RESEARCH LETTER

Isolation and characterization of a novel *Enterococcus faecalis* bacteriophage ϕEF24C as a therapeutic candidate

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Abstract

Vancomycin-resistant *Enterococcus faecalis* (VRE) has become a significant threat in nosocomial settings. Bacteriophage (phage) therapy is frequently proposed as a potential alternative therapy for infections caused by this bacterium. To search for candidate therapeutic phages against *Enterococcus faecalis* infections, 30 *Enterococcus faecalis* phages were isolated from the environment. One of these, virulent phage ϕEF24C, which has a broad host range, was selected for analysis. The plaque-forming ability of ϕEF24C was virtually unaffected by differences in the clinical host strains. Furthermore, the phage had a shorter latent period and a larger burst size than ordinary tailed phages, indicating that ϕEF24C has effective lytic activity against many *Enterococcus faecalis* strains, including VRE. Morphological and genomic analyses revealed that ϕEF24C is a large myovirus (classified as family *Myoviridae* morphotype A1) with a linear double-stranded DNA genome of *c.* 143 kbp. Analyses of the N-terminal amino acid sequences of the virion proteins, together with the morphology and the genome size, speculated that ϕEF24C is closely related to other myoviruses of Gram-positive bacteria that have been used experimentally or practically for therapy or prophylaxis. Considering these results, ϕEF24C may be a potential candidate therapeutic phage against *Enterococcus faecalis* infections.

Introduction

The continuous overuse or misuse of effective antibiotics has led to the ongoing emergence of new antibiotic-resistant pathogenic bacteria. The continuous vicious cycle between increasing bacterial resistance and new drug development needs intervention. Bacteriophage (phage) therapy harnesses a live prokaryotic virus as a bioagent to target and destroy disease-causing bacteria, and is predicted to be a practicable alternative therapy because of its long history of successful use in the east (Ho, 2001; Sulakvelidze *et al.*, 2001; Matsuzaki *et al.*, 2005). Phage therapy has recently begun to be proven superior to conventional chemotherapy for certain uses (Lederberg, 1996; Barrow & Soothill, 1997; Clark & March, 2006; Fox, 2006; Marza *et al.*, 2006).

Enterococcus is an environmental bacterium and is considered to be useful and harmless due to its commensalisms to humans. However, some enterococcal species occasionally cause a variety of diseases in humans (Kayser, 2003; Fujita, 2005). Recently, infections with vancomycin-resistant *Enterococcus* (VRE) have become a threat in nosocomial settings, with an increased incidence reported worldwide (Fujita, 2005; Deshpande *et al.*, 2007). In particular, *Enterococcus faecalis* is a major causative agent of enterococcal infections, and is the second most isolated VRE species (Kayser, 2003; Fujita, 2005). Therefore, the significance of its future medical impact is of great concern.

Despite increasing medical concern about VRE and the expectations for phage therapy, no characterized *Enterococcus faecalis* phage has been available. In this study, 30 *Enterococcus faecalis* phages were isolated from environmental water samples, and a virulent phage with a broad host range, ϕEF24C, was selected and biologically characterized.

Materials and methods

Culture media

All the reagents and constituents of culture media used were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise stated. Heart infusion broth (HIB) was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). Tryptic soy broth (TSB) and the constituents of Luria–Bertani (LB) medium (yeast extract and trypton) were obtained from Becton Dickinson and Company (Sparks, MD). LB medium was described (Sambrook *et al.*, 1989). For the phage plaque formation assay, solid medium containing 1.5% or 0.5% agar was used for the lower and upper layers, respectively.

Bacterial strains

Thirty *Enterococcus faecalis* strains (non-VRE strains), named EF1–EF30, were isolated at the Clinical Laboratory Center in Kochi Medical School Hospital (KMSH), Kochi, Japan, between 15 October and 20 December 1999. Ten *Enterococcus faecium*, named EFum1–EFum10, were also isolated at KSMH. These clinical isolates were identified using the MicroScan WalkAway system (Dade Behring, West Sacramento, CA). Four VRE strains, named VRE1–VRE4, were kindly donated by Hokushin General Hospital, Nagano City, Nagano Prefecture, Japan (Oana *et al.*, 2001). *Enterococcus faecalis* (ATCC19433) and *Escherichia coli* B (ATCC11303) were purchased from the American Type Culture Collection (Manassas, VA). *Staphylococcus aureus* IID671 (209P) was obtained from the Research Institute for Microbial Diseases, Osaka University. *Staphylococcus aureus* SA37 has been described elsewhere (Matsuzaki *et al.*, 2003). Culture media for *Enterococcus faecalis*, *Enterococcus faecium*, *S. aureus*, and *Escherichia coli* were HIB or TSB, TSB, TSB, and LB, respectively. The bacteria used in this experiment were cultured at 37 °C in the appropriate medium.

