Isolation of Nine Bacteriophages Shown Effective against *Erwinia amylovora* in Korea

Jungkum Park, Byeori Kim, Sujin Song, Yong Whan Lee, and Eunjung Roh

Crop Protection Division, National Institute of Agricultural Sciences, Rural Development Administration, Wanju 55365, Korea

(Received on November 30, 2021; Revised on April 17, 2022; Accepted on April 19, 2022)

*Erwinia amylovora* is a devastating bacterial plant pathogen that infects Rosaceae including apple and pear and causes fire blight. Bacteriophages have been considered as a biological control agent for preventing bacterial infections of plants. In this study, nine bacteriophages (ΦFifi011, ΦFifi044, ΦFifi051, ΦFifi067, ΦFifi106, ΦFifi287, ΦFifi318, ΦFifi450, and ΦFifi451) were isolated from soil and water samples in seven orchards with fire blight in Korea. The genetic diversity of bacteriophage isolates was confirmed through restriction fragment length polymorphism pattern analysis. Host range of the nine phages was tested against 45 *E. amylovora* strains and 14 *E. pyrifoliae* strains and nine other bacterial strains. Among the nine phages, ΦFifi044 and ΦFifi451 infected and lysed *E. amylovora* only. And the remaining seven phages infected both *E. amylovora* and *E. pyrifoliae*. The results suggest that the isolated phages were different from each other and effective to control *E. amylovora*, providing a basis to develop biological agents and utilizing phage cocktails.

**Keywords**: bacteriophage, biological control agent, *Erwinia amylovora*, fire blight

---

*Corresponding author.
Phone) +82-63-238-3284, FAX) +82-63-238-3838
E-mail) rosalia51@korea.kr

ORCID
Eunjung Roh
https://orcid.org/0000-0003-2999-5817

Handling Editor: Chang-Jin Park

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0) which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Articles can be freely viewed online at www.ppjonline.org.
Isolation of Nine Bacteriophages against *E. amylovora*

Isolation of Nine Bacteriophages against *E. amylovora*

249

acids and proteins. Additionally, bacteriophages are present in the surrounding environment of soil, air, and water and infect only target bacteria without any negative effects on humans or plants. Accordingly, phage therapy is non-toxic, eliminating just target bacteria, and requires small doses (Essa et al., 2020; Loc-Carrillo and Abedon, 2011; Principi et al., 2019).

Although bacteriophage use may lead to the emergence of resistant bacteria, and some of them have a narrow host range, combination of the phages should solve the problem. According to studies, combination of phages reduces the occurrence of phage-resistant bacteria as well as the bacterial population (Doss et al., 2017; Gayder et al., 2020). Born et al. (2011) reported that phage cocktails (L1/Y2 and L1/S6) are effective in controlling bacterial growth. Synergistic effects of phage cocktails is expected to affect different receptors of bacteria. When phages infect host bacteria, receptor binding proteins (RBPs) of phages bind to host receptors. Because of phage-host interactions, host specificity of phages varies at the level of genus, species, or even strains of host. Therefore, RBPs of phages are important to infect host bacteria (Dunne et al., 2018; Ge et al., 2020; Stone et al., 2019).

The key aspect of phage therapy to control fire blight is securing bacteriophages for all strains of target bacteria, *E. amylovora*, and use of combinations suppresses the appearance of resistant bacteria. In this study, the lytic activities, host specificity, and genetic diversity of nine bacteriophages isolated from soil and water samples in orchards that showed fire blight disease were evaluated.

