

Study on isolation, characterization of bacteriophages against multidrug-resistant enterococci, and their synergy with bacteriocins and endolysins

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Title : Study on isolation, characterization of bacteriophages against multidrug-resistant enterococci, and their synergy with bacteriocins and endolysins
(多剤耐性腸球菌に対するバクテリオファージの単離、特性評価、ならびにバクテリオシンおよびエンドリシンとの相乗作用に関する研究)

Category : Kou

Thesis Summary

Enterococci are gram-positive, cocci-shaped, facultative anaerobic bacteria. Enterococci are commonly found in various foods, including vegetables, fermented sausages, and cheeses. Furthermore, they are considered undesirable organisms in processed meats, as they can lead to food spoilage. They are characterized by their ability to survive in harsh conditions like high salt concentrations (6.5%) and a wide range of temperatures and pH levels. *E. faecalis* and *E. faecium* are the main enterococcal species responsible for various life-threatening infections, including urinary tract infections, bacteremia, nosocomial infections, and endocarditis. They may also be associated with foodborne illness due to their enteric habitat and capacity to enter the food chain, as well as their resistance to a broad spectrum of antibiotics, virulence genes, and ability to form biofilms on both biotic and abiotic surfaces.

Firstly, *E. faecalis* used was isolated from various Egyptian and Japanese food sources, including raw milk and vegetables. These isolates were characterized and showed resistance to a wide range of antibiotics like kanamycin, gentamicin, rifampicin, and ciprofloxacin. Furthermore, all isolates showed the ability to form biofilm and were classified as strong, moderate, and weak biofilm producers. Five bacteriophages (phages) were isolated from different food samples, including cow faeces, compost, and raw milk. A phage, named vB_EfKS5, was selected and further characterized based on its wide host range, high reproducibility, and productivity within different enterococcal hosts. This phage lysed 70.85% of *E. faecalis* tested isolates and 42.85% of *E. faecium*. Phage vB_EfKS5 showed high reproducibility with an efficiency of plating of 0.5 or higher, which showed the potential of this phage in controlling different hosts of *E. faecalis*. The genome of phage vB_EfKS5 is double-stranded DNA, 59,246 bp in length, and has 125 open reading frames. It does not contain any virulence, resistance, or lysogenic genes. Four major groups were identified and had functions related to DNA replication and protein regulation, DNA packaging, structure, lysis of the host, and additional functions. This phage showed stability at different temperatures up to 70°C and pH ranging from 3 to 12. It could reduce the biofilm biomass of *E. faecalis* with different multiplicities of infection. The combination of phage vB_EfKS5 with nisin reduced the total viable counts of *E. faecalis* in broth and milk, revealing synergistic actions.

Secondly, phages vB_EfKS5 and PEF9 are lytic phages with lytic activities against *Enterococcus* spp. and encode endolysins designated as LysEf-5 and LysEf-9, respectively. LysEf-5 and LysEf-9 consist of 714 aa and 724 aa, and their molecular masses are 26.4 and 26.5 kDa, respectively. Both belong to the *N*-acetylmuramyl-L-alanine amidase family of endolysins. LysEf-5 and LysEf-9 showed 99% identity. LysEf-9 was selected for further characterization and analysis. The modular structure of LysEf-9 consisted of two basic structures: the binding domains, which are responsible for binding to the host surface receptors, and the enzymatically active domain, which plays the main role in the lysis activity of endolysin. LysEf-9 was able to reduce the turbidity of *E. faecalis* JCM 7783 with different concentrations, and the concentration of 125 µg/ml reduced the OD₆₀₀ from 0.6 to 0.3. The host range experiment showed the wide spectrum of LysEf-9 as it infected 12 out of 15 *E. faecalis* and 3 out of 6 *E. faecium*. Also, it showed specificity to enterococci, as it did not lyse any other tested species.

Thirdly, a bacteriocin-producing *Enterococcus* strain was isolated from raw milk collected from the Kyushu University farm. The culture-free supernatant, crude bacteriocin, was isolated and partially purified using the ammonium sulfate precipitation method. The resistant mutant of phage vB_EfKS5 was isolated and used as a host strain, besides *E. faecalis* JCM 7783 and *E. faecium* FHC 26. The crude bacteriocin showed lytic activity against 17 out of 20 tested enterococci and an isolated phage-resistant mutant. Using LysEf-9 with the crude bacteriocin reduced the viable count of *E. faecalis* JCM 7783 by 2.45 log after 24 h. In addition, LysEf-9 with phage vB_EfKS5 retarded the growth of the phage-resistant mutant. LysEf-9 lowered the viable count of *E. faecalis* JCM 7783, *E. faecium* FHC26, and the phage-resistant mutant in the 48-h biofilm by 3 log, 2 log, and 1.5 log, respectively. The combination of LysEf-9 with crude bacteriocin reduced the total viable count of the indicator strains by at least 3 logs.

Finally, a phage, named vB_EfS_C1, was isolated against *E. faecium*. The genomic analysis of the phage revealed that its length is 86,777 bp and it contains lysis-related genes like endolysin, holin, lysozyme, and endopeptidase. A competent cell from *E. faecalis* JH2-2 was prepared with high transformant efficiency. Two plasmids, pMG3c-pCas-9 and pRNA-pCas-9, were constructed using Gibson assembly and transformed into *E. coli* BL21. A spacer consisting of 20 bp downstream of the PAM site was introduced into the plasmid and transformed into *E. coli* BL21 and then into *E. faecalis* JH2-2 competent cells. The site-specific mutation in the phage tail spike protein and recombinase protein was tested using a CRISPR plasmid and mutagenesis, such as ultraviolet (UV) and chemical mutagens. These trials aimed to enhance the phage lysis efficiency and expand its host range.

This study successfully isolated and characterized phages, endolysins, and crude bacteriocins against drug-resistant enterococci and phage-resistant mutants and provided a framework for improving phage-based control through genomic characterization and modification.