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BIOTECHNOLOGY

Development of a Bacteriophage Cocktail against *Pectobacterium carotovorum* Subsp. *carotovorum* and Its Effects on *Pectobacterium* Virulence

Hyeongsoon Kim,[a] Minsik Kim,[b] Sam-Nyu Jee,[c] Sunggi Heu,[d] Sangryeol Ryu[a]

[a]Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul, South Korea

[b]Department of Food and Nutrition, Brain Korea 21 FOUR, College of Human Ecology, Yonsei University, Seoul, South Korea

[c]Highland Agriculture Research Institute, National Institute of Crop Science, Pyeongchang-gun, South Korea

[d]Department of Agriculture, Forestry and Bioresources, Seoul National University, Seoul, South Korea

Hyeongsoon Kim and Minsik Kim have contributed equally to this article. Author order was determined in order of increasing seniority.

ABSTRACT *Pectobacterium carotovorum* subsp. *carotovorum* is a necrotrophic plant pathogen that secretes plant cell wall-degrading enzymes (PCWDEs) that cause soft rot disease in various crops. Bacteriophages have been under consideration as harmless antibacterial agents to replace antibiotics and copper-based pesticides. However, the emergence of bacteriophage resistance is one of the main concerns that should be resolved for practical phage applications. In this study, we developed a phage cocktail with three lytic phages that recognize colanic acid (phage POP12) or flagella (phages POP15 and POP17) as phage receptors to minimize phage resistance. The phage cocktail effectively suppressed the emergence of phage-resistant *P. carotovorum* subsp. *carotovorum* compared with single phages in *in vitro* challenge assays. The application of the phage cocktail to napa cabbage (*Brassica rapa* subsp. *pekinensis*) resulted in significant growth retardation of *P. carotovorum* subsp. *carotovorum* ($P < 0.05$) and prevented the symptoms of soft rot disease. Furthermore, phage cocktail treatments of young napa cabbage leaves in a greenhouse environment indicated effective prevention of soft rot disease compared to that in the nonphage negative control. We isolated 15 phage-resistant mutants after a phage cocktail treatment to assess the virulence-associated phenotypes compared to those of wild-type (WT) strain Pcc27. All mutants showed reduced production of four different PCWDEs, leading to lower levels of tissue softening. Ten of the 15 phage-resistant mutants additionally exhibited decreased swimming motility. Taken together, these results show that the phage cocktail developed here, which targets two different types of phage receptors, provides an effective strategy for controlling *P. carotovorum* subsp. *carotovorum* in agricultural products, with a potential ability to attenuate *P. carotovorum* subsp. *carotovorum* virulence.

IMPORTANCE *Pectobacterium carotovorum* subsp. *carotovorum* is a phytopathogen that causes soft rot disease in various crops by producing plant cell wall-degrading enzymes (PCWDEs). Although antibiotics and copper-based pesticides have been extensively applied to inhibit *P. carotovorum* subsp. *carotovorum*, the emergence of antibiotic-resistant bacteria and demand for harmless antimicrobial products have emphasized the necessity of finding alternative therapeutic strategies. To address this problem, we developed a phage cocktail consisting of three *P. carotovorum* subsp. *carotovorum*-specific phages that recognize colanic acids and flagella of *P. carotovorum* subsp. *carotovorum*. The phage cocktail treatments significantly decreased *P. carotovorum* subsp. *carotovorum* populations, as well as soft rot symptoms in napa cabbage. Simultaneously, they resulted in virulence attenuation in phage-resistant *P. carotovorum* subsp. *carotovorum*, which was represented by decreased PCWDE production and

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Address correspondence to Sangryeol Ryu, sangryu@snu.ac.kr.

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decreased flagellum-mediated swimming motility. These results suggested that preparations of phage cocktails targeting multiple receptors would be an effective approach to biocontrol of *P. carotovorum* subsp. *carotovorum* in crops.

KEYWORDS *Pectobacterium carotovorum* subsp. *carotovorum*, bacteriophage cocktail, phage receptor, soft rot disease, virulence attenuation

Pectobacterium carotovorum subsp. *carotovorum* is a ubiquitous necrotrophic phytopathogen that is responsible for soft rot disease in various crops and ornamental plants during their cultivation, transport, and storage (1). *P. carotovorum* subsp. *carotovorum* produces and secretes plant cell wall-degrading enzymes (PCWDEs), including pectate lyase (Pel), polygalacturonase (Peh), cellulase (Cel), and protease (Prt), which are the major virulence factors of *P. carotovorum* subsp. *carotovorum* (2). These pathogens primarily degrade cell walls by using Pel and penetrate host tissue through natural pores (e.g., lenticels and stomata) or wounds, which consequently leads to tissue maceration in tubers, rhizomes, and leaves (1, 3). *P. carotovorum* subsp. *carotovorum* has been reported as a causative agent of many infectious crop disease outbreaks and is ranked among the top 10 pathogens of importance in agriculture (4–7). Copper-based pesticides and antibiotics have been applied to protect agricultural products from *Pectobacterium* spp. (former *Erwinia* spp.) infections (8–11). However, a novel therapeutic strategy for the biocontrol of *P. carotovorum* subsp. *carotovorum* needs to be developed due to the spread of bacterial resistance against chemotherapeutics and the public's desire to use safer and ecologically friendly biological agents (12).

Bacteriophages (phages) are viruses that specifically infect host bacteria. They hijack the host machinery for self-replication and subsequently lyse their host bacterium to release progeny viral particles (13). Phages have several advantages for agricultural application as alternative antimicrobial agents compared to conventional chemical biocides: harmlessness to humans and symbiotic bacteria, relatively lower development and production costs than antibiotics, and self-dosing (14, 15). Several therapeutic uses of bacteriophages for the biocontrol of *Pectobacterium* have been studied. Phages PP1, DU_PP13B, ϕA38, and ϕ41 were characterized and successfully protected lettuce and potato tubers from *P. carotovorum* subsp. *carotovorum* infections (16–18). However, these cases of single-phage treatments have several limitations, such as narrow host ranges and the easy development of bacterial resistance (19).

Antiphage mechanisms in bacteria, including spontaneous mutations, restriction-modification systems, and CRISPR-Cas adaptive immunity, have been studied (20). Bacteria may alter or eliminate their cell surface components that function as phage receptors through spontaneous mutations, which block the adsorption of phages. For instance, we recently revealed that a spontaneous mutant resistant to colanic acid (CA)-recognizing phage POP72 contained a missense mutation within a putative *wzc* gene that is involved in the biosynthesis of the capsular polysaccharide CA (21). *Salmonella enterica* serovar Typhimurium strain LT2(c) could block phage SPC35 adsorption through phase-variable glucosylation of the receptor O antigen (22), and phage 117-resistant *Klebsiella* prevented phage adsorption by introducing mobile genetic elements into the coding region of the *wcaJ* gene, which is associated with CA biosynthesis (23). The use of phage cocktails has been considered to be an effective strategy to broaden host ranges and to overcome phage resistance mechanisms in the biocontrol of foodborne pathogens or phytopathogens. Kim et al. and Bai et al. developed phage cocktails targeting different phage receptors, and they successfully retarded the emergence of phage-resistant *Salmonella* Typhimurium in *in vitro* challenge assays (24, 25). Czajkowski et al. and Zaczek-Moczydłowska et al. reported the efficacy of phage mixtures that were able to suppress *Pectobacterium* growth in semi-*in planta* experiments on potato tubers (26, 27).

Phage resistance induced by strong selective pressure may trigger trade-off costs, including attenuated virulence and reduced fitness within heterogeneous populations

(28). Phage-mediated selection induced a modified lipopolysaccharide (LPS) composition in *Pectobacterium atrosepticum*, which resulted in reduced motility and virulence while unexpectedly improving abiotic-surface adhesion (29). Phage-resistant *Ralstonia solanacearum* strains that were isolated from phage combination-treated tomatoes exhibited defective growth rates and reduced competitive abilities compared to those of the phage-susceptible ancestral pathogen (30). In contrast, phenotypic changes beneficial to bacterial survival, such as increased antibiotic resistance and exopolysaccharide production, were reported in *Escherichia coli* and *Pseudomonas fluorescens* (31, 32). These various trade-offs associated with phage resistance inevitably occur in pathogens during the arms race with predator phages. Therefore, it is important to understand the fitness costs associated with phage treatments when formulating phage cocktails.

