Development of bacteriophage cocktail against *Pectobacterium carotovorum* subsp. *carotovorum* and its effects on virulence of the resistant bacteria

Hyeongsoon Kim[1, †], Minsik Kim[2, †], Sam-Nyu Jee[3], Sunggi Heu[4], and Sangryeol Ryu[1*]

[1]Department of Food and Animal Biotechnology, Department of Agricultural Biotechnology, Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Korea

[2]Department of Food and Nutrition, BK21 FOUR, College of Human Ecology, Yonsei University,
Seoul 03722, Korea

[3]Highland Agriculture Research Institute, National Institute of Crop Science, Pyeongchang-gun 25342, Korea

[4]Department of Agriculture, Forestry and Bioresources , Seoul National University, Seoul 08826, Korea

* Corresponding author.

Mailing address: Department of Food and Animal Biotechnology, Seoul National University, Seoul 08826, Korea

Phone: 82-2-880-4856. Fax: 82-2-873-5095. E-mail: sangryu@snu.ac.kr

[†]These authors have contributed equally to this work

Table S1. Phage receptor analysis of *Pectobacterium* POP phages

Tn5 mutant strain	Locus_tag of homologous gene in Pcc21[b]	Related cellular apparatus	Phage[a]					
			POP11	POP12	POP13	POP14	POP15	POP17
wcaA::Tn5[c]	*Pcc21_RS06680*	Colanic acid	-	-	-	-	+	+
gmd::Tn5[c]	*Pcc21_RS06710*	Colanic acid	-	-	-	-	+	+
cpsG::Tn5[c]	*Pcc21_RS06735*	Colanic acid	-	-	-	-	+	+
flhA::Tn5	*Pcc21_RS13355*	Flagella	+	+	+	+	-	-
flhD::Tn5	*Pcc21_RS13415*	Flagella	+	+	+	+	-	-

[a] +, presence of plaques; -, absence of plaques

[b] Homologous genes in Pcc21 were indicated for reference because the whole genome of Pcc27 was not sequenced yet.

[c] (1)

Table S2. Phage susceptibility test against phage-resistant mutants[a]

Name of bacterial isolate	POP12	POP15	POP17
Pcc27	+	+	+
Pcc^{POP12}	-	+	+
Pcc^{POP17}	+	-	-
Pcc^{R1}	-	+	+
Pcc^{R2}	-	+	+
Pcc^{R3}	-	+	+
Pcc^{R4}	-	+	+
Pcc^{R5}	-	+	+
Pcc^{R6}	-	+	+
Pcc^{R7}	-	+	+
Pcc^{R8}	-	+	+
Pcc^{R9}	-	+	+
Pcc^{R10}	-	+	+
Pcc^{R11}	-	-	-
Pcc^{R12}	-	-	-
Pcc^{R13}	-	-	-
Pcc^{R14}	-	-	-
Pcc^{R15}	-	-	-