Phage isolation

Water samples were collected from nine different water channels in Kochi City, Kochi Prefecture, Japan. After particle removal by paper filters, the water sample was filtered through 0.45 μm pore membranes. Next, 5 mL of 2 × HIB medium and 0.1 mL of overnight-cultured non-VRE strain were added to 5 mL of the filtered water sample, and the mixture was incubated at 37 °C overnight. The culture was then filtered through a 0.45 μm pore size membrane. Single plaque isolation was performed at least three times on the respective strains.

Measurement of phage infectious ability

The host range of the isolated phages was determined by the streak test, which was an assay performed by streaking phages on TSB-based-double-layered agar plates inoculated with *Enterococcus faecalis* and then checking for the presence or the absence of plaque formation.

To check the polyvalency of the phage, *Enterococcus faecium*, *S. aureus*, and *Escherichia coli* were inoculated on double-layered agar plates containing the appropriate medium, and a streak test was performed.

The plaque-forming ability of phage ϕEF24C against each *Enterococcus faecalis* strain was measured as the efficiency of plating (EOP), which was set as 1 for EF24. In addition, the adsorption rate, latent period, and burst size were determined relative to EF24, essentially as described elsewhere (Adams, 1959). All incubations for these analyses were carried out in TSB medium at 37 °C.

Preparation of purified phages

Enterococcus faecalis strain EF24 was cultured with the phage in 1 L of TSB medium at 37 °C until lysis was complete. Phage lysate was centrifuged (10 000 ***g***, 4 °C, 10 min). The supernatant was collected and mixed with NaCl (final concentration, 0.5 M) and polyethylene glycol 6000 (Sigma-Aldrich Co., MI; final concentration, 10%). The mixture was incubated for 30 min at 4 °C and then centrifuged (10 000 ***g***, 4 °C, 30 min). The resulting precipitated phage was resuspended in TM buffer (10 mM Tris-HCl and 5 mM $MgCl_2$, pH 7.2) and treated with 50 μg mL^{-1} DNase I (Type II; Sigma-Aldrich) and 50 μg mL^{-1} RNase A (Type IA; Sigma-Aldrich) for 1 h at 37 °C. The phage suspension was then placed on top of a discontinuous CsCl gradient (ρ = 1.3, 1.5 and 1.7) and centrifuged (50 000 ***g***, 4 °C, 2 h). The collected phage band was placed between ρ = 1.3 and 1.7, and then centrifuged again (50 000 ***g***, 4 °C, 2 h). After phage band collection, the purified phage sample was treated differently for each experiment.

For the preparation of phage for electron microscopic observation, the purified phage sample was dialyzed against AAS (0.1 M ammonium acetate, 10 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.2) (4 °C, 1 h). For preparation for proteome analysis, genome extraction, and pulsed-field gel electrophoresis (PFGE), the purified phage sample was diluted four times with AAS and was pelleted by centrifugation (100 000 ***g***, 4 °C, 1 h). After removal of the supernatant, the pellet was stored at − 80 °C until use.

Electron microscopy

The dialyzed phage was fixed in 5% formalin, and was then negatively stained with 2% uranyl acetate (pH 4.0). Electron micrographs were taken with a Hitachi H-7100 transmission electron microscope at 100 kV.

Analysis of phage proteins

The phage pellet was dissolved with 500 μL of 1 × Laemmli sample buffer (62.5 mM Tris-HCl pH 6.8, 5%

2-mercaptoethanol, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.01% bromophenol blue) and boiled for 5 min. The phage proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% acrylamide). Prestained protein markers (broad range) for SDS-PAGE (Nacalai Tesque) were used as molecular weight (MW) standards. The MW of six phage proteins was estimated from the visualized gel.