In this study, we developed a *P. carotovorum* subsp. *carotovorum* phage cocktail consisting of three phages that recognize different phage receptors (CA and flagella). The phage cocktail treatment more effectively retarded both *P. carotovorum* subsp. *carotovorum* growth and soft rot disease progression in napa cabbage (*Brassica rapa* subsp. *pekinensis*) compared to single-phage treatments. The attenuated virulence of phage-resistant *P. carotovorum* subsp. *carotovorum* due to phage cocktail treatment could indicate the usefulness of phage cocktails as alternative biocontrol agents against *P. carotovorum* subsp. *carotovorum* infections.

RESULTS

Receptor analysis of *Pectobacterium* phages to develop phage cocktails. Phage cocktails, which consist of multiple phages that recognize various phage receptors, can retard the development of phage-resistant bacteria (25, 33). Six *Pectobacterium* phages with clear plaques were isolated from sewage by using WT *P. carotovorum* subsp. *carotovorum* strain Pcc27 as the host bacterium. As all *Pectobacterium* phages that were previously isolated by our group recognize CA as a phage receptor (21), we attempted to obtain phages that target phage receptors other than CA. We conducted spot assays to determine the phage receptors by using three Pcc27 mutants with insertions of transposon Tn*5* (*cpsG*::Tn*5*, *wcaA*::Tn*5*, and *gmd*::Tn*5*) that could not synthesize CA (21, 34). Among the six phages, four phages (POP11, POP12, POP13, and POP14) could not infect the three transposon mutants (Table S1 in the supplemental material). The sensitivity of Pcc27 against the four phages was restored upon plasmid complementation with the *wcaA* gene even without IPTG (isopropyl-β-D-thiogalactopyranoside) induction (Fig. 1; Fig. S1). This result suggested that POP11, POP12, POP13, and POP14 recognized CA as a phage receptor. However, POP15 and POP17 formed single plaques on the lawns of the three transposon mutants, which indicated that these two phages recognized an apparatus other than CA on the Pcc27 cell surface.

A Tn*5* insertional mutant library in the Pcc27 background was constructed and screened for phage POP15 and POP17 resistance to identify phage receptor(s) (35, 36). We acquired two POP17-resistant bacterial strains from the screening. Through partial DNA sequencing, we found that these two mutants had a Tn*5* insertion in a putative *flhA* (homology to *Pcc21_RS13355*) or putative *flhD* (homology to *Pcc21_13415*) gene, respectively. The putative *flhA* gene in *Erwinia amylovora* is associated with flagellum biosynthesis, and the putative *flhD* gene encodes a master regulator that controls the expression of flagellum genes in *Pectobacterium carotovorum* subsp. *carotovorum* (formerly *Erwinia carotovora* subsp. *carotovora*) (37, 38). When the *flhA* gene was complemented under the control of the IPTG-inducible promoter, the susceptibility of the *flhA*::Tn*5* mutant against phages POP15 and POP17 was restored to the WT level (Fig. 1). These results suggested that phages POP15 and POP17 recognize flagella as phage receptors.

Host range determination of *Pectobacterium* phages. The host ranges of six phages were determined by using 70 *Pectobacterium* strains that were isolated in South Korea (39, 40). Phage POP12 had the broadest host range compared to the other CA-recognizing phages and showed clear plaques or bacterial growth inhibition zones on *P. carotovorum* subsp. *carotovorum* isolates (43 of 47 strains), *P. carotovorum* subsp. *brasiliensis* isolates (12 of 17 strains), and *P. carotovorum* subsp. *odoriferum* isolates

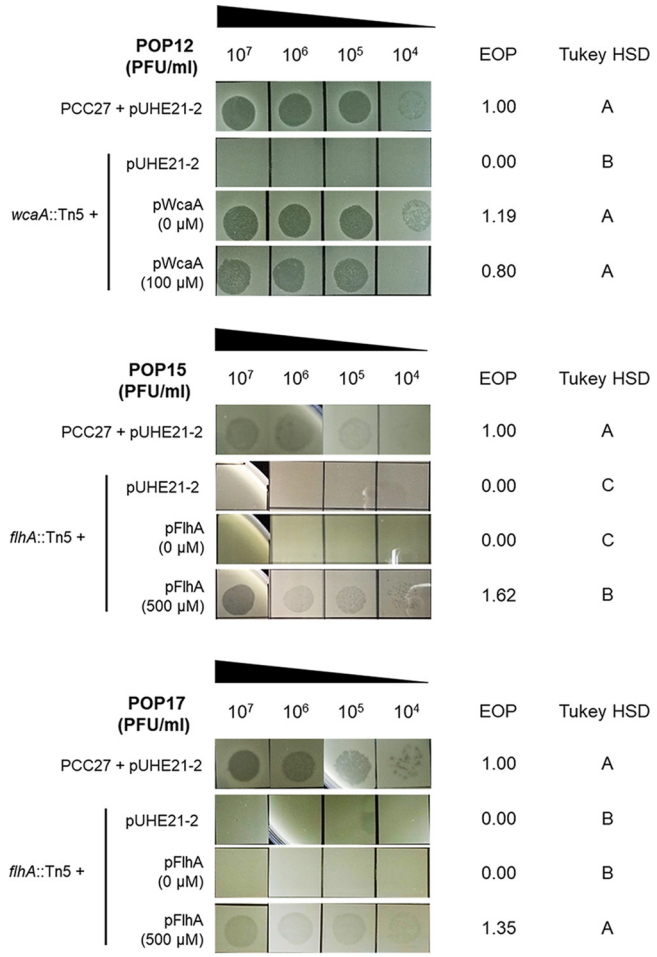

FIG 1 Determination of phage receptors for *Pectobacterium*-targeting phages. Transposon mutants with *wcaA* or *flhA* genes disrupted by Tn*5* insertion did not form plaques in the phage spot assays. Complementation of each gene in the Tn*5* mutants restored phage susceptibility. The IPTG concentrations are indicated in parentheses. Efficiency of plating (EOP) was calculated by dividing the titer of the phage on each indicated strain by the titer of the same phage on WT strain Pcc27 harboring pUHE21-2. Significant differences among the experimental groups are marked with letters to the right. One representative result from triplicate experiments is shown. HSD, honestly significant difference.

(4 of 4 strains) (Fig. S2). Phages POP15 and POP17 showed relatively narrower host ranges than POP12, but both flagellum-dependent phages could form plaques or inhibition zones against *P. carotovorum* subsp. *carotovorum* strains (e.g., Pcc19 and Pcc92) and two *Pectobacterium atrosepticum* strains that are insusceptible to POP12. Phage POP15 could infect a *P. carotovorum* subsp. *carotovorum* strain, Pcc22, that is resistant to POP12 and POP17, and phage POP17 could form plaques or inhibition zones against *P. carotovorum* subsp. *brasiliensis* strains (e.g., E10, E12, and E42) that are resistant to POP12 and POP15 (Fig. S2). The results of the host range assays of POP15 and POP17 indicated that the use of flagellum-dependent phages together with POP12 could broaden the host spectrum. Thus, we mixed POP12, POP15, and POP17 to construct a phage cocktail in which CA- and flagellum-recognizing phages were mixed at the same concentration.