[a] +, presence of plaques; -, absence of plaques

Table S3. PCWDEs activity of phage resistant mutants

Name of bacterial isolate	PCWDE activity (mm)[a]			
	Pel	Peh	Cel	Prt
Pcc27	20.5 ± 1.05	18.1 ± 0.62	14.0 ± 0.40	22.8 ± 1.81
Pcc^{POP12}	17.5 ± 0.33	14.1 ± 0.47	12.1 ± 0.19	18.5 ± 0.76
Pcc^{POP17}	20.0 ± 0.66	14.8 ± 0.26	13.2 ± 0.28	23.8 ± 0.27
Pcc^{R1}	14.5 ± 1.29^{A}	15.2 ± 0.82	11.9 ± 0.21	16.3 ± 0.45
Pcc^{R2}	14.5 ± 1.02	16.7 ± 0.15	11.9 ± 0.19	17.4 ± 1.94
Pcc^{R3}	12.0 ± 1.21^{C}	14.2 ± 0.41	9.7 ± 0.13^{C}	5.7 ± 0.30^{C}
Pcc^{R4}	13.2 ± 1.16^{C}	14.7 ± 0.18	10.7 ± 0.38	5.8 ± 0.37^{C}
Pcc^{R5}	15.4 ± 0.12	16.7 ± 0.30	12.1 ± 0.15	19.2 ± 1.09
Pcc^{R6}	14.1 ± 1.31^{B}	15.0 ± 1.66	10.5 ± 1.14^{B}	7.4 ± 1.85^{C}
Pcc^{R7}	12.1 ± 0.60^{C}	13.9 ± 0.53	10.0 ± 0.18^{B}	11.8 ± 1.41^{C}
Pcc^{R8}	13.0 ± 1.28^{C}	14.7 ± 0.29	10.5 ± 0.69^{B}	9.9 ± 2.20^{C}
Pcc^{R9}	15.6 ± 0.95	15.8 ± 0.07	11.3 ± 0.67	15.9 ± 0.83
Pcc^{R10}	10.7 ± 0.49^{C}	12.8 ± 0.7	6.6 ± 0.79^{C}	5.3 ± 0.25^{C}
Pcc^{R11}	13.8 ± 1.06^{B}	15.0 ± 1.11	11.2 ± 0.49	14.8 ± 1.96^{B}
Pcc^{R12}	13.7 ± 0.39^{B}	15.0 ± 0.65	10.9 ± 0.24	12.3 ± 0.42^{C}
Pcc^{R13}	14.8 ± 0.82	14.4 ± 0.50	11.1 ± 0.2	14.1 ± 0.92^{C}
Pcc^{R14}	8.8 ± 1.75^{C}	8.7 ± 1.77^{C}	6.1 ± 0.16^{C}	5.7 ± 0.11^{C}
Pcc^{R15}	14.6 ± 1.17	14.6 ± 0.90	11.1 ± 0.70	13.9 ± 1.77^{C}

[a]The length of clear haloes from inoculated holes indicated enzyme activities. The data represent the average values and standard deviations of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey post-tests. The significance of difference among Pcc^{POP12} and phage-resistant mutants (Pcc^{R1} to Pcc^{R15}) was marked with letters as follows: A, $P < 0.05$; B, $P < 0.01$; C, $P < 0.001$.

