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Characterization of *Enterococcus faecium* bacteriophage IME-EFm5 and its endolysin LysEFm5

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ABSTRACT

Due to the worldwide prevalence of antibiotic resistant strains, phages therapy has been revitalized recently. In this study, an *Enterococcus faecium* phage named IME-EFm5 was isolated from hospital sewage. Whole genomic sequence analysis demonstrated that IME-EFm5 belong to the *Siphoviridae* family, and has a double-stranded genome of 42,265 bp (with a 35.51% G+C content) which contains 70 putative coding sequences. LysEFm5, the endolysin of IME-EFm5, contains an amidase domain in its N-terminal and has a wider bactericidal spectrum than its parental phage IME-EFm5, including 7 strains of vancomycin-resistant *E. faecium*. The mutagenesis analysis revealed that the zinc ion binding residues (H27, H132, and C140), E90, and T138 are required for the catalysis of LysEFm5. However, the antibacterial activity of LysEFm5 is zinc ion independent, which is inconsistent with most of other amidase members. The phage lysin LysEFm5 might be an alternative treatment strategy for infections caused by multidrug-resistant *E. faecium*.

1. Introduction

Enterococcus faecium is a Gram-positive opportunistic pathogen that commonly inhabits the digestive tract, oral cavity, and vaginal tract of humans and animals. In a healthy individual, *E. faecium* has no adverse effect on the host. However, the bacterium can cause life-threatening conditions in immune-compromised patients (de Been et al., 2013; Edgeworth et al., 1999; Johnson, 1994) and can cause endocarditis, bacteremia, urinary tract infections, and meningitis (Jett et al., 1994). In recent years, the abuse of antibiotics has enhanced the spread of antibiotic-resistant bacterial strains (including vancomycin-resistant strains), which has resulted in their dominance in human and animal microorganisms (Huycke et al., 1998; Poh et al., 2012). In the last decade, hospital-acquired infections caused by enterococci have increasingly been associated with antibiotic-resistant *E. faecium* compared with other *Enterococcus spp.* (Brueggemann et al., 2007).

Virulent bacteriophages (phages) are bacterial viruses that can specifically infect and lyse host bacteria (Wittebole et al., 2014). Although phage therapy was explored as an antimicrobial agent for the treatment of bacterial infectious diseases in the early 1920s, the development of phage therapy was hampered by the advent of antibiotics (Sulakvelidze et al., 2001). Recently, the rise of antibiotic-resistant bacteria has led to an increasing interest in phage therapy, as evidenced by the number of published reviews (Chan et al., 2013; Nobrega et al., 2015). Endolysin is encoded by the phage genome at the end of the phage lytic life cycle to lyse the host cell (Gu et al., 2010). Lysin belongs to the family of muralytic enzymes that directly destroy peptidoglycan of the bacterial cell wall (Gu et al., 2010). Recent research has suggested that lysin provides an effective approach to control bacterial infections, including multidrug-resistant strains (Gu et al., 2010; Kong and Ryu, 2015; Linden et al., 2015; Schmelcher et al., 2015a, 2015b).

In recent years, the treatment of infections caused by *E. faecium* has become increasingly difficult given the prevalence of multidrug-resistant *E. faecium* strains, especially vancomycin-resistant, raising serious concerns within the medical community (Chen et al., 2015). Few studies on *E. faecium* lysin have been reported (Yoong et al., 2004). Thus, in this study, a novel lytic

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bacteriophage against *E. faecium* was isolated from the hospital sewer system, and the lysin of this phage was studied.

2. Results

2.1. Identification and antibiotic resistance of E. faecium strains

The 16S rRNA and SodA gene fragments of putative *E. faecium* isolates were amplified by PCR, and their sizes were approximately 733 bp and 214 bp, respectively, according to gel electrophoresis and sequencing. BLAST analysis showed that the SodAs of these strains were more than 99% identical with those of *E. faecium* strains, such as Aus0085, NRRLB-2354, DO, and Aus0004 (GenBank Accession number: CP006620, CP004063, CP003583 and CP003351, respectively).

The antibiotic susceptibilities of these 23 *E. faecium* isolates indicated that most of the strains were sensitive to chloramphenicol and resistant to ampicillin, nitrofurantoin, erythromycin, ciprofloxacin, streptomycin, rifampicin and tetracycline (Table S1). In particular, 9 strains were vancomycin-resistant.

RAPD with primer 5′- AACGCGCAAC-3′ indicated genetic diversity among the *E. faecium* isolates, as seen in Fig. S1. However, there were also several isolates with highly similar fingerprints (e.g., E016 and E030; E038 and J1; 327, 353, 378, 383 and 483), and there was limited correspondence with the antibiotic susceptibility pattern.

2.2. Isolation and characterization of the phage IME-EFm5

E. faecium phage IME-EFm5 was isolated from the Changchun sewage by plaque purification. When incubated with *E. faecium* 4P-SA, IME-EFm5 formed clear plaques (1–2 mm diameter) on lawns of 4P-SA (Fig. S2). Apart from 4P-SA, IME-EFm5 was unable to lyse other *E. faecium* strains and other species, as seen in Table S2.