Genomic and morphological characterization of POP12, POP15, and POP17. We conducted whole-genome sequencing of the three phages chosen for the phage cocktail to investigate whether the genes associated with bacterial virulence, antibiotic resistance, and phage lysogen decisions were present in the phage genomes. Bioinformatic analysis revealed that phages POP12, POP15, and POP17 contained 170,838 bp, 153,445 bp, and

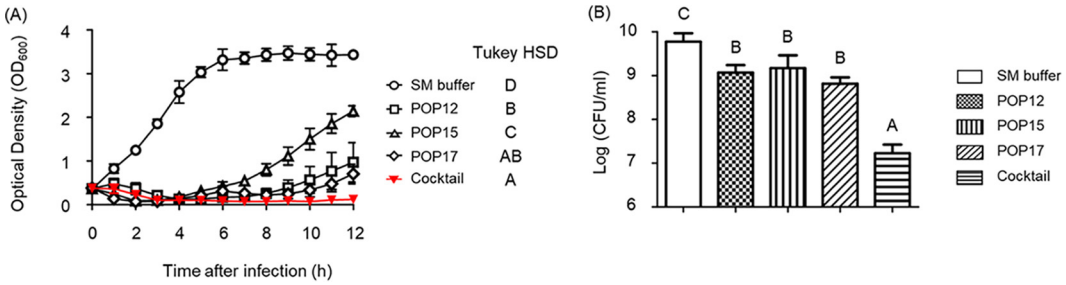

FIG 2 Bacterial challenge assay with phage POP12, POP15, or POP17 or the phage cocktail comprised of all three phages. Wild-type Pcc27 was infected with phages at MOIs of 1. (A) The bacterial growth was monitored hourly by measuring the optical densities (OD_{600}). (B) The viable bacterial populations were enumerated at 12 h postinfection. SM buffer was added to the Pcc27 culture as a negative control. The mean values with standard deviations (SD) from triplicate experiments are shown. Statistical analysis was conducted by one-way analysis of variance (ANOVA) with Tukey's multiple-comparison tests among the experimental groups 12 h postinfection. Significant differences among the experimental groups are marked with letters ($P < 0.001$).

155,176 bp of double-stranded DNA, respectively, and that the %GC contents were 36.3%, 51.7%, and 51.9%, respectively (Fig. S3). Genome annotation by GeneMark.hmm and the RAST annotation engine suggested that phages POP12, POP15, and POP17 had 257, 301, and 308 putative open reading frames (ORFs), respectively (41, 42). No genes associated with lysogen formation, toxins, or bacterial virulence factors were identified, which implies the lytic nature and safety of these three phages.

Morphological observations by transmission electron microscopy (TEM) revealed that the three phages belonged to the family *Myoviridae* (Fig. S4). Phage POP12 had an elongated icosahedral head (125.6 $\pm$ 2.6 nm long and 85.6 $\pm$ 0.9 nm wide) and contractile tail (122.2 $\pm$ 1.9 nm). Phages POP15 and POP17 had isometric heads and contractile, long tails. The head diameter of POP15 was 69.5 $\pm$ 2.7 nm, and that of POP17 was 94.9 $\pm$ 0.7 nm. The tail length of POP15 was 100.7 $\pm$ 5.0 nm, whereas that of POP17 was 129.0 $\pm$ 5.7 nm.

Bacterial challenge assays with phage cocktails. We conducted *in vitro* bacterial challenge assays to evaluate the efficacy of single phages or phage cocktails in the inhibition of bacterial growth. Single phages or phage cocktails were added to the strain Pcc27 culture at an MOI of 1. The growth of Pcc27 was rapidly suppressed by phage POP15 but resumed at 4 h postinfection. Phages POP12 and POP17 each inhibited bacterial growth until 8 h postinfection. In contrast, treatment with phage cocktails inhibited the emergence of phage-resistant mutants for at least 12 h postinfection (Fig. 2A). The numbers of viable *P. carotovorum* subsp. *carotovorum* cells 12 h postinfection with the phage cocktail were also significantly lower than those of the single-phage treatments, as well as the negative control ($P < 0.001$) (Fig. 2B). These results indicated that the phage cocktail could effectively inhibit host growth and delay the emergence of phage-resistant mutants.

Application of phage cocktails to prevent soft rot in napa cabbage. The bactericidal effects of single phages and the phage cocktail against *P. carotovorum* subsp. *carotovorum* infection were evaluated by using napa cabbage. Each sample was artificially inoculated with 3×10^5 CFU/mL of rifampicin-resistant Pcc27 ($Pcc27^{RifR}$) and was then treated with single phages or a phage cocktail at a multiplicity of infection (MOI) of 10^3. When phages were not applied, the number of $Pcc27^{RifR}$ cells in napa cabbage increased up to approximately 7-log CFU/cm^2 within 24 h, and consequently, the sample exhibited typical soft rot symptoms. In contrast, the phage cocktail treatment, rather than single-phage treatments, significantly inhibited the growth of $Pcc27^{RifR}$ ($P = 0.032$) and the progression of disease in napa cabbage after 24 h of incubation (Fig. 3). The growth of $Pcc27^{RifR}$ was also retarded by a phage POP12 treatment up to 16 h of incubation, but the number of recovered $Pcc27^{RifR}$ populations at 24 h was higher than that of the cocktail-treated group and showed no significant difference from the negative control ($P = 0.075$) (Fig. 3A). In addition, soft rot symptoms began

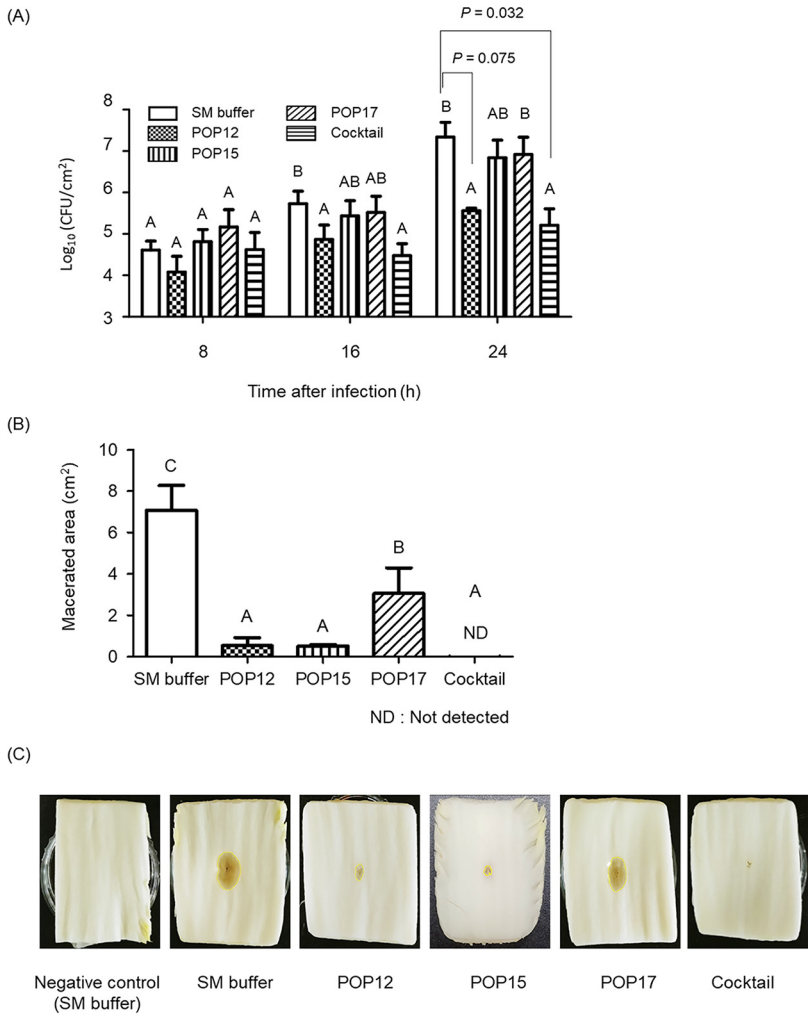

FIG 3 Retardation of soft rot disease development in napa cabbage by phage treatment. Each artificially inoculated crop sample was treated with or without phages at an MOI of 10^3 and incubated in a humid chamber. (A) The numbers of *P. carotovorum* subsp. *carotovorum* cells were determined at the indicated time points. (B) Macerated areas of napa cabbage were measured after 24 h of incubation using ImageJ. (C) The symptoms of soft rot were monitored after 24 h of incubation. A sample inoculated with SM buffer was used as a negative control, and strain Pcc27^{RifR} treated with SM buffer was used as a nonphage control. Each column in panels A and B represents the mean value from triplicate experiments, and error bars indicate the standard deviations. One-way analysis of variance (ANOVA) with Tukey's multiple-comparison test was performed for comparing the CFU values and macerated areas among the groups at the indicated time points. Significant differences among the experimental groups are marked with letters ($P < 0.001$). Representative results from triplicate experiments are shown in panel C.

after 24 h of incubation in POP12-treated napa cabbage (Fig. 3C). A single-phage treatment with phage POP15 or POP17 did not significantly affect the growth of Pcc27^{RifR} cells in napa cabbage (Fig. 3A), and the symptoms of soft rot were also noticeable (Fig. 3C). Comparable protective effects were also achieved against soft rot disease in napa cabbage artificially inoculated with *P. carotovorum* subsp. *carotovorum* strain Pcc19 or Pcc21 (Fig. S5).