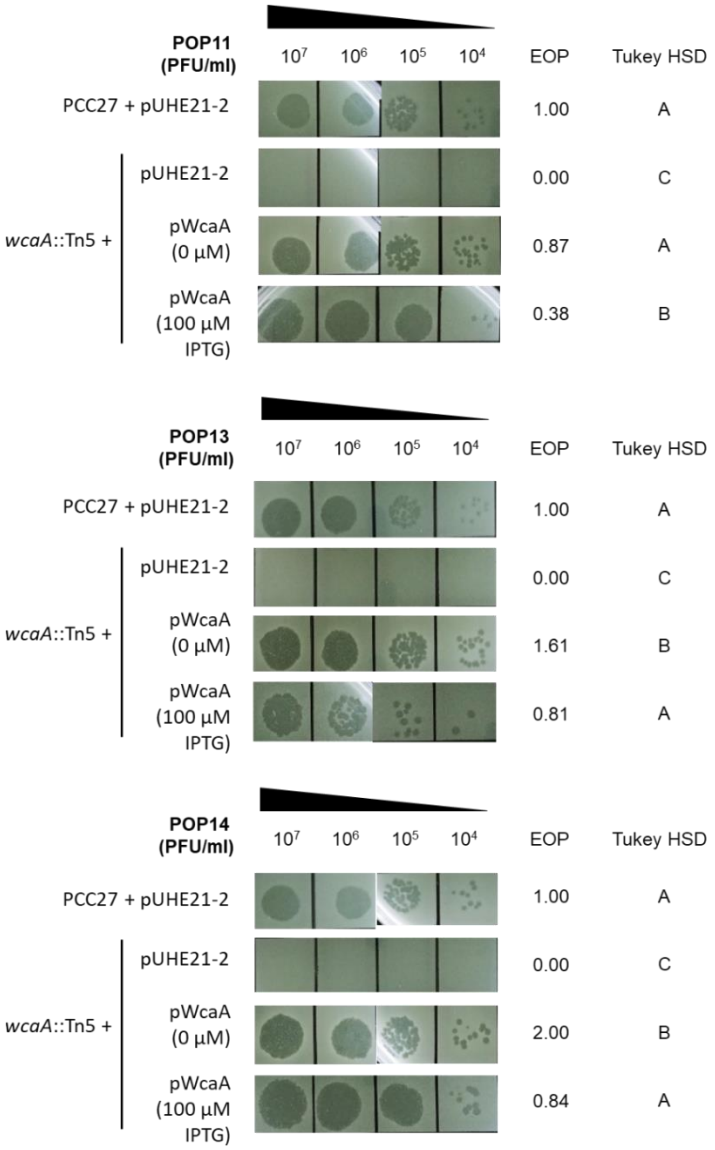


Fig S1. Determination of phage receptor for *Pectobacterium*-targeting phages. Transposon mutants with disrupted *wcaA* by Tn5 insertion did not form plaques in the phage spot assay. Complementation of *wcaA* gene restored the phage susceptibility. The IPTG concentrations are indicated in parentheses. EOP (Efficiency of Plating) was calculated by dividing the titer of the phage on each indicated strain by the titer of the same phage on Pcc27 harboring pUHE21-2. The significant differences among the experimental groups are marked with letters. One representative result of triplicates experiments is shown.