The separated phage proteins were also blotted on a polyvinylidene difluoride membrane (Bio-Rad Japan, Tokyo, Japan) and visualized by Coomassie brilliant blue R250 staining. The bands were excised and the N-terminal amino acid residues were sequenced using a model 492 protein sequencer (Applied Biosystems, Foster City, CA). The N-terminal sequences of these virion proteins, designated Band 1–6 from the top of the gel, have been deposited in the UniProt database (accession numbers P85225, P85226, P85227, P85228, P85229, and P85230, respectively).

The N-terminal amino acid sequences were analyzed by protein BLAST of National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). An in-house BLAST search against genome sequences of the other phages was then performed using the In Silico Molecular Cloning Genomics Edition (In Silico Biology Inc., Yokohama, Japan).

Extraction of genomic nucleic acid

After the phage pellet had been suspended in lysis solution [0.4 μg mL^{-1} proteinase K, 1% SDS, 40 mM EDTA (Wako, Osaka, Japan)], the suspension was incubated at 55 °C for 3 h. An equal volume of water-saturated phenol was added, gently vortexed, and the mixture was centrifuged (20 000 ***g***, 4 °C, 5 min). The upper aqueous layer was collected. This procedure was performed twice. The nucleic acids were precipitated by ethanol twice and resuspended in water.

Enzyme treatments

Phage nucleic acids (2 μg) were treated with 1 μg mL^{-1} DNAse I, RNAse A or Bal31 (Takara Bio, Kyoto, Japan) for 1 h at 37 °C, and were also treated with the following 17 restriction enzymes, AccI, BamHI, ClaI, DraI, EcoRI, EcoRV, HaeIII, HindIII, KpnI, MboI, PstI, SacI, SalI, Sau3AI, SmaI, XbaI (all Takara Bio), or SfaNI (New England Biolab, Beverly, MA), at 2 U μL^{-1} for about 8 h at 37 °C. Following electrophoresis of samples in 0.6% or 0.8% (w/v) agarose, nucleic acids were stained with ethidium bromide (1 μg mL^{-1}).

PFGE

After the phage pellet had been suspended at *c.* 10^8 pfu mL^{-1} in phosphate buffered-saline, 500 μL of phage suspension was mixed with 500 μL of 2% (w/v) NuSieve GTG agarose (FMC BioProducts, Philadelphia, PA), dispensed into plug molds and solidified. The plugs were punched out of the molds into a small volume of digestion buffer (500 mM EDTA, 10 mM Tris pH 8.0, 1% w/v SDS and 1 mg mL^{-1} of proteinase K) and incubated at 50 °C overnight. The digestion buffer was decanted, and the samples were washed three times using TE buffer and then incubated in TE buffer (4 °C, 1 h). The plugs were placed in wells of 1% Pulsed Field Certified agarose (Bio-Rad Japan) in 0.5 × TBE and overlaid with molten 0.5% NuSieve GTG agarose. Lambda Ladder PFG Markers (New England Biolab) were used as the MW standards. The samples were electrophoresed using a CHEF-DRII System (Bio-Rad Japan) at 6 V cm^{-1} with pulse ramps from 1 to 25 s for 24 h at 14 °C in 0.5 × TBE buffer. Following electrophoresis, nucleic acids were stained with 1 × SYBR Gold (Molecular Probes Inc., Eugene, OR) for 30 min.

Results and discussion

Isolation of *Enterococcus faecalis* phage ϕEF24C

Thirty phages were isolated, using 16 *Enterococcus faecalis* strains as hosts. Each phage was named as a ϕ[host strain used for phage isolation and purification][isolation location of phage]. For instance, ϕEF24C was isolated from location C, using *Enterococcus faecalis* strain EF24. According to the results of the streak test (plaque formation test), ϕEF17H and ϕEF24C had the broadest host ranges (specificity for 91% and 89% of tested hosts, respectively) (see supplementary material), irrespective of the bacterial origin (e.g. a patient's clinical profile). Phage ϕEF24C was selected for further biological characterization.

Infectious activity of phage ϕEF24C

The sensitivity of different bacterial strains to a phage is often considerably different because of phage-protection systems possessed by the hosts (restriction–modification system, adsorption interference, etc.) (Gachechiladze *et al.*, 1991; O'Flaherty *et al.*, 2005). Generally, bacterial strains with stronger phage-protection systems tend to show lower efficiencies of plating. However, as the EOP of ϕEF24C was relatively constant for all sensitive bacterial strains (Fig. 1), it was considered that the phage was able to proliferate efficiently in the host strains without significant influence of phage-protection systems.