Next, single phages or the phage cocktail were applied by foliar spraying onto young napa cabbage leaves in a greenhouse to assess their antibacterial effects against Pcc27. The three-phage cocktail treatment protected young leaves from soft rot disease better than the single-phage treatments (Fig. 4A). The percentage of rotten leaves with the phage cocktail treatment was 7.75%, but the values obtained after applying single phages ranged from 22.54% to 33.1% (Fig. 4A). Moreover, the qualitative ordinal scale of the soft rot disease indicated that the disease severity of the phage cocktail-treated

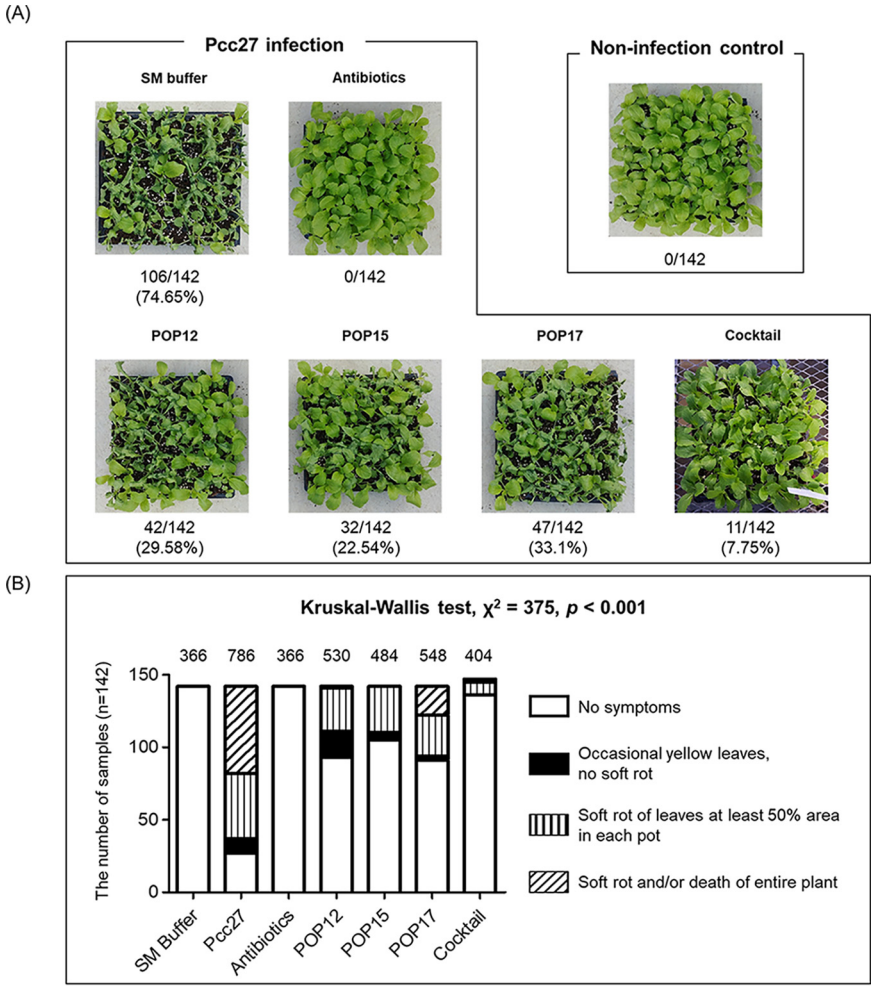

FIG 4 Effect of the phage cocktail in preventing soft rot in young leaves of napa cabbage grown in the greenhouse. (A) Each phage or phage cocktail was sprayed onto young leaves at an MOI of 100. *P. carotovorum* subsp. carotovorum Pcc27 was added to experimental groups at 1×10^6 CFU/pot 1 day after phage treatments, and the samples were incubated for 30 h in a humidity chamber. The disease symptoms were examined 2 days after transfer of the samples to the greenhouse. Antibiotics were used as a positive control, and SM buffer was used as a nonphage negative control. The rates of rotten napa cabbage are indicated below the images. One representative result from the triplicate experiments is shown. (B) The extent of soft rot was evaluated based on visual assessment of symptom severity in leaves using a four-point scale. Statistical analysis was performed by using nonparametric Kruskal-Wallis analysis. The chi-square (χ^2) test result is presented above each bar. A lower chi-square value indicates weaker symptoms of soft rot.

samples was similar to that of antibiotic-treated samples (Fig. 4B). These results suggested that phage cocktail treatments exhibited better antimicrobial effects in preventing soft rot disease than single-phage treatments, at least in the laboratory and greenhouse tests performed.

Virulence evaluation of phage-resistant mutants. Attenuation of bacterial virulence caused by phage infections has been well reported in various foodborne pathogens (43–45). However, studies on the association of bacterial virulence with phage resistance have been reported for only a few plant pathogens, such as *P. atrosepticum* and *R. solanacearum* (29, 46, 47). To determine whether the *P. carotovorum* subsp. *carotovorum* virulence was altered by phage infection, we obtained 80 colonies from the phage cocktail treatment and purified them by sequential streaking on fresh LB plates at least three times. The susceptibility of each clone against single phages was tested with spot assays. Fifteen clones (Pcc^{R1} to Pcc^{R15}) were insusceptible to phage POP12, and five of the clones (Pcc^{R11} to Pcc^{R15}) were additionally resistant to phage POP15 and POP17 infections (Table S2). In