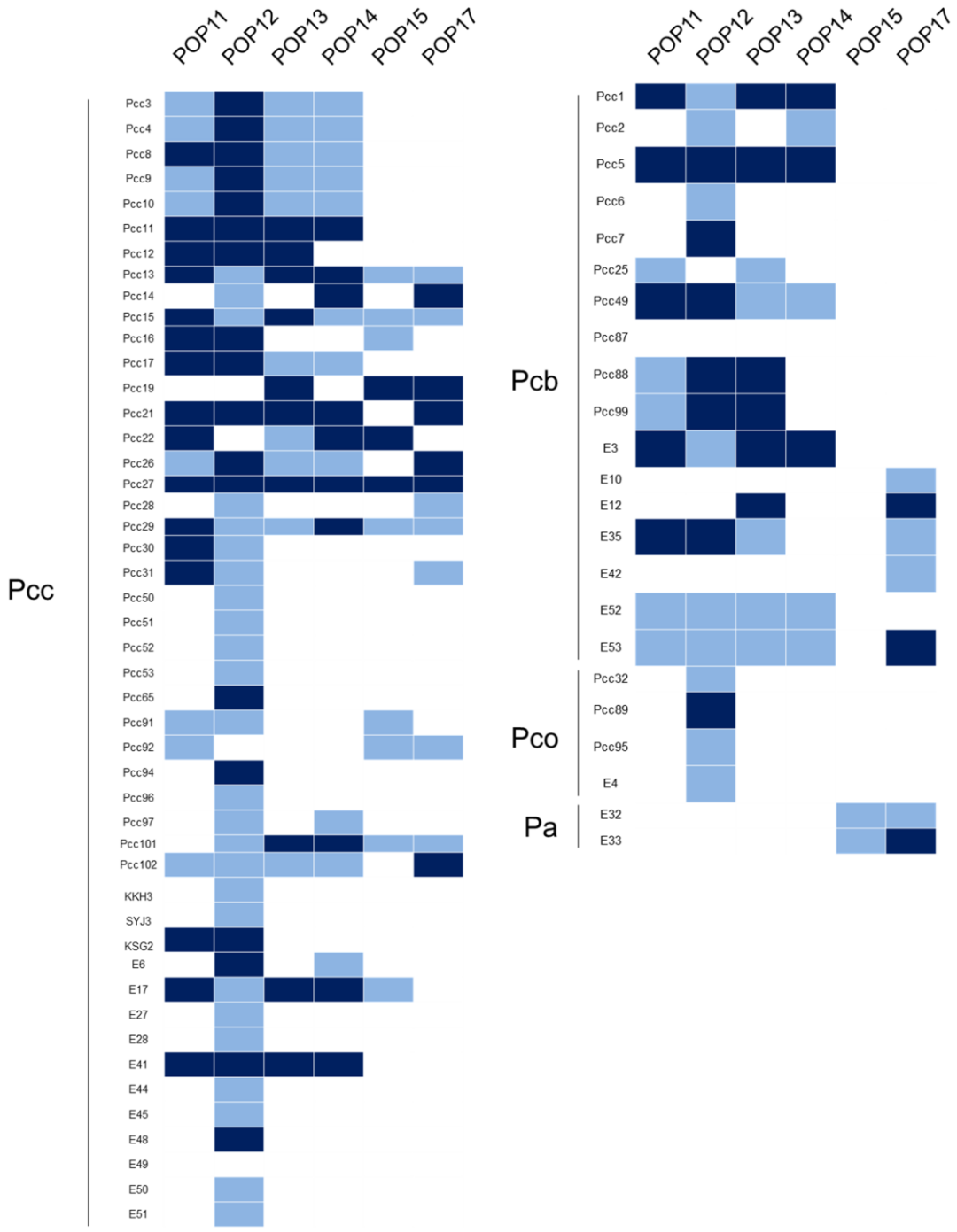
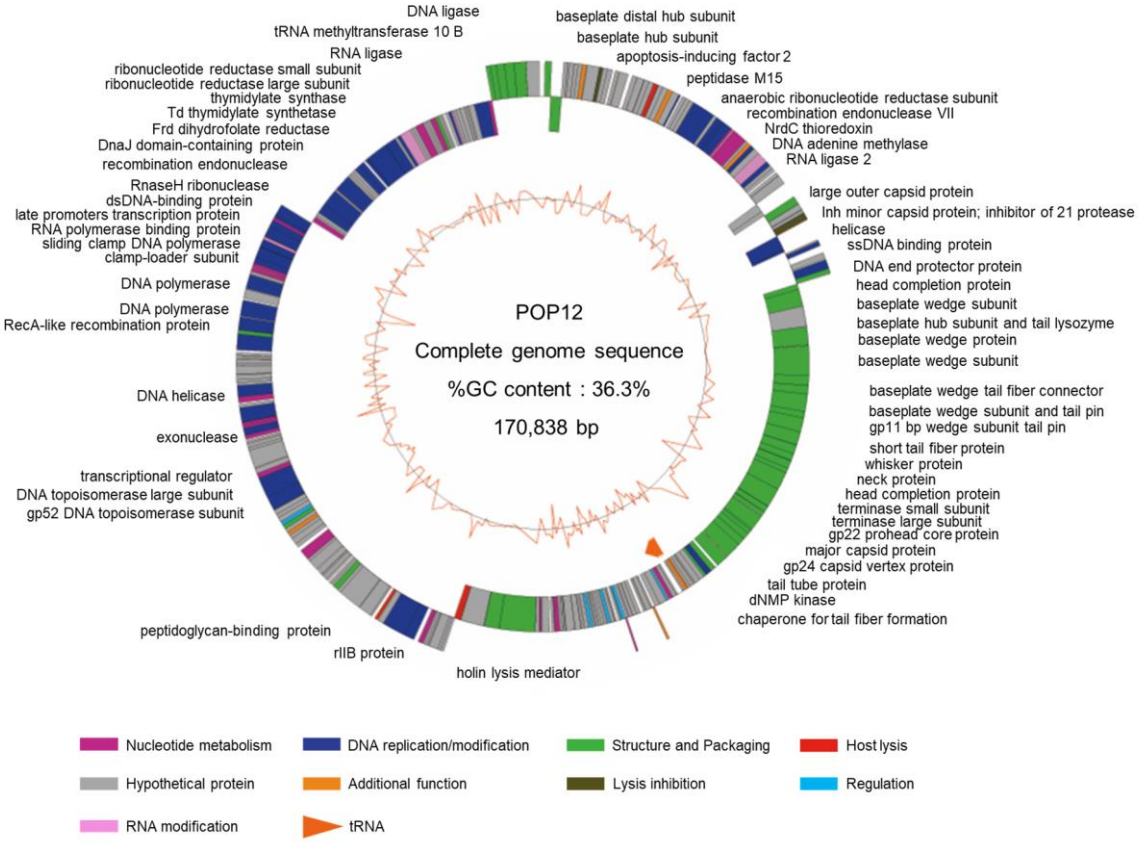


