrupt biofilm and its effect is more evident on the high biofilm-producer strains. In addition, it is relevant to notice that EF1TV acts on biofilm produced by both plaque-forming and non-plaque-forming strains.

Finally, from the analysis of two spontaneous EF1TV-resistant mutants we identified *epaR* as a gene associated to the receptor implicated in phage adsorption. The *epaR* gene encodes a putative glycosyltransferase that has already been described as required for φNPV1 adsorption [29]. These results suggest that either the polysaccharide produced by the *epa* cluster or the EpaR protein itself could be the receptor of EF1TV, even if this hypothesis will be the subject of future complementation experiments aimed to confirm the role of this gene cluster in the phage infectivity. This information is relevant for the formulation of phage cocktails using phages having different receptor specificities in order to reduce the appearance of phage resistant mutants.

In conclusion, EF1TV collects the characteristics that are considered significant for use in phage therapy ranging from the lytic nature to the biofilm-degrading potential, and for these reasons could be a promising tool for therapeutic applications.

Declarations

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Competing Interests: None declared.

Ethical Approval: Not required.

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Figure captions

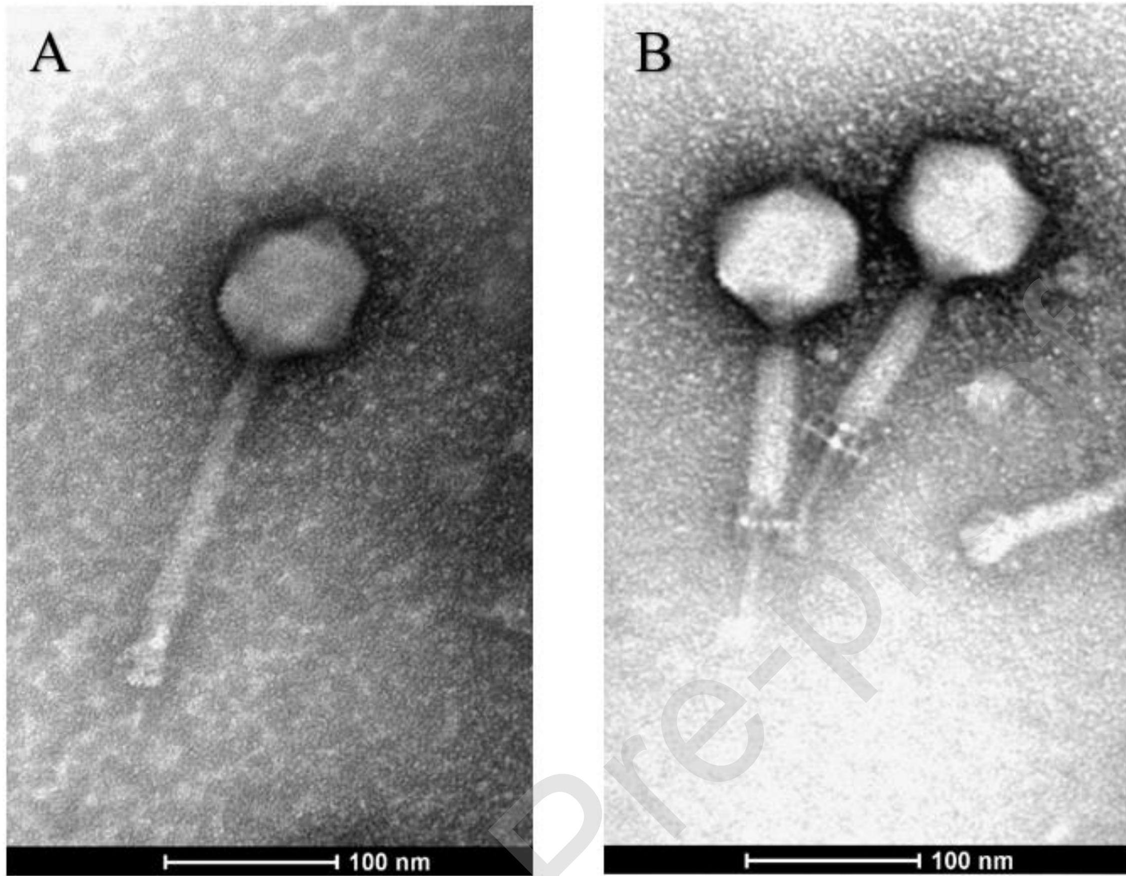

Figure 1. Transmission electron micrographs of EF1TV phage particles, with extended (A) or contracted tail (B). Bars represent 100 nm.