To obtain *E. amylovora*-specific phages in Korea, environmental samples were collected from apple and pear orchards in which fire blight was observed in 2020. Each sample (10 ml or 10 g) was mixed with 10 ml of tryptic soy broth (TSB) and 1 ml of *E. amylovora* strain YKB 14808 (approximately 10^9 colony-forming unit [CFU]/ml). The samples mixed with *E. amylovora* were incubated overnight in a shaking incubator for enrichment of bacteriophages. All samples were centrifuged at 10,000 × g for 10 min at 4°C and filtered using a 0.22 μm PVDF syringe filter (Millipore, Bedford, MA, USA) for removal of bacteria cells and other debris. Dotting assays and plaque assays were carried out as described previously with minor modifications (Park et al., 2018). Presence of bacteriophages was confirmed by dotting assays. When 4 ml of soft agar (TSB containing 0.4% agar) was heated by microwave and cooled to 40°C, 20 μl of overnight cultured *E. amylovora* strain YKB 14808 was mixed and poured onto tryptic soy agar (TSA) plates. After the soft agar layer was solid, 10 μl of the environmental samples incubated with *E. amylovora* were added and incubated at 28°C overnight. Based on dotting assay results, bacteriophages were purified by plaque picking and passaged by plaque assays at least five times. In plaque assays, 4 ml of soft agar, 20 μl of overnight cultured *E. amylovora* strain YKB 14808, and 100 μl of bacteriophage (10^3 plaque-forming unit [PFU]/ml) were mixed and poured on TSA. After incubation at 28°C overnight, differently sized and shaped single plaques were isolated. The 29 environmental samples were collected from nine orchards of three cities to isolate bacteriophages with genetic diversity. Among them, nine bacteriophages were isolated from seven samples. Information of samples and isolated bacteriophages is listed in Table 1. Three bacteriophages (ΦFifi011, ΦFifi106, and ΦFifi287) were isolated from soils and three bacteriophages (ΦFifi044, ΦFifi450, and ΦFifi451) were isolated from the water in the pear orchard in Anseong. One bacteriophage, ΦFifi051, was isolated from soils and two bacteriophages (ΦFifi067 and ΦFifi318) were isolated from water in the apple orchard in Chungju. Plaques of bacteriophages are shown in Fig. 1. Two phages (ΦFifi044 and ΦFifi451) formed big and clear plaques. The plaque diameter of two phages ranged from 2 to 4 mm. Seven phages (ΦFifi011, ΦFifi450, ΦFifi051, ΦFifi067, ΦFifi106, ΦFifi287, and ΦFifi318) formed smaller but clear plaques. The plaque diameter of seven phages irregularly ranged from 0.5 to 2 mm. The plaque

| Location | Orchard | Host plant | Sample | Isolated phages |
|----------|---------|------------|--------|-----------------|
| Anseong  | A       | Pear       | Soil   | ΦFifi011        |
|          | B       | Pear       | Water  | ΦFifi044, ΦFifi450, ΦFifi451 |
|          | C       | Pear       | Soil   | ΦFifi106        |
|          | D       | Pear       | Soil   | ΦFifi287        |
| Chungju  | E       | Apple      | Soil   | ΦFifi051        |
|          | F       | Apple      | Water  | ΦFifi067        |
|          | G       | Apple      | Water  | ΦFifi318        |
diameter of previously reported Erwinia phages generally range from 0.5 to 4 mm; phage phiEa2809 formed variable sized plaques (0.5-2 mm), H5K and phiEaP-8 formed plaques with an average diameter of 1-2 mm, and Pea1 formed plaques with an average diameter of 2-4 mm (Lagonenko et al., 2015; Nagy et al., 2015; Park et al., 2018; Ritchie and Klos, 1977).

To examine the genetic diversity of bacteriophages, genomic DNA of nine bacteriophages was extracted with a phage DNA isolation kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer’s instructions. One μg genomic DNA was digested with restriction enzyme AseI (NEB, Ipswich, MA, USA) or HindIII (Takara, Tokyo, Japan) in a final volume of 20 μl at 37°C for 2 h. Two μl of LoadingSTAR (Dyne Bio Inc., Seongnam, Korea) was mixed with 10 μl of samples and loaded on an 0.7% agarose gel (70 V, 2 h). Restriction fragment length polymorphism (RFLP) analysis of the nine bacteriophages is shown in Fig. 2. All phages showed different DNA patterns in AseI except for ΦFifi044 and ΦFifi451. Because ΦFifi044 and ΦFifi451 showed similar RFLP patterns by AseI, the DNA patterns of two phages were additionally compared by HindIII. The two phages showed different DNA patterns. And clustering based on RFLP pattern for the nine bacteriophages were carried out. The difference of nine phages was confirmed through the dendrogram depicting clustering in RFLP of nine bacteriophages based on Neighbor joining generated in Toallab Quant v.13.2 algorithm (Supplementary Fig. 1).