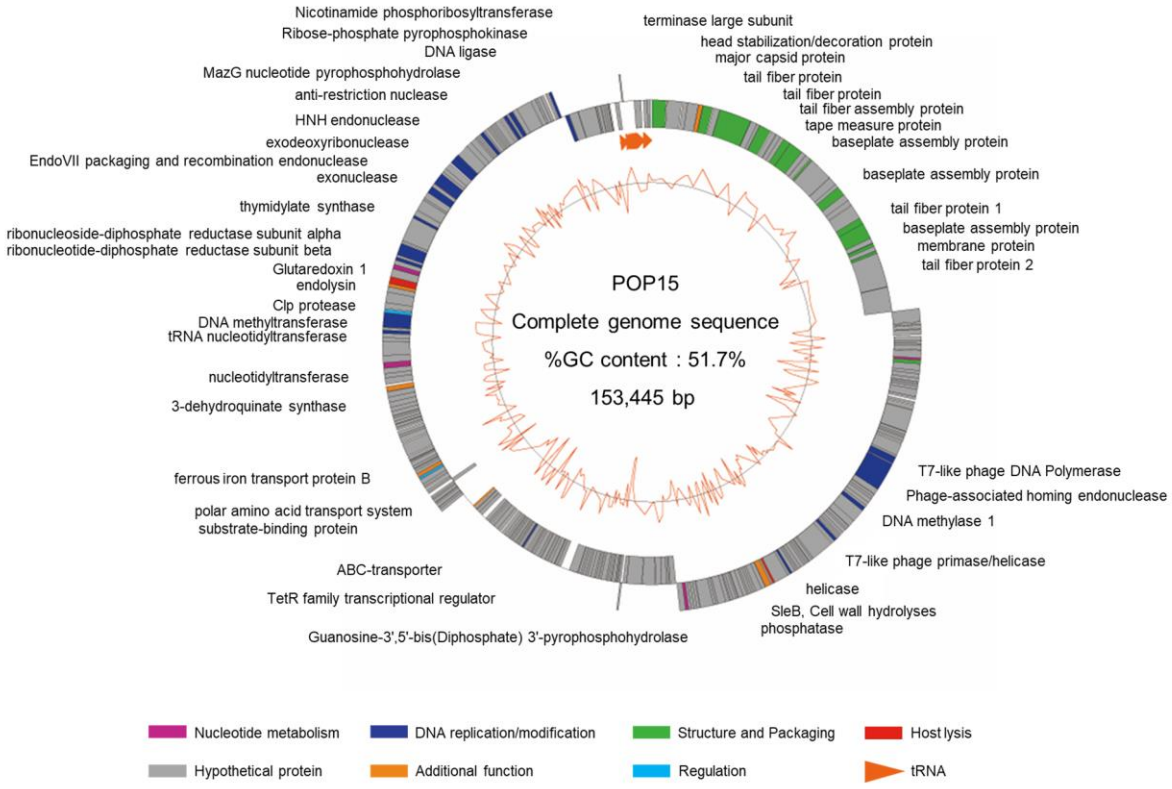
Fig S2. Host ranges of *Pectobacterium* POP phages. The host ranges were determined by spot assays. A navy color indicates that a phage could form a single plaque on a bacterial strain. A sky blue color indicates an inhibition zone on the bacterial strain.