As seen from Fig. 1, IME-EFm5 has an isometric, icosahedral head and a long non-contractile tail. The diameter of the isometric head was approximately 48 nm, and the tail length was approximately 160 nm. These structural characteristics of IME-EFm5 suggest that it is a member of the *Caudovirales*, *Siphoviridae* family, according to the guidelines of the International Committee on Taxonomy of Viruses (ICTV, 2005). As shown in Fig. S3, the SDS-PAGE gel revealed that the phage contains many structural proteins. The size of the most abundant major structural protein was approximately 36 kDa. The genome of the phage IME-EFm5 could only be digested with DNase I, which indicated that it was double-stranded DNA (Fig. S4).

When the MOI was 0.01, the phage IME-EFm5 titer was the highest, reaching approximately $>10^9$ PFU/mL (Fig. 2A). Thus, the one-step growth curve of IME-EFm5 propagated on the 4P-SA strain in BHI broth was determined at an MOI of 0.01. As seen in Fig. 2B, the one-step growth curve revealed that the latent period was approximately 30 min, and the release period was approximately 70 min.

2.3. Overview of the phage IME-EFm5 genome

The sequencing indicated that IME-EFm5 has a double-stranded, terminally non-redundant genome of 42,265 bp with a low G+C content of 35.51 mol%. The complete genome of IME-EFm5 encodes 70 putative open reading frames (ORFs). The arrangement of these putative ORFs was mapped at the whole-genome level, as seen in Fig. 3A.

All predicted proteins were examined for similarity to known bacterial and phage sequences deposited in the National Center for Biotechnology Information (NCBI) databases. Table S3 shows that 57 ORFs were significantly similar to other proteins in GenBank, of which 43 ORFs showed homology with those of the *E. faecium* phage IME-EFm1 (GenBank Accession number: KJ010489) (Wang et al., 2014). Additionally, the IME-EFm5 genome only exhibited similarity at the whole genome level with IME-EFm1, as seen in Fig. 3B and C. The query cover of IME-EFm5 with IME-EFm1 was 64%, and the identity of their common gene fragments was 89%.

ORF32 and ORF31 of IME-EFm5 showed 97% and 95% similarity with the putative holin and N-acetylmuramoyl-L-alanine amidase of IME-EFm1, respectively. These two ORFs may compose the host lysis module of IME-EFm5.

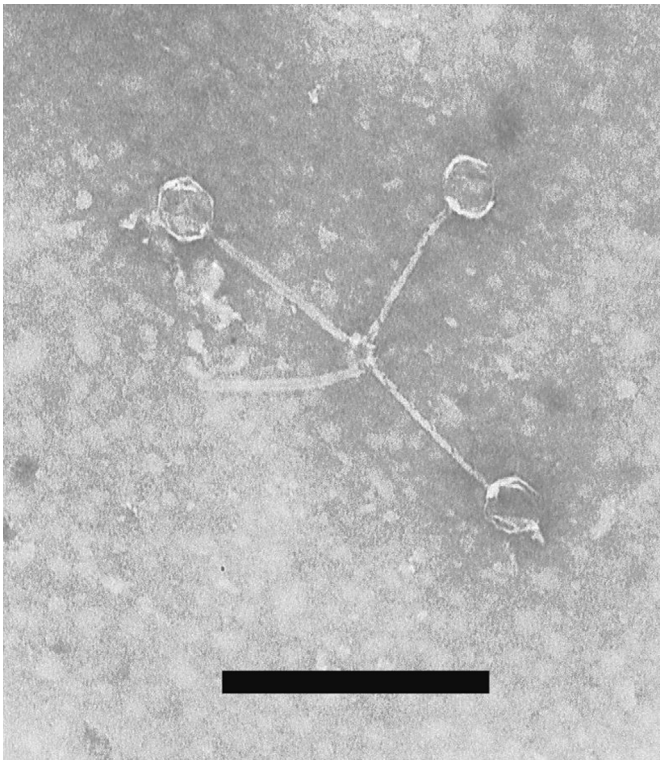

Fig. 1. The morphology of phage IME-EFm5. IME-EFm5 phage was negatively stained with 2% phosphotungstic acid (PTA) (2% (wt/vol)) and examined by transmission electron microscopy (TEM) at an accelerating voltage of 80 kV. The scale bars represent 200 nm.

2.4. Sequence alignment of the LysEFm5 and homologous proteins

ORF31 of IME-EFm5 (LysEFm5), the putative lysin protein, consisted of 341 amino acids (approximately 37 kDa). The amino acid sequence of LysEFm5 was analyzed by BLAST on the NCBI database. In addition to the 95% (323/341) identity with the amidase of IME-EFm1, LysEFm5 also showed similarity to the amidase of bacteria or lysin of other phages, including the N-acetylmuramoyl-L-alanine amidase of *E. faecium* (NCBI Reference Sequence: WP_047927174.1, 214/341, 63% identity) and *E. faecalis* (NCBI Reference Sequence: WP_048947726.1, 98/184, 53% identity), the lysins of *Brochothrix* phage NF5 (101/195, 52% identity) and *Staphylococcus warneri* phage ØWMY (79/174, 45%) (Fig. 4A). The foregoing 1–185 amino acids belong to an amidase domain. Additionally, the 186–341 amino acid residue domain of LysEFm5 showed identity with the C-terminal of the putative glycosyl-hydrolase I of *E. faecium* (98/160, 61%, GenBank accession numbers: WP_034867121) and glycosyl-hydrolase II of *E. faecalis* (95/139, 68%, GenBank accession numbers: WP_016624531) (Fig. 4B).