To confirm the host range of the nine bacteriophages, E. amylovora strains, closely related E. pyrifoliae strains and other bacteria were used. E. amylovora and E. pyrifoliae were isolated from fire blight-infected apple and pear trees in diverse locations of Korea from 2015 to 2020 (Anseong, Asan, Chungju, Cheonan, Cheorwon, Chuncheon, Eumseong, Goseong, Gwangju, Hongcheon, Hwacheon, Icheon,
Isolation of Nine Bacteriophages against *E. amylovora*

Iksan, Jecheon, Jincheon, Mungyeong, Paju, Pocheon, Yanggu, and Yeoncheon) (Supplementary Table 1). All *Erwinia* strains were identified based on polymerase chain reaction (PCR) with species-specific primers, and identifications were confirmed using 16S rRNA sequence analysis. *E. amylovora* strains were identified by PCR by amplifying pEA29 using A (5′-CGGTTTTTTAAGCTGGG-3′)/B (5′-GGGCAAAATCTCGGATT-3′) primers (Bereswill et al., 1992). PCR conditions were as follows. Initial denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 50 s, and a final extension at 72°C for 7 min followed by 30 cycles for pEA29. *E. pyrifoliae* strains were identified by PCR by amplifying *cps* genes using CPS1 (5′-CGCGGAAGTGGTGAGAA-3′)/CPS2c (5′-GAACAGATGTGCCGAGTA-3′) primers (Kim et al., 2001). PCR conditions were as follows. Initial denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 50 s, and a final extension at 72°C for 7 min followed by 30 cycles for *cps* genes. PCR amplification was conducted using SafeDry Taq PCR Premix (Cellsafe, Yongin, Korea) with primers in a final volume of 20 μl.

For confirming the identification of bacteria, DNA from isolates was amplified by PCR using 16S rRNA universal primers 1492R (5′-GGTTACCTTGTTACGACTT-3′)/27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and sequenced using 16S rRNA universal primers 907R (5′-RCCGTCAATTCMTTTRAGTTT-3′)/785F (5′-GGATTAGATACCCTGGTA-3′) followed by Macrogen Co. (Seoul, Korea). The nine isolated bacteriophages were examined in regard to their host range through dotting assays against 45 strains of *E. amylovora*, 14 strains of *E. pyrifoliae*, and nine other bacteria (*Dickeya zeae*, *Escherichia coli*, *P. agglomerans*, *Pectobacterium carotovorum*, *P. fluorescens*, *Xanthomonas campestris*, *Bacillus thuringiensis*, *Enterococcus faecalis*, and *Staphylococcus xylosus*). Bacteria were grown in TSA or TSB at 28°C, but *E. coli* strain ATCC 43895, *E. faecalis* strain ATCC 35038, and *S. xylosus* strain ATCC 29971 were grown in TSA or TSB at 37°C. A volume of 4 ml of soft agar mixed with 20 μl of overnight cultured *E. amylovora* strain YKB 14808 was poured on agar plates and 10 μl bacteriophage aliquots (10⁸ PFU/ml) were added and incubated overnight. The results of host range tests of the nine phages are shown in Fig. 3. ΦFifi044 and ΦFifi451 were only effective against *E. amylovora* strains. On the other hand, the other seven phages were effective against both *E. amylovora* and *E. pyrifoliae* strains. All nine phages were not effective against other tested bacterial strains. *Erwinia* phages reported so far can be divided into two groups according to the host range. The first is a group with a narrow host range specific to *E. amylovora*, such as Pea1, 2, 5, and Henal (Besarab et al., 2020; Ritchie and Klos, 1977). The second group includes phages with a wide host range, including the *Pantoea* spe-

---

**Fig. 3.** Heatmap showing the host range of nine isolated phages. Susceptibility of bacteriophages against *Erwinia amylovora*, *E. pyrifoliae*, and other bacterial strains is indicated by a color scale, from white to red (clearing of 1 to 4; 0 = no lysis).
cies. Phage phiEa2809, vB_EamM_Y3, Deimos-Minion, Simmy50, RAY, Special G, Bosolaphorus, Desertfoxx, MadMel, and Mortimer are broad host range phages infecting E. amylovora, P. agglomerans, and Pantoaea vagans strains (Buttimer et al., 2018; Lagonenko et al., 2015). Erwina and Pantoaea species belong to Erwiniaaceae bacteria. Therefore, most reported phages were tested for host range against Erwina and Pantoaea strains (Sharma et al., 2019). In Korea, four E. amylovora bacteriophages were reported. phiEaP-8, with a narrow host range, is effective against only E. amylovora and E. pyrifoliae strains (Park et al., 2018). Phage pEa_SNUABM_12, 47, and 50, with a broad host range, is effective against E. amylovora, E. pyrifoliae, Erwinia spp., and Serratia maarcescens strains (Kim et al., 2020). ФFifi044 and ФFifi451 infected and lysed E. amylovora only. This is the first report on the isolation of E. amylovora-specific bacteriophages in Korea. The other seven phages were specific to E. amylovora and E. pyrifoliæ, similar to some previously reported phages. E. pyrifoliæ was reported and classified as a novel species causing black shoot blight disease of pear and apple in Korea, and the symptoms are similar to those of fire blight (Kim et al., 1999). In Korea, these phages may be useful as biological control agents because E. amylovora and E. pyrifoliæ can coexist in the same environment.