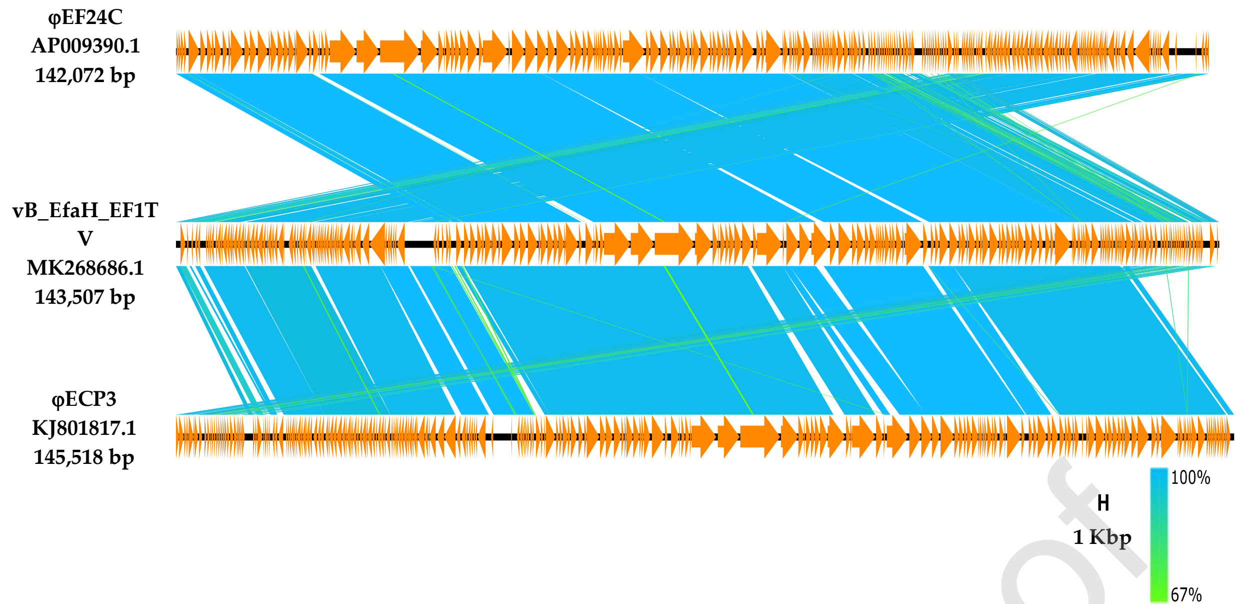

Figure 2. Genome comparison of EF1TV with the φEF24C (upper part) and the φECP3 (lower part) bacteriophages. ORFs are represented by orange arrows. The level of nucleotide identity between phage genomes is indicated by a colour gradient.

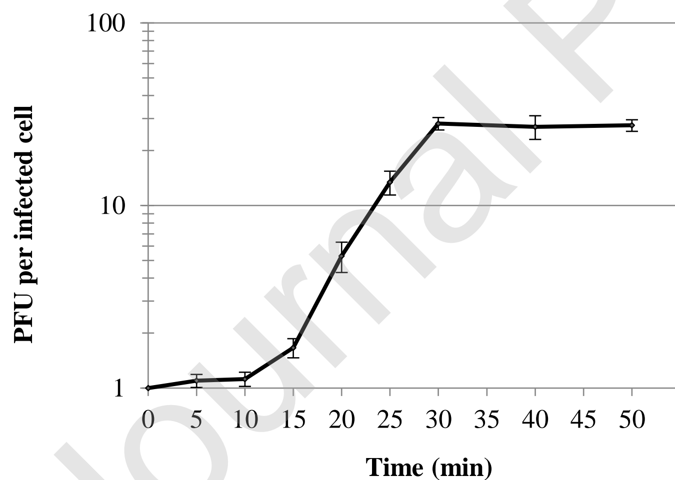

Figure 3. One-step growth curve of EF1TV on *E. faecalis* 1/1112. PFU per infected cell at different time points are shown. Each data point is the mean of three experiments ± SD.