Moreover, although most phages display host specificity to particular bacterial species and strains, some phages can infect more than one species of bacteria (i.e. polyvalent phage) (Pantůček *et al.*, 1998; Khan *et al.*, 2002; O'Flaherty *et al.*, 2005). However, ϕEF24C showed neither lysis activity nor lysis-from-without activity against the tested bacteria other than *Enterococcus faecalis* (10 *Enterococcus faecium*

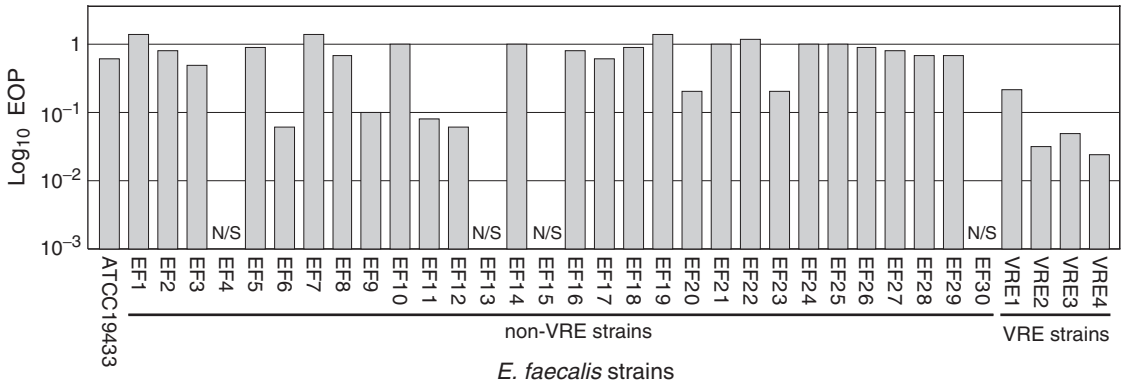


Fig. 1. EOP of ϕEF24C against various bacterial strains. N/S represents nonsensitivity of the host to ϕEF24C.

strains, two *S. aureus* strains, and one *Escherichia coli* strain). Thus, ϕEF24C seems to be specific to only *Enterococcus faecalis*.

The adsorption rate, latent period, and burst size of phage are thought to be biological parameters for phage infection measurements. Comparing these parameters of ϕEF24C against EF24, the adsorption rate within 5 min was about 90%, the latent period was about 30 min, and the burst size was about 110–120. As medians of latent period and burst size in tailed phage are typically 40–60 min and 50–100, respectively, ϕEF24C was regarded as having relatively effective lytic activity (Ackermann & DuBow, 1987).

Morphology of phage ϕEF24C

ϕEF24C was morphologically classified into the family *Myoviridae*, morphotype A1 (an icosahedral head and a long contractile sheathed tail) (Fig. 2). The head diameter was 93 ± 7 nm (mean $\pm$ SD) ($n = 15$). The extended tail length was 204 ± 9 nm ($n = 15$), and the contracted sheath length was 106 ± 11 nm ($n = 15$). Some large myoviruses of Gram-positive bacteria such as *Staphylococcus* phage K, *Listeria* phage A511, and *Lactobacillus* phage LP65 have similar morphological characteristics to ϕEF24C in head shape, tail striation and base plate (tail tip globular structure) (Rees & Fry, 1981; Zink & Loessner, 1992; Chibani-Chennoufi *et al.*, 2004).

Structural proteins of phage ϕEF24C

Based on the morphological similarity and the results of protein BLAST search at NCBI (data not shown), the following phage genome data were retrieved from the GenBank database for an in-house BLAST search: *Lactobacillus plantarum* phage LP65 (accession AY682195); *Listeria* phages A511 *orf1*, *orf2*, *cps*, *orf3*, *orf4*, *orf5*, *orf6*, *orf7*, *tsh*, *orf8*, and *orf9* genes (accession X91069), P100 (accession DQ004855); *Staplylococcus* phages G1 (accession AY954969), K (accession AY176327), Twort (accession AY954970). The N-terminal