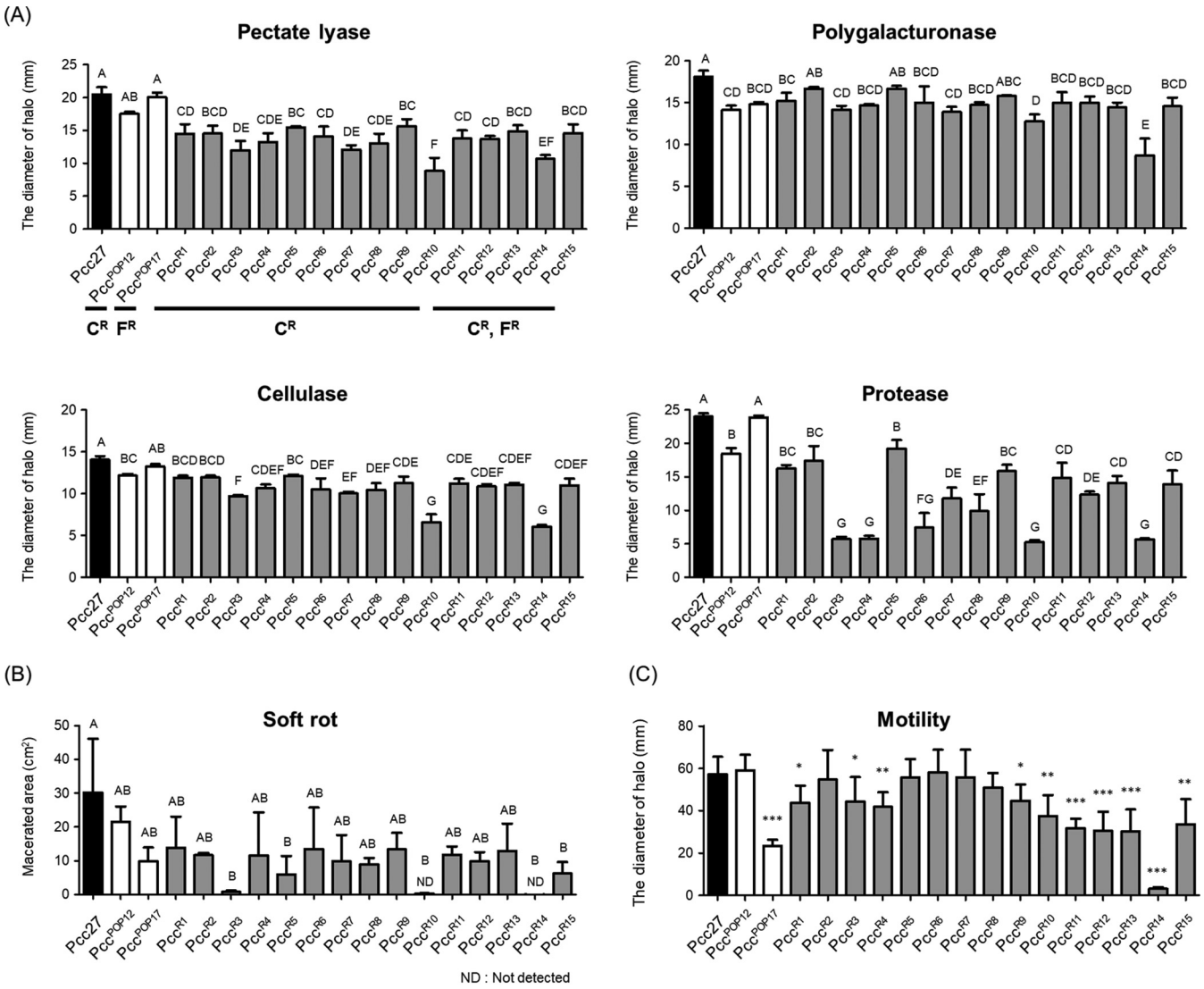

FIG 5 Attenuated virulence of phage-resistant mutants evaluated by extracellular enzyme assay (A), virulence assay in napa cabbage (B), and motility assay (C). (A) Each column indicates the diameters of the haloes around the wells that were measured to represent the enzyme activities (details in Materials and Methods). (B) Macerated areas of napa cabbage were measured after 24 h of incubation using ImageJ. (C) The swimming haloes of bacterial growth on 0.3% agar plates were measured after 24 h of incubation. The mean values with SD from triplicate experiments are shown. Significant differences among the experimental groups are marked with letters and asterisks. C^R, resistant to CA-recognizing phage; F^R, resistant to flagellum-recognizing phage; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

addition to the 15 mutants isolated after phage cocktail treatment (Pcc^{R1} to Pcc^{R15}; also referred to as "phage-resistant mutants" below), we obtained POP12- or POP17-resistant mutants (Pcc^{POP12} and Pcc^{POP17}) from single-phage treatments for use as controls in the virulence comparison.

Among the important virulence factors of *P. carotovorum* subsp. *carotovorum* are PCWDEs, which are secreted through the type II secretion system (48). We evaluated the production of four different PCWDEs in the 15 phage-resistant mutants by performing extracellular enzyme assays and virulence assays using napa cabbage. Interestingly, all 15 mutants (Pcc^{R1} to Pcc^{R15}) and Pcc^{POP12} produced smaller amounts of PCWDEs than WT Pcc27 (Fig. 5A; Fig. S6 and Table S3). We then compared the PCWDE activities of the 15 phage-resistant mutants to those of Pcc^{POP12}, which presented lower PCWDE activities than Pcc^{POP17}. Ten mutants exhibited lower Pel activities than Pcc^{POP12}, and the levels of Cel and Prt production were also reduced in 6 and 11 of the 15 phage-resistant *P. carotovorum* subsp. *carotovorum* strains, respectively (Fig. 5A). However, all of the 15 mutants except

Pcc^{R14} had Peh activities similar to that of Pcc^{POP12} (Table S3). In accordance with the much lower production of PCWDEs than for the other phage-resistant mutants (Fig. 5A), napa cabbage inoculated with the Pcc^{R10} and Pcc^{R14} strains showed no symptoms of soft rot (Fig. 5B; Fig. S6). Three more mutants (Pcc^{R3}, Pcc^{R5}, and Pcc^{R15}) also showed significant reduction in soft rot symptoms compared to those in napa cabbage inoculated with Pcc^{POP17}, and the rotten-tissue areas of napa cabbage inoculated with the other 10 phage-resistant *P. carotovorum* subsp. *carotovorum* strains were smaller than those of the WT Pcc27- or Pcc^{POP12}-inoculated controls (Fig. 5B; Fig. S6).

The group of mutants that were resistant to all three phages (Pcc^{R11} to Pcc^{R15}) and the group of mutants that were resistant only to phage POP12 (Pcc^{R1} to PccR^{10}) both exhibited similarly low levels of PCWDE production and soft rot symptoms. These results indicated that phage cocktail treatments could impair the production of PCWDEs more than single-phage treatments, leading to the alleviation of soft rot symptoms.

Because flagellum-mediated motility was recognized as another essential virulence determinant in *P. carotovorum* subsp. *carotovorum* (2, 49, 50), we also assessed the swimming motilities of WT strain Pcc27 and phage-resistant mutants. Among 15 mutants resistant to the phage cocktail, 10 showed significant reductions in motility compared to that of the WT Pcc27 (Fig. 5C). In particular, all five mutants (Pcc^{R11} to Pcc^{R15}) that evaded infection by flagellum-recognizing phages POP15 and POP17 exhibited further decreases in motility compared with that of the other mutants that were resistant only to POP12 (particularly Pcc^{R1}, Pcc^{R3}, Pcc^{R4}, Pcc^{R9}, and Pcc^{R10}). The Pcc^{R14} strain was even nonmotile (Fig. 5C; Table S2). This result implied that phage cocktail treatments could confer strong selection pressure on WT Pcc27 and increase fitness costs, such as the accumulation of phage resistance mutations. Taken together, the virulence assessments of phage-resistant mutants revealed that the use of a phage cocktail that targeted two different receptors could effectively attenuate the virulence of *P. carotovorum* subsp. *carotovorum*.

DISCUSSION

P. carotovorum subsp. *carotovorum* is a widespread plant pathogen that causes quality depreciation and loss of agricultural products. Phages have been considered to be attractive antimicrobial agents for the biocontrol of *P. carotovorum* subsp. *carotovorum*, but the emergence of phage resistance should be overcome to provide practical phage therapies. In this study, we developed a phage cocktail by considering the host range spectra and types of phage receptors to constrain the emergence of phage resistance. The CA-targeting phage POP12, with the broadest host range, and two flagellum-recognizing phages that can complement the host spectrum of POP12 were selected to formulate the cocktail.

We tested napa cabbage, which is a major isolation source of *Pectobacterium* spp. in South Korea (4, 51). The results of the semi-*in planta* bioassay suggested that our phage cocktail, which targeted two phage receptors, reduced the pathogen population in napa cabbage and alleviated soft rot symptoms more significantly than single-phage treatments. Interestingly, no symptoms of soft rot were observed in phage cocktail-treated napa cabbage (Fig. 3C), even though the average number of Pcc27^{RifR} cells that were recovered from the phage cocktail-treated napa cabbage was more than 10^5 CFU/cm^2 (Fig. 3A), which is sufficient for disease progression (52), suggesting that phage-resistant *P. carotovorum* subsp. *carotovorum* produces smaller amounts of PCWDEs (Fig. 5A).