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Figure 4

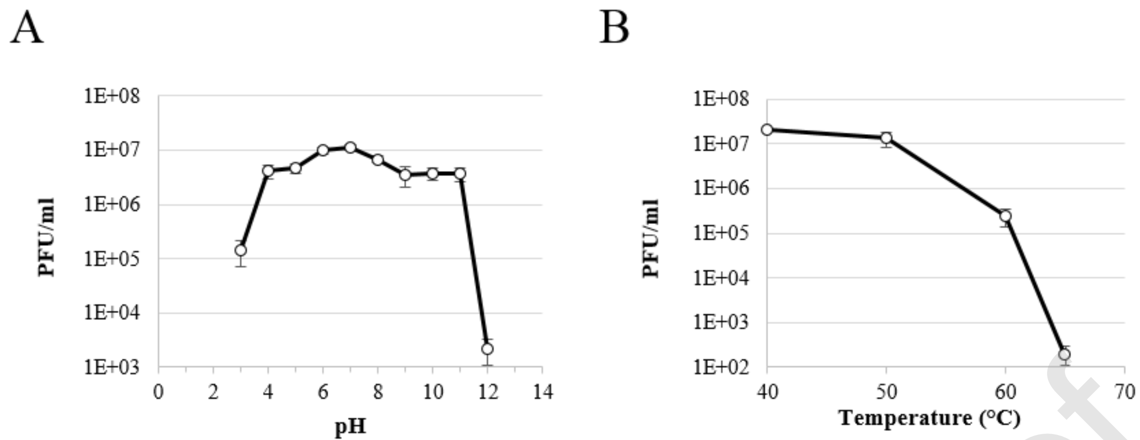

Figure 4. Effect of pH and temperature on the stability of EF1TV. A) Phage was incubated for 1 h at different pH values before determining the number of active infectious phage particles. (B) Surviving phage after 1 h exposure to different temperatures. Each data point is the mean of three experiments. Standard deviation is reported by vertical lines.

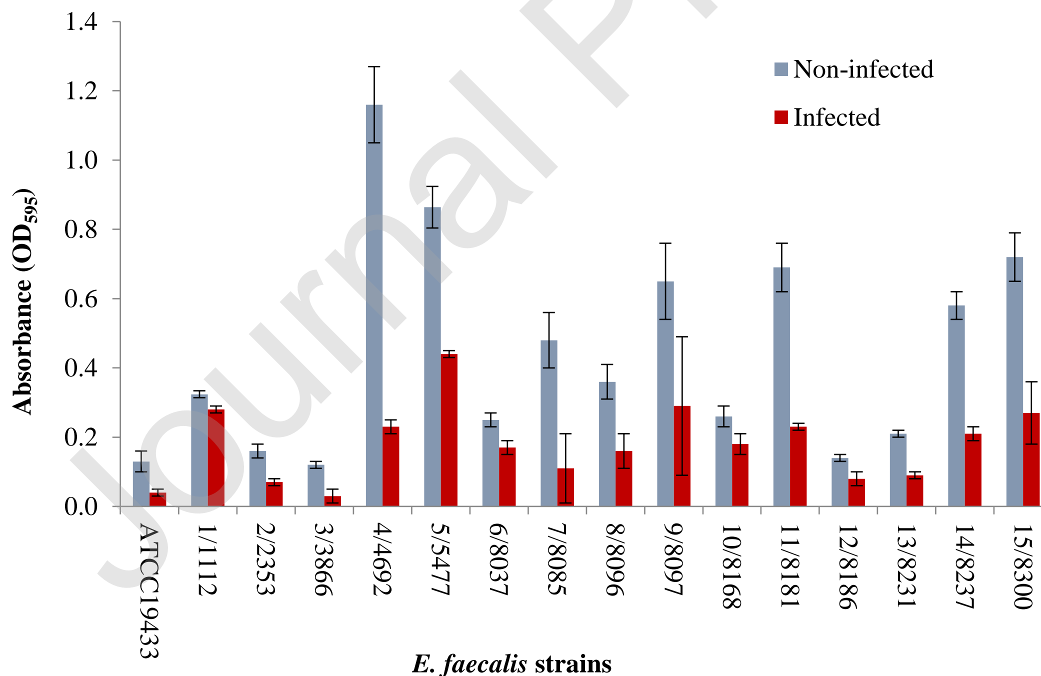

Figure 5. Effect of EF1TV on *E. faecalis* biofilm. Crystal violet stained biofilms of non-infected (gray bars) and EF1TV-infected cells (red bars) were measured by absorbance at 595

nm. The blank value ($OD_{595} = 0.2$) was subtracted from the OD_{595} values of the bacterial strains. Differences in OD_{595} values between infected and non-infected bacteria are significant at $P<0.01$ for all strains (one-way ANOVA). Standard deviations are shown as vertical lines.