(A)


DNA ligase
tRNA methyltransferase 10 B
RNA ligase
ribonucleotide reductase small subunit
ribonucleotide reductase large subunit
thymidylate synthase
Td thymidylate synthetase
Frd dihydrofolate reductase
DnaJ domain-containing protein
recombination endonuclease
RnaseH ribonuclease
dsDNA-binding protein
late promoters transcription protein
RNA polymerase binding protein
sliding clamp DNA polymerase
clamp-loader subunit
DNA polymerase
DNA polymerase
RecA-like recombination protein
DNA helicase
exonuclease
transcriptional regulator
DNA topoisomerase large subunit
gp52 DNA topoisomerase subunit
peptidoglycan-binding protein
rIIB protein
holin lysis mediator
baseplate distal hub subunit
baseplate hub subunit
apoptosis-inducing factor 2
peptidase M15
anaerobic ribonucleotide reductase subunit
recombination endonuclease VII
NrdC thioredoxin
DNA adenine methylase
RNA ligase 2
large outer capsid protein
Inh minor capsid protein; inhibitor of 21 protease
helicase
ssDNA binding protein
DNA end protector protein
head completion protein
baseplate wedge subunit
baseplate hub subunit and tail lysozyme
baseplate wedge protein
baseplate wedge subunit
baseplate wedge tail fiber connector
baseplate wedge subunit and tail pin
gp11 bp wedge subunit tail pin
short tail fiber protein
whisker protein
neck protein
head completion protein
terminase small subunit
terminase large subunit
gp22 prohead core protein
major capsid protein
gp24 capsid vertex protein
tail tube protein
dNMP kinase
chaperone for tail fiber formation
POP12
Complete genome sequence
%GC content : 36.3%
170,838 bp
Nucleotide metabolism
DNA replication/modification
Structure and Packaging
Host lysis
Hypothetical protein
Additional function
Lysis inhibition
Regulation
RNA modification
tRNA

47

(B)


Nicotinamide phosphoribosyltransferase
Ribose-phosphate pyrophosphokinase
DNA ligase
MazG nucleotide pyrophosphohydrolase
anti-restriction nuclease
HNH endonuclease
exodeoxyribonuclease
EndoVII packaging and recombination endonuclease
exonuclease
thymidylate synthase
ribonucleoside-diphosphate reductase subunit alpha
ribonucleotide-diphosphate reductase subunit beta
Glutaredoxin 1
endolysin
Clp protease
DNA methyltransferase
tRNA nucleotidyltransferase
nucleotidyltransferase
3-dehydroquinate synthase
ferrous iron transport protein B
polar amino acid transport system
substrate-binding protein
ABC-transporter
TetR family transcriptional regulator
Guanosine-3',5'-bis(Diphosphate) 3'-pyrophosphohydrolase
terminase large subunit
head stabilization/decoration protein
major capsid protein
tail fiber protein
tail fiber protein
tail fiber assembly protein
tape measure protein
baseplate assembly protein
baseplate assembly protein
tail fiber protein 1
baseplate assembly protein
membrane protein
tail fiber protein 2
T7-like phage DNA Polymerase
Phage-associated homing endonuclease
DNA methylase 1
T7-like phage primase/helicase
helicase
SleB, Cell wall hydrolyses
phosphatase
POP15
Complete genome sequence
%GC content : 51.7%
153,445 bp
Nucleotide metabolism
DNA replication/modification
Structure and Packaging
Host lysis
Hypothetical protein
Additional function
Regulation
tRNA

48

(C)