Gill and Abedon proposed several factors that are associated with successful phage therapy in plants, such as the location or niche where target bacteria exist, density of target bacteria, adequate solution for phage diffusion, and environmental conditions that can allow phage amplification (53). In the *in vitro* challenge assay performed in liquid broth, treatment with phage POP15 or POP17 efficiently prevented the growth of *P. carotovorum* subsp. *carotovorum* cells for up to 4 or 8 h, respectively (Fig. 2A). However, the same treatment with 100 times more PFU of phage was not able to restrict the growth of *P. carotovorum* subsp. *carotovorum* cells in napa cabbage, and consequently, noticeable soft rot symptoms were observed (Fig. 3C). This might be due to the optimized conditions, such as nutrition and temperature, for *P. carotovorum*

subsp. *carotovorum* in the *in vitro* assay, which allowed superior propagation and consequently better bactericidal activities of the phages than in napa cabbage.

Considering that the three phages were applied under the same experimental conditions, we speculated that the more efficient control of *P. carotovorum* subsp. *carotovorum* cells by POP12 in napa cabbage would originate from the characteristics of POP12, such as the efficiency of adsorption to Pcc27. Indeed, phage POP12, recognizing CA, was adsorbed to WT Pcc27 more rapidly than the flagellotropic phages, POP15 and POP17 (Fig. S7A).

A mixture of validamycin and streptomycin was used as a positive control in the greenhouse test, which resulted in effective prevention of the disease (Fig. 4). The overuse of aminoglycoside antibiotics like validamycin and streptomycin is, however, a serious problem due to its negative impacts on plants, food, and humans (54). Over 40,000 tons of validamycin have been produced annually to prevent sheath blight disease in rice plants. Streptomycin, which is recognized as critically and highly important for human medication by the World Health Organization (WHO), is used at a rate of approximately 80,000 pounds per year to prevent phytopathogen infections (54–57). Because the preventive effect of the phage cocktail was comparable to those of the antibiotics tested in the present study (Fig. 4B), phage cocktails are suggested as important alternatives and/or adjuvants to minimize the use of antibiotics.

An understanding of how simultaneous application of multiple phages affects the acquisition of phage resistance in bacteria is necessary to formulate effective phage combinations. Intriguingly, we isolated 15 phage-resistant mutants that exhibited different patterns of susceptibility against the three phages. All mutants were resistant to the CA-recognizing phage POP12. Similar to our previously reported study, in which a spontaneous mutant resistant to another CA-targeting phage, POP72, produced a smaller amount of CA than WT Pcc27 (21), this result suggested that the 15 mutants might be deficient in CA biosynthesis. Among them, 5 mutant strains (Pcc^{R11} to Pcc^{R15}) that were also resistant to flagellum-dependent phages had decreased motility (Fig. 5C; Table S2). Wright et al. reported that simultaneous treatments with multiple phages targeting different receptors often led to single receptor-specific mutations in host bacteria (58), because it is a burden for bacteria to acquire resistance against all phage components at once. Interestingly, two flagellotropic phages, POP15 and POP17, adsorbed better to Pcc27 when CA was absent (Fig. S7B), which indicated that CA might act as a physical obstacle for phage POP15 and POP17 infections. These results implied that POP12 first affected WT Pcc27 extensively and had a primary impact on the development of phage resistance, and then POP15 and POP17 affected the resultant CA-lacking resistant mutants and WT host as secondary or auxiliary components of the phage cocktail.

The malfunction or altered functions of phage receptor molecules as a cost for phage resistance often lead to virulence attenuation in bacterial pathogens (59). In the present study, all 15 mutants (Pcc^{R1} to Pcc^{R15}), which had in common resistance to the CA-targeting phage POP12, exhibited decreased PCWDE production and showed reduced soft rot symptoms in napa cabbage compared to the soft rot symptoms caused by infection with WT Pcc27 (Fig. 5A; Fig. S6 and Table S3). Because the PCWDE activity was also lowered in Pcc^{POP12}, the POP12 resistance that was probably driven by the loss of CA might have had a large effect on the virulence attenuation in the 15 mutants. Many genes are involved in the production of CA and PCWDEs (34, 60), but their known regulation mechanisms are not enough to elucidate the regulatory link between CA and PCWDE synthesis. Thus, the causal relationship between *P. carotovorum* subsp. *carotovorum* virulence and CA production needs to be investigated in further studies by using mutants with targeted gene deletions.

The two flagellotropic phages, POP15 and POP17, also played roles as components of the phage cocktail, not only by lysing the susceptible *P. carotovorum* subsp. *carotovorum* cells but also by contributing to further virulence attenuation in the resistant mutants by inducing restricted motility, similar to Pcc^{POP17} (Fig. 5C). Flagellum-mediated motility is reported to be an important determinant for *P. carotovorum* subsp.

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Reference or source
Pectobacterium carotovorum subsp. *carotovorum* strains		
Pcc27	*P. carotovorum* subsp. *carotovorum* isolate Pcc27; wild type, host for phages POP12, POP15, and POP17	39
Pcc27^{RifR}	Spontaneous rifampicin-resistant mutant of Pcc27	21
Pcc27/pUHE21-2	Pcc27 with pUHE21-2 *lacI*^{q}	21
wcaA::Tn*5* strain	Pcc27 with transposon insertion in putative *wcaA*	21
gmd::Tn*5* strain	Pcc27 with transposon insertion in putative *gmd*	21
cpsG::Tn*5* strain	Pcc27 with transposon insertion in putative *cpsG*	21
flhA::Tn*5* strain	Pcc27 with transposon insertion in putative *flhA*	This study
flhD::Tn*5* strain	Pcc27 with transposon insertion in putative *flhD*	This study
wcaA::Tn*5*/pUHE21-2	*wcaA*::Tn*5* strain with pUHE21-2 *lacI*^{q}	21
wcaA::Tn*5*/pWcaA	*wcaA*::Tn*5* strain complemented with *wcaA* gene from Pcc27	21
flhA::Tn*5*/pUHE21-2	*flhA*::Tn*5* strain with pUHE21-2 *lacI*^{q}	This study
flhA::Tn*5*/pFlhA	*flhA*::Tn*5* strain complemented with *flhA* gene from Pcc27	This study
Escherichia coli strains		
DH5α/λ_{pir}	ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*/λ_{pir}	76
MFD$_{pir}$	MG1655 RP4-2-Tc::[ΔMu1::Δ*aac(3)IV*-Δ*aphA*-Δ*nic35*-ΔMu2::*zeo*] Δ*dapA*::(*erm-pir*) Δ*recA*	36
Plasmids		
pUHE21-2 *lacI*^{q}	rep$_{pMB1}$ *lacI*^{q}; inducible Lac promoter, Amp^{r}	77
pWcaA	pUHE21-2 *lacI*^{q}::*PCC21_RS06680*; Amp^{r}	21
pFlhA	pUHE21-2 *lacI*^{q}::*PCC21_RS13355*; Amp^{r}	This study

[a]Amp^{r}, ampicillin resistant.

carotovorum virulence because flagellum deficiencies affect the adhesion to and penetration of phytopathogens into plant tissue (2, 61). Although both Pcc^{POP12} and 10 of the 15 phage-resistant mutants obtained after phage cocktail treatment were resistant to phage POP12 only, the mutant Pcc^{POP12} obtained after phage POP12 treatment alone was fully functional in terms of its swimming motility, whereas five of the resistant mutants (Pcc^{R1}, Pcc^{R3}, Pcc^{R4}, Pcc^{R9}, and Pcc^{R10}) that were isolated after phage cocktail treatment exhibited significantly impaired motilities (Fig. 5C; Table S2). This result further supported the concept that phage cocktail treatments impose strong selective pressure on WT Pcc27, which leads to greater attenuation of bacterial virulence. Phenotypic variations among phage-resistant bacteria can originate from genetic changes like stochastic gene expression related to receptor biosynthesis (62) and/or phase variations (63). Through these diverse evolutionary trajectories, phage cocktail treatments could trigger the attenuation of *P. carotovorum* subsp. *carotovorum* virulence at various levels. Further genetic and physiological studies are needed to elucidate reasons for the attenuated virulence of the phage-resistant mutants.