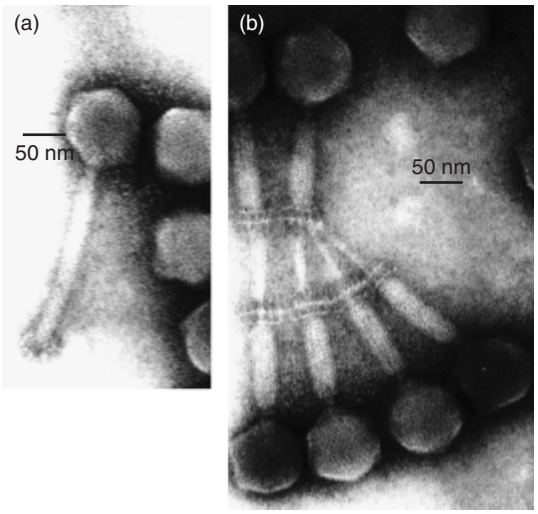
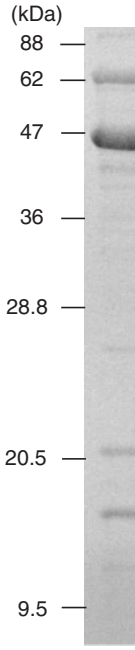


Fig. 2. Transmission electron micrographs of negatively stained ϕEF24C virions. ϕEF24C with an extended tail (a) and with an contracted tail (b) are shown.

amino acid sequences of six phage proteins were analyzed by the in-house BLAST search and their functions were determined (Fig. 3). Band 1 showed 80% similarity to the major tail sheath protein of *Staphylococcus* phages K and G1 and *Listeria* phages P100 and A511, and 60% similarity to that of *Lactobacillus* phage LP65. Band 2 showed 89.5% similarity to major capsid proteins of *Listeria* phages P100 and A511, 78.9% similarity to those of *Staphylococcus* phages K, G1, and Twort, and 73.7% similarity to those of *Lactobacillus* phage LP65. Band 5 only showed 90% similarity to the unknown function ORF8 of *Listeria* phage A511 (Loessner & Scherer, 1995). Bands 3, 4, and 6 did not show similarities to any other proteins.

Major capsid proteins, which are highly conserved among viruses, are frequently used for taxonomical and phylogenic classifications (Loessner *et al.*, 1994; Hambly *et al.*, 2001; Bamford *et al.*, 2005). Hence, the close relation of ϕEF24C



Band	N-terminal sequence	MW (kDa)	Speculated function
1	AVEQFPRKKV	62	Tail sheath protein
2	GFTTGYGITP ESQTDAAAL	46	Major capsid protein
3	AIATNNSRVY	26	Unknown
4	AKEILNIEDL	21	Unknown
5	ASVGNQTVHT	16	Unknown
6	MNYPKXXKVV	13	Unknown

Fig. 3. SDS-PAGE profile of ϕEF24C virion proteins and their N-terminal sequences. The left figure shows the separation of ϕEF24C virion proteins by SDS-PAGE. In the right table, the estimated MWs and the N-terminal amino acid sequences of six bands are shown. 'X' in the N-terminal sequence indicates 'not determined'. The speculated function of each band is also shown, details described in the text.

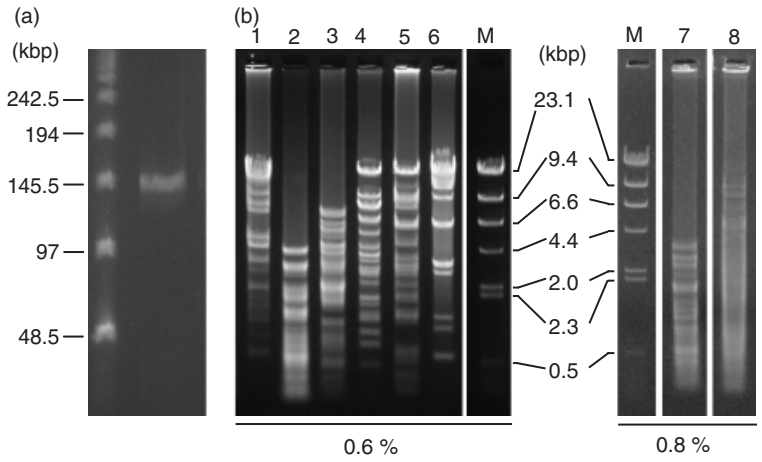


Fig. 4. (a) Pulsed-field gel electrophoretogram of the ϕEF24C genome. The left and right lanes indicate the Lambda Ladder PFG markers and the ϕEF24C genome, respectively. (b) Electrophoretogram showing the patterns of ϕEF24C genome digestion by eight restriction endonucleases. Lane M represents the λ-HindIII digest marker, with fragment sizes indicated in the middle. In the left and right panels, the digested phage DNA was separated electrophoretically in 0.6% and 0.8% agarose gel, respectively. Lanes 1, 2, 3, 4, 5, 6, 7, and 8 represent the phage DNA digestion patterns produced by ClaI, DraI, HindIII, KpnI, PstI, SacI, AccI, and SfaNI, respectively. The digestion patterns produced with AccI and SfaI are blurred, which is considered to be attributed to the characteristics of the highly fragmented DNA of the large genome.