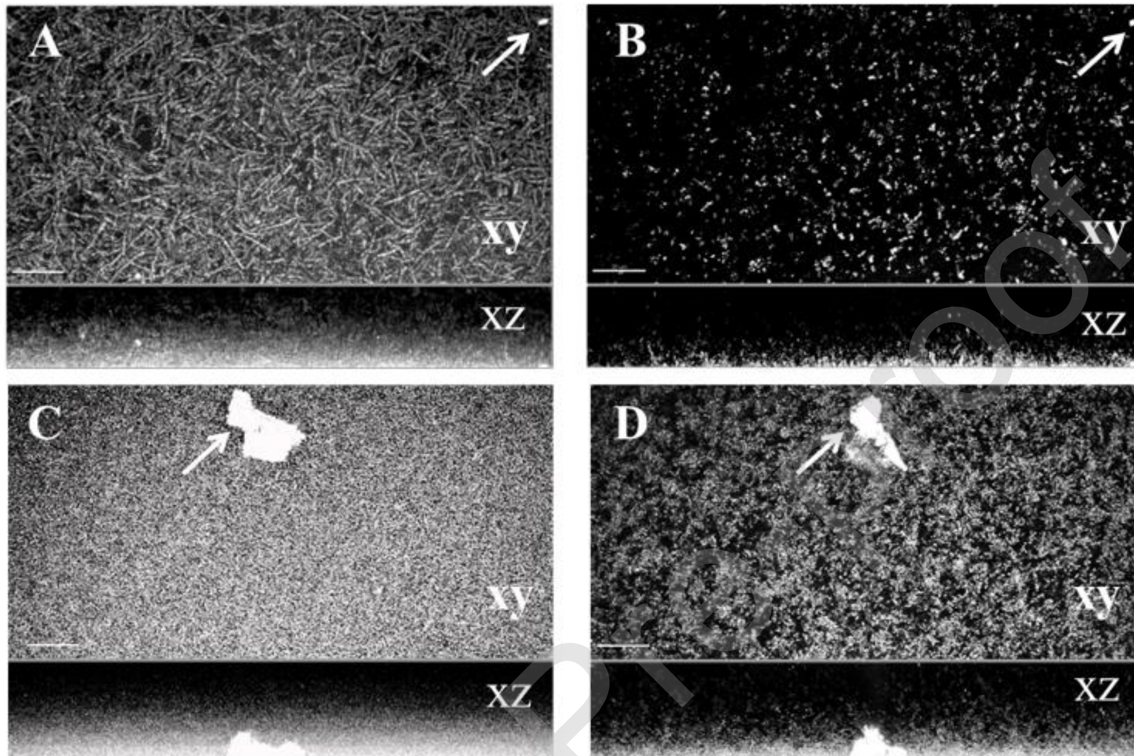

Figure 6. 3D Confocal images of biofilms of *E. faecalis* strains 4/4692 and 11/8181 and effect of EF1TV infection. The biofilm 3D structure was directly analyzed by reflection *in vivo* without staining. The same microscopic field was observed before and after infection. (A) 3-day old biofilm of strain 4/4692, thickness x-z 40µm. (B) Strain 4/4692 infected with EF1TV and observed after 72 h, thickness x-z 10µm. (C) 3-day old biofilm of strain 11/8181; thickness x-z 40µm. (D) Strain 11/8181 infected with EF1TV and observed after 72 h; thickness x-z = 20µm. The arrows indicate the position of arbitrary reference points used to assure that the same field was observed. Magnification 60x. Bars represent 20 µm.

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Table 1

Bacterial strains[a]		Origin	Spot Test	EOP[b]
E. faecalis	ATCC 19433	Lab collection	++	1.00
“	ATCC 29212	Lab collection	+	
“	1/1112	Blood stream	++	1.00
“	2/2353	Wound swab	+	
“	3/3866	Urinary tract	+	
“	4/4692	Urinary tract	++	0.47
“	5/5477	Urinary tract	++	0.66
“	6/8037	Blood stream	+	
“	7/8085	Blood stream	+	
“	8/8096	Venous catheter	++	0.50
“	9/8097	Blood stream	++	1.00
“	10/8168	Blood stream	+	
“	11/8181	Blood stream	+	
“	12/8186	Blood stream	+	
“	13/8231	Blood stream	++	0.44
“	14/8237	Blood stream	+	
“	15/8300	Central venous	++	1.00
E. faecium	DSM 20477	catheter	-	n.d.
“	16/2933	DSMZ	-	“
“	17/4303	Blood stream	-	“
“	18/4988	Blood stream	-	“
“	19/5288	Blood stream	-	“
“	20/5323	Blood stream	-	“
S. aureus	ATCC 29213	Blood stream	-	“
		Lab collection		

[a]The *E. faecalis* and *E. faecium* strains, except ATCC29212, ATCC19433 and DSM 20477, are clinical isolates obtained from Careggi University Hospital of Florence, Italy. [b]EOP was calculated as the titer (PFU ml^{-1}) on the test strain/titer (PFU ml^{-1}) on *E. faecalis* 1/1112. Blank cells indicate that no plaques were detected (EOP < $1x10^{-7}$). (-) absence of lysis, (+) turbid spot, (++) clear spot, n.d., not determined.

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