In conclusion, we designed a bacteriophage cocktail that consists of three phages that recognize two different phage receptors (CA and flagella) in *P. carotovorum* subsp. *carotovorum* to delay the emergence of bacterial resistance. The cocktail could efficiently retard the growth of *P. carotovorum* subsp. *carotovorum* and the subsequent progress of tissue softening in napa cabbage. Moreover, phage cocktail treatment attenuated *P. carotovorum* subsp. *carotovorum*'s virulence, including PCWDE production and bacterial motility. These results suggest that the preparation of phage cocktails that target multiple phage receptors and an understanding of the fitness trade-offs that are generated by phage resistance would be helpful for the development of effective alternative strategies to control *P. carotovorum* subsp. *carotovorum* with phages instead of using conventional antibiotics.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *Pectobacterium carotovorum* subsp. *carotovorum* isolates were provided by the Rural Development Administration (RDA) at Wanju-gun, South Korea. All bacterial strains and plasmids used in this study are listed in Table 1. *P. carotovorum* subsp. *carotovorum* strains were grown in LB (Luria-Bertani) broth and plates (1.5% [wt/vol] agar) at 30°C. *Escherichia*

TABLE 2 Primers used in this study

Purpose, primer	Sequence (5′–3′)[a]
Plasmid construction	
Pcc27_*wcaA*_F_BamHI	ATAGGATCCATGTCAACAAATAATTTAGTCAGTGTTATTATT
Pcc27_*wcaA*_R_HindIII	ATAAAGCTTTGAACGCAAGTCAATCATTTTATTTTTTCC
Pcc27_*flhA*_F_EcoRI	ATAGAATTCCCGGATGCACTGGATTTTGCT
Pcc27_*flhA*_R_HindIII	ATAAAGCTTCTGCAGCCAGAGAATGCATCG
Sequence confirmation	
Pcc27_*flhA*_F_confirm	ACCGACTTCAGCAATACGTC
Pcc27_*flhA*_R_confirm	CGGTGCAACCAGATCCTTAT
pUHE21-2_F1	AGATTCAATTGTGAGCGGATAAC
pUHE21-2_R3	GGTCATTACTGGATCTATCAACA
tpnRL17–1	AACAAGCCAGGGATGTAACG
tpnRL13–2	CAGCAACACCTTCTTCACGA

[a]Restriction enzyme sites are underlined.

coli strain MFD$_{pir}$ was grown at 37°C in LB broth and plates that were supplemented with 0.3 mM diaminopimelic acid (DAP). Antibiotics were used at the following concentrations: ampicillin (Amp), 50 μg/mL; carbenicillin (Car), 100 μg/mL; kanamycin (Kan), 50 μg/mL; and rifampicin (Rif), 50 μg/mL. IPTG (isopropyl β-D-1-thiogalactopyranoside) was added at a concentration of 100 or 500 μM.

Bacteriophage isolation and propagation. Bacteriophages were isolated from sewage by using *P. carotovorum* subsp. *carotovorum* strain Pcc27 as a bacterial host. Five milliliters of sewage was mixed with 5 mL of 2× LB broth, and the mixture was incubated overnight with the host strain at 30°C. The culture was centrifuged at 13,000 × *g* for 10 min and filtered to exclude bacterial cells. The filtered supernatants were serially diluted 10-fold and spotted onto host bacterial lawns (see below). The plates were incubated overnight at 30°C, and the phage plaques that formed were observed. Single plaques were picked with a sterile tip and eluted in a sodium chloride-magnesium sulfate (SM) buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 8 mM $MgSO_4 \cdot 7H_2O$). Dilution, spotting, and plaque picking were sequentially repeated at least three times to purify a single phage. For phage propagation, the phage lysate was added to the exponentially growing host bacterial culture at a multiplicity of infection (MOI) of 1 and incubated for 3 h. The propagated phages were precipitated with polyethylene glycol (PEG) 6000 and concentrated by using CsCl density gradient ultracentrifugation (78,500 × *g* for 2 h at 4°C) (64).

Bacteriophage spot assay. The bacterial lawn was prepared as described elsewhere (64). Briefly, 100 μL of cultured host cells was inoculated into 5 mL of LB soft agar (0.4% agar), supplemented with the appropriate antibiotic and IPTG if necessary. This mixture was poured onto LB agar plates and solidified for 30 min. Ten microliters of serially diluted (10-fold) phage lysates was spotted on the bacterial lawn and dried for 20 min at room temperature. The plates were incubated for 12 h at 30°C, and the phage plaques were monitored.

Screening phage-resistant mutants from the transposon Tn*5* insertional-mutant library of *P. carotovorum* subsp. *carotovorum*. Phage-insensitive *P. carotovorum* subsp. *carotovorum* mutants were screened from the Tn*5* transposon insertional mutant library of *P. carotovorum* subsp. *carotovorum* as previously described, with some modifications (35, 36). The suicide vector pRL27 containing Tn*5* was transferred from donor *E. coli* MFD$_{pir}$ cells to recipient Pcc27 cells by conjugation as follows. Donor and recipient strains at the early log phase were harvested and washed three times with 10 mM $MgSO_4$. A mixture of donor and recipient cells (3:1, vol/vol) was spotted onto LB agar plates supplemented with DAP and incubated for 24 h at 30°C for conjugation. The transconjugants were resuspended in LB broth, and the cell dilutions were mixed with phage POP17 (10^9 PFU/mL). After incubation at room temperature for 15 min, the mixture was plated on LB/Kan plates and incubated for 24 h at 30°C. Each emerging colony was isolated, and the absence of remaining phages in the cells was verified by spotting the culture supernatant on a bacterial lawn. Determining transposon insertion sites is similar to cloning of plasposons (65). The genomic DNA that was extracted from the phage-resistant Tn*5* mutants was digested with the restriction enzyme BamHI and circularized by T4 ligase (Roche). *E. coli* strain DH5α/λ_{pir} was transformed with a portion of the ligated mixture by heat shock, and the transformants were selected on LB agar/Kan plates. Plasmid DNA containing Tn*5* was extracted from the selected transformants, and the locus of the transposon insertion site was determined by sequencing with transposon-specific primers tpnRL17-1 and tpnRL 13-2 (Table 2) (35). The nucleotide sequence was compared to the sequence of *Pectobacterium carotovorum* subsp. *carotovorum* strain Pcc21 (GenBank accession number CP003776) as a reference for the analysis.

Sequencing of phage DNA and bioinformatics analysis. Phage DNA was extracted by the phenol-chloroform method as previously described (66). The purified phage DNA was sequenced using a Genome Sequencer FLX titanium sequencer (Roche, Mannheim, Germany) and assembled with GS *De Novo* Assembler software (Roche) at LabGenomics, Inc., and Sanigen Co. Ltd., South Korea. The ORFs were predicted by using Glimmer3 (67), GeneMarkS (41), Fgenesb software (Softberry, Inc., Mount Kisco, NY), and the RAST annotation server (http://rast.nmpdr.org/) (42, 68). The annotated data were assorted using Artemis (69). The tRNA sequence in the phage genome was analyzed with the tRNAscan-SE

program (70). The predictions of protein functions were performed with NCBI BLASTp and InterProScan (71, 72).

TEM analysis. The three purified phages were morphologically characterized by transmission electron microscopy (TEM) analysis as described by Kim and Ryu (64). Briefly, 5 μL of high-titer phage stock (approximately 1×10^{11} PFU/mL) was placed on carbon-coated copper grids and negatively stained with 2% aqueous uranyl acetate (pH 4.0). Phage POP12 was observed with TEM (Carl Zeiss LEO 913AB) at a 100-kv acceleration voltage at the National Institute of Agricultural Sciences (Wanju-gun, South Korea). Phages POP15 and POP17 were observed with TEM (energy-filtered [EF]-TEM) (JEM-1010; JEOL, Japan) at an acceleration voltage of 80 kV at the NICEM (Seoul, South Korea). These phages were classified morphologically by using the International Committee on Taxonomy of Viruses (ICTV) classification (73).