Control of E. amylovora infections is necessary to protect apples and pears and reduce economic losses. Chemical controls such as antibiotics and copper compounds have the potential to affect the environment and humans by giving rise to resistant bacteria as well as inhibiting the growth of all microorganisms to which they are exposed, including those beneficial to plants. Bacteriophages are considered a suitable biological control because of their host specificity and environmental friendliness. Therefore, nine bacteriophages effective against E. amylovora were isolated and characterized regarding plaque morphology, genetic diversity, and host range. The results of gDNA pattern analysis show that the nine phages are different from each other, and this diversity is considered a positive factor for the synergistic effect of a phage cocktail. According to the host range and plaque morphology, the nine phages are divided into two groups. In particular, ФFifi044 and ФFifi451 belonging to the first group are noteworthy in that they are the first E. amylovora specific phages reported in Korea. Further studies will provide information on the characteristics of phages for application as biocontrol agents.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

We thank Dr. Young-Kee Lee for providing the YKB strains of E. amylovora and E. pyrifoliæ. This work was supported by the Rural Development Administration (RDA; grant number PJ01496501).

Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

References

Aćimović, S. G., Zeng, Q., McGhee, G. C., Sundin, G. W. and Wise, J. C. 2015. Control of fire blight (Erwinia amylovora) on apple trees with trunk-injected plant resistance inducers and antibiotics and assessment of induction of pathogenesis-related protein genes. Front. Plant Sci. 6:16.

Berestwill, S., Pahl, A., Bellemann, P., Zeller, W. and Geider, K. 1992. Sensitive and species-specific detection of Erwinia amylovora by polymerase chain reaction analysis. Appl. Environ. Microbiol. 58:3522-3526.

Besarab, N. V., Akhremchuk, A. E., Zlatohurska, M. A., Romaniuk, L. V., Valentovich, L. N., Tovkach, F. I., Lagonenko, A. L. and Evtushenkov, A. N. 2020. Isolation and characterization of Henal - a novel Erwinia amylovora bacteriophage. FEMS Microbiol. Lett. 367:fnaa070.

Born, Y., Fieseler, L., Marazzi, J., Lurz, R., Dufy, B. and Loesner, M. J. 2011. Novel virulent and broad-host-range Erwinia amylovora bacteriophages reveal a high degree of mosaicism and a relationship to Enterobacteraeae phages. Appl. Environ. Microbiol. 77:5945-5954.

Buttimer, C., Born, Y., Lucid, A., Loessner, M. J., Fieseler, L. and Coffey, A. 2018. Erwinia amylovora phage vB_EamM_Y3 represents another lineage of hairy Myoviridae. Res. Microbiol. 169:505-514.

Dagher, F., Olishhevskaya, S., Philion, V., Zheng, J. and Déziel, E. 2020. Development of a novel biological control agent targeting the phytopathogen Erwinia amylovora. Helion 6:e05222.

Doss, J., Culbertson, K., Hahn, D., Camacho, J. and Barekzi, N. 2017. A review of phage therapy against bacterial pathogens of aquatic and terrestrial organisms. Viruses 9:50.

Dunne, M., Hupfeld, M., Klumpp, J. and Loesner, M. J. 2018. Molecular basis of bacterial host interactions by Gram-positive targeting bacteriophages. Viruses 10:397.

Essa, N., Rossitto, M. and Fiscarelli, E. V. 2020. Phages and phage therapy: past, present and future. Microbiol. Med. 35:8709.

Gayder, S., Parcey, M., Castle, A. J. and Svircev, A. M. 2019. Host range of bacteriophages against a world-wide collection
of *Erwinia amylovora* determined using a quantitative PCR assay. *Viruses* 11:910.

Gayder, S., Parcey, M., Nesbitt, D., Castle, A. J. and Svircev, A. M. 2020. Population dynamics between *Erwinia amylovora*, *Pantoea agglomerans* and bacteriophages: exploiting synergy and competition to improve phage cocktail efficacy. *Microorganisms* 8:1449.