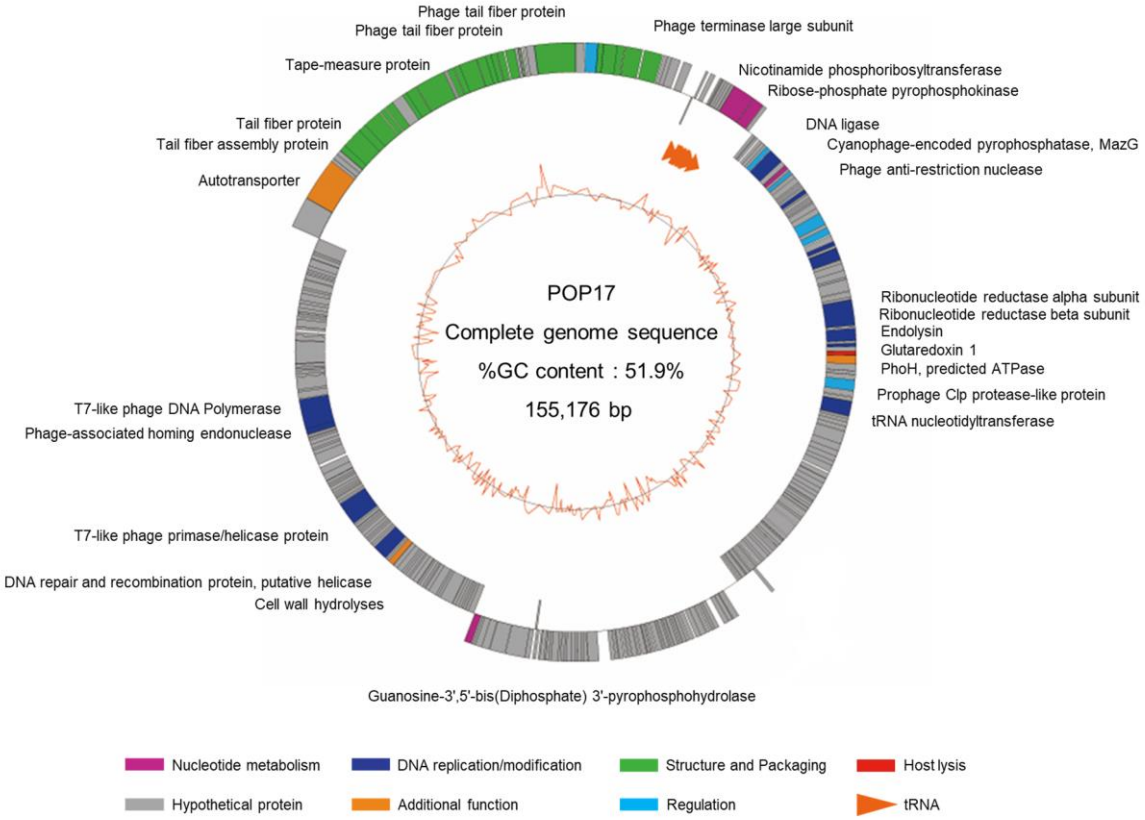
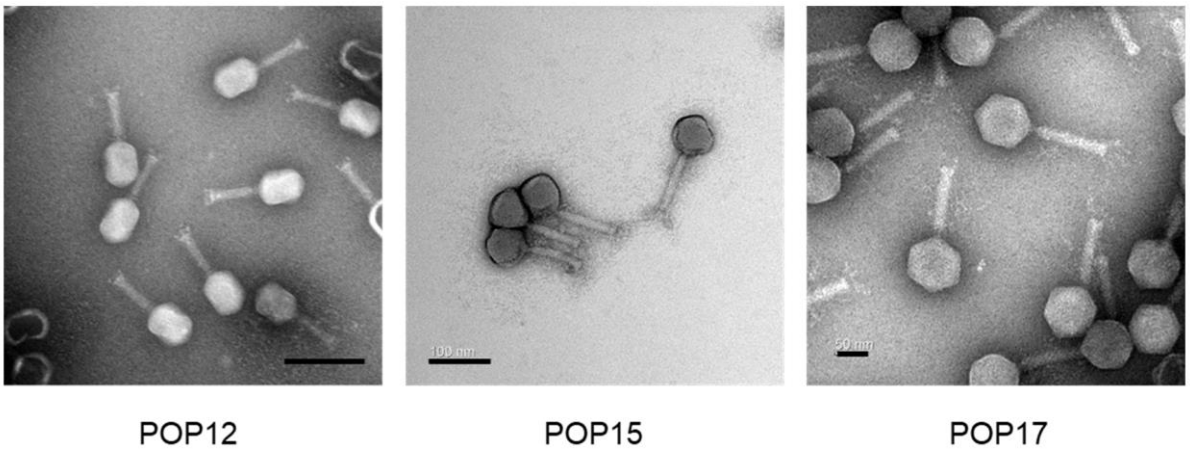


Fig S3. Whole-genome maps of phages (A) POP12, (B) POP15, (C) POP17. The predicted ORFs with the corresponding gene products are presented in colors based on their functions. The inner orange line indicates the %GC content of the phage genome.