Bacterial challenge assays. A phage cocktail consisting of three phages (POP12, POP15, and POP17) was prepared at a ratio of 2:1:1. Host Pcc27 cultures at the early exponential phase were infected with each phage or phage cocktail at an MOI of 1 (3×10^9 PFU/mL). The optical densities at 600 nm were measured every hour for 16 h. The cultures were sampled at 12 h post-phage infection and plated onto LB agar medium to count the numbers of viable cells. SM buffer instead of phages was added to the negative control. The experiments were conducted in triplicate.

Biocontrol assays on crop models using a phage cocktail. The napa cabbage used in this study was purchased from a local market in Seoul, South Korea. Samples were cut into equal sizes (approximately 10 cm by 7 cm) and sanitized with 1% sodium hypochlorite for 5 min. After washing with sterilized water for 5 min and air drying, the samples were stabbed with a sterilized needle. Ten microliters of Pcc27^{RifR} bacterial culture (10^7 CFU/mL) or sterilized water (negative control) was inoculated into the wounds. After air drying for 10 min, single phages or the prepared phage cocktail was spotted on the Pcc27^{RifR}-inoculated wound at an MOI of 10^3. The samples were stored in a plastic box to maintain humidity and incubated at 30°C for 24 h. At the indicated time points, the numbers of Pcc27^{RifR} cells were measured as described by Bai et al. (25). The crop samples were transferred to sterile bags (Filtra-Bag with open top, code SCTO7012A; Labplas) containing 100 mL of sterilized buffered peptone water (BPW) and homogenized with a stomacher (BagMixer 400 Laboratory Blender, Interscience) for 1 min. Sample debris was removed, and the bacterial cells in the supernatants were collected via centrifugation (13,000 $\times$ *g* for 1 min at 4°C). The cell pellets were resuspended in phosphate-buffered saline (PBS). A serially diluted (10-fold) cell suspension was plated onto LB agar/Rif plates, and the colonies were counted after 12 h of incubation at 30°C. The macerated areas of napa cabbage samples were measured using ImageJ.

Greenhouse trials. The greenhouse trials to determine the prophylactic effect of phages as biocontrol agents were kindly supported by the Highland Agriculture Research Institute (Pyeongchang-gun, South Korea). Seeds of napa cabbage (*Brassica rapa* subsp. *pekinensis*) were purchased in a local market in Pyeongchang-gun. These seeds were planted in plastic pots (2 cm by 2 cm) and cultivated for 1 month. The young napa cabbage leaves were sprayed with a single phage or phage cocktail (1×10^8 PFU/pot). SM buffer instead of phages was used as a nonphage negative control, and antibiotics (validamycin-A [15%] plus streptomycin [5%]) purchased from the local market were sprayed as a positive control. After air drying for 1 day, serially diluted (10-fold) Pcc27 cells (1×10^6 CFU/pot) with 10 mM $MgSO_4$ were sprayed onto the young leaves. The inoculated samples were placed in a humidity chamber (28°C and 90% relative humidity) for 30 h and then transferred to the greenhouse. The extent of soft rot was evaluated 2 days after incubation based on visual assessment of symptom severity in leaves using the following four-point scale: 0, no symptoms; 1, occasional yellow leaves, no soft rot; 2, soft rot of leaves of at least 50% of area in each pot; and 3, soft rot and/or death of entire plant. Leaves that turned dark green over at least 50% of their total area were considered diseased. The day/night temperatures in the greenhouse ranged from 15 to 30°C.

Isolation of phage-resistant *P. carotovorum* subsp. *carotovorum* mutants. Mutants with resistance to the phage cocktail were isolated by high-titer overlay as previously described (64). The WT Pcc27 (10^7 CFU/mL) culture was mixed with the phage cocktail at an MOI of 10^3 in 5 mL of soft LB soft agar. The mixture was poured onto LB agar plates and solidified, and the plates were incubated at 30°C for 24 h. The emerging colonies were streaked three times onto LB plates to isolate a single colony. At each streaking step, the newly formed colonies were tested by spot assays to identify phage resistance.

Extracellular enzyme assays. Extracellular enzyme assays were conducted as described by Chatterjee et al. and Lee et al., with some modifications (2, 74). The media were prepared as follows. The Pel assay plate contained 1% polygalacturonic acid (PGA), 1% yeast extract, 0.38 μM calcium chloride, and 100 mM Tris/HCl, pH 8.5; the Peh assay plate contained 1% PGA, 1% yeast extract, 2.2 mM EDTA and 110 mM sodium acetate, pH 5.5; the Cel assay plate contained 1% carboxymethyl cellulose and 25 mM sodium phosphate, pH 7.0; and the Prt assay plate contained 1% skim milk and 0.1% yeast extract. All plates were supplemented with 0.8% agarose and 0.2% sodium azide. In each plate, wells were created with a no. 2 cork borer (Sigma-Aldrich), and the bottoms of the wells were covered with 0.8% (vol/vol) molten agarose containing 0.2% sodium azide. The *P. carotovorum* subsp. *carotovorum* cell cultures at the stationary phase grown in LB medium were centrifuged (16,000 $\times$ *g* for 1 min), and 30 μL of the supernatant containing extracellular enzymes was applied to each well. Then, the plates were incubated at 30°C for 16 h. For the Peh and Pel assay plates, 4 mL of 4 N HCl was poured, and the diameters of the clear zones were measured. The Cel assay plates were stained with 0.1% Congo red solution for 30 min and washed three times with 1 M NaCl until clear zones were visible. The haloes on the Prt assay plates were measured after 36 h of incubation without any further treatment.

Infection assay of phage cocktail-resistant bacteria in napa cabbage. Napa cabbage samples were prepared by using the same method as described for the biocontrol assays. Amounts of 10 μL of

P. carotovorum subsp. *carotovorum* bacterial culture and each phage-resistant-mutant culture (5×10^7 CFU/mL) were inoculated into the wounds. After air drying for 10 min, the samples were stored in a plastic box to maintain humidity and incubated at 30°C for 36 h. Macerated areas of napa cabbage were measured using ImageJ.

Motility assay. The swimming motilities of the *P. carotovorum* subsp. *carotovorum* strains were evaluated as described by Choi et al. with some modifications (75). Amounts of 1 μL of the overnight-cultured *P. carotovorum* subsp. *carotovorum* cells were inoculated onto semisolid LB agar plates (0.3% agar) by stabbing. The diameters of the bacterial growth zones were measured after plate incubation at 30°C for 24 h.

Adsorption assays. The phage adsorption assays were conducted as previously described with some modifications (22). Briefly, *P. carotovorum* subsp. *carotovorum* cells were harvested at an optical density at 600 nm (OD_{600}) of 1.0 (5×10^8 CFU/mL) and washed with LB medium. Each phage was added to a bacterial suspension at an MOI of 0.01 and the suspension aliquoted in equal volumes into five microtubes. During phage adsorption for 15 min at 30°C, each tube was centrifuged (16,000 $\times$ *g* for 1 min at 4°C) at the indicated time points, and the supernatants were immediately filtered (0.22-μm pore size; Millipore). The numbers of unadsorbed phage particles in the filtrates were determined by spot assays using WT Pcc27 and the *cpsG*::Tn*5* mutant as the indicator strains.

Statistical analysis. Statistical Package for Social Science (SPSS) 25 software was used for statistical analysis. Statistical analysis was conducted by one-way analysis of variance (ANOVA) with Tukey's multiple-comparison tests among the experimental groups. The visual assessment of the extent of soft rot whose results are shown in Fig. 4B was statistically analyzed by nonparametric Kruskal-Wallis test. Statistical analysis for the adsorption assay whose results are shown in Fig. S7B was performed by using the unpaired *t* test.

Data availability. The complete genome information of the three phages chosen for the phage cocktail was registered in the NCBI GenBank database under accession numbers MT560058 for POP12, MT560059 for POP15, and MT552976 for POP17.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.5 MB.

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H.K., M.K., and S.R. conceived and designed the experiments. H.K. performed the experiments and analyzed the data. H.K., M.K., and S.R. wrote the paper. S.-N.J. supported the greenhouse experiments. S.H. provided H.K. and M.K. with the *Pectobacterium carotovorum* strains.

We declare no conflicts of interest.

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