Ge, H., Hu, M., Zhao, G., Du, Y., Xu, N., Chen, X. and Jiao, X. 2020. The "fighting wisdom and bravery" of tailed phage and host in the process of adsorption. *Microbiol. Res.* 230:126344.

Kim, S. G., Lee, S. B., Giri, S. S., Kim, H. J., Kim, S. W., Kwon, J., Park, J., Roh, E. and Park, S. C. 2020. Characterization of novel *Erwinia amylovora* jumbo bacteriophages from *Eneladusvirus* genus. *Viruses* 12:1373.

Kim, W.-S., Gardan, L., Rhim, S.-L. and Geider, K. 1999. *Erwinia pyrifoliae* sp. nov., a novel pathogen that affects Asian pear trees (*Pyrus pyrifolia* Nakai). *Int. J. Syst. Bacteriol.* 49:899-906.

Kim, W.-S., Hildebrand, M., Jock, S. and Geider, K. 2001. Molecular comparison of pathogenic bacteria from pear trees in Japan and the fire blight pathogen *Erwinia amylovora*. *Microbiology* 147:2951-2959.

Lagonenko, A. L., Sadovskaya, O., Valentovich, L. N. and Evtushenkov, A. N. 2015. Characterization of a new Vil-like *Erwinia amylovora* bacteriophage phiEa2809. *FEMS Microbiol. Lett.* 362:fnv031.

Lee, M. S., Lee, I., Kim, S. K., Oh, C.-S. and Park, D. H. 2018. *In vitro* screening of antibacterial agents for suppression of fire blight disease in Korea. *Res. Plant Dis.* 24:41-51 (in Korean).

Lec-Carrillo, C. and Abedon, S. T. 2011. Pros and cons of phage therapy. *Bacteriophage* 1:111-114.

Mechan Llontop, M. E., Hurley, K., Tian, L., Bernal Galeano, V. A., Wildschutte, H. K., Marine, S. C., Yoder, K. S. and Violat, B. A. 2020. Exploring rain as source of biological control agents for fire blight on apple. *Front. Microbiol.* 11:199.

Mikiciński, A., Sobieczewski, P., Pulawska, J. and Maciorowski, R. 2016. Control of fire blight (*Erwinia amylovora*) by a novel strain 49M of *Pseudomonas graminis* from the phyllosphere of apple (*Malus* spp.). *Eur. J. Plant Pathol.* 145:265-276.

Nagy, J. K., Schwarzinger, I., Künstler, A., Pogány, M. and Király, L. 2015. Penetration and translocation of *Erwinia amylovora*-specific bacteriophages in apple: a possibility of enhanced control of fire blight. *Eur. J. Plant Pathol.* 142:815-827.

Park, D. H., Yu, J.-G., Oh, E.-J., Han, K.-S., Yea, M. C., Lee, S. J., Myung, I.-S., Shim, H. S. and Oh, C.-S. 2016. First report of fire blight disease on Asian pear caused by *Erwinia amylovora* in Korea. *Res. Plant Dis.* 100:1946.

Park, J., Lee, G. M., Kim, D., Park, D. H. and Oh, C.-S. 2018. Characterization of the lytic bacteriophage phiEaP8 effective against both *Erwinia amylovora* and *Erwinia pyrifoliae* causing severe diseases in apple and pear. *Plant Pathol. J.* 34:445-450.

Principi, N., Silvestri, E. and Esposito, S. 2019. Advantages and limitations of bacteriophages for the treatment of bacterial infections. *Front. Pharmacol.* 10:513.

Ritchie, D. F. and Klos, E. J. 1977. Isolation of *Erwinia amylovora* bacteriophage from aerial parts of apple trees. *Phytopathology* 67:101-104.

Sharifazizi, M., Harighi, B. and Sadeghi, A. 2017. Evaluation of biological control of *Erwinia amylovora*, causal agent of fire blight disease of pear by antagonistic bacteria. *Biol. Control* 104:28-34.

Sharma, R., Pielstick, B. A., Bell, K. A., Nieman, T. B., Stubbs, O. A., Yeates, E. L., Baltrus, D. A. and Grose, J. H. 2019. A novel, highly related jumbo family of bacteriophages that were isolated against *Erwinia*. *Front. Microbiol.* 10:1533.

Stone, E., Campbell, K., Grant, I. and McAuliffe, O. 2019. Understanding and exploiting phage-host interactions. *Viruses* 11:567.