to the other large phages of Gram-positive bacteria can be speculated upon.

Genomic nucleic acids of phage ϕEF24C

A tailed phage typically possesses double-stranded (ds) DNA as its genomic nucleic acid. ϕEF24C nucleic acids were sensitive to DNAse I (endonuclease), Bal31 nuclease (exonuclease) (data not shown), and eight restriction enzymes (see Fig. 4 (b)), but not to RNase A. The restriction patterns were not altered by heat treatment for 5 min at 65 °C (data not shown), suggesting that there was no sticky end in the phage genome. The genome size was estimated to be about 143 kbp using PFGE (Fig. 4 (a)). Based on these

results, it was concluded that the ϕEF24C genome consists of linear dsDNA that is *c.* 143 kbp long. The genome of ϕEF24C was slightly larger than those of the following related large myoviruses of Gram-positive bacteria: *Staphylococcus* phages K (127 345 bp), G1 (138 715 bp) and Twort (130 706 bp), *Lactobacillus* phage LP65 (131 573 bp), and *Listeria* phage P100 (131 384 bp) (Chibani-Chennoufi *et al.*, 2004; O'Flaherty *et al.*, 2004; Carlton *et al.*, 2005; Kwan *et al.*, 2005).

Phages often have a genome that contains highly modified bases produced by phage-coded enzymes, for example, 5-hydroxymethyl cytosine or 5-hydroxymethyl uracil. Such modified DNAs are highly resistant to host restriction enzymes (Huang *et al.*, 1982). However, because the ϕEF24C genome DNA was sensitive to various restriction enzymes, including SfaNI (an endonuclease derived from *Enterococcus faecalis*) (Fig. 4 (b)), it does not seem to be highly modified. Therefore, it is possible that ϕEF24C synthesizes powerful inhibitors of host restriction–modification systems, and this may be one of the reasons for its broad host range (Bickle & Kruger, 1993; García & Molineux, 1999; O'Flaherty *et al.*, 2004).

Development of a therapeutic phage and *Enterococcus feacalis* phage ϕEF24C

ϕEF24C has broad host specificity and an infectious ability that is relatively stronger than ordinary tailed phages. Furthermore, its infectious efficiency does not seem to be affected by phage-protection systems of the host. The analysis of virion proteins suggested that ϕEF24C is closely related to the large myoviruses of Gram-positive bacteria, such as *Staphylococcus* phage K and *Listeria* phages A511 and P100, which have been used experimentally or practically for therapy and prophylaxis (Carlton *et al.*, 2005; O'Flaherty *et al.*, 2005; Gill *et al.*, 2006; Hagens & Loessner, 2007). Considering these, ϕEF24C may be a putative therapeutic phage candidate.

In the development of modern therapeutic phages, a scientific rationale is strongly required. In particular, a newly isolated phage needs to be examined in detail. Assuming the phage to be a drug, its form and mode of action must be characterized. One of the simple effective methods is basic genomic analysis, because the phage genome reflects the biological characteristics of the phage, such as its morphology and life cycle. Importantly, genome analysis also allows assessment of the safety of the phage by checking for the absence or the presence of undesirable genes, such as pathogenic and lysogenic genes, thus reducing the risk of adverse effects (Merril *et al.*, 2006). Therefore, the authors are currently undertaking a genome project that characterizes the ϕEF24C phage genome. The preliminary sequence data and its analysis indicate no such disadvantageous genes. Moreover, the therapeutic effectiveness of the phage must also be tested *in vivo*, because phage multiplication depends on host bacterial growth (i.e., bacteriocidal effectiveness differs *in vitro* and *in vivo*). Thus, animal experiments are also currently in preparation.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Isolated *Enterococcus faecalis* phages and their host specificity.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2007.00996.x (This link will take you to the article abstract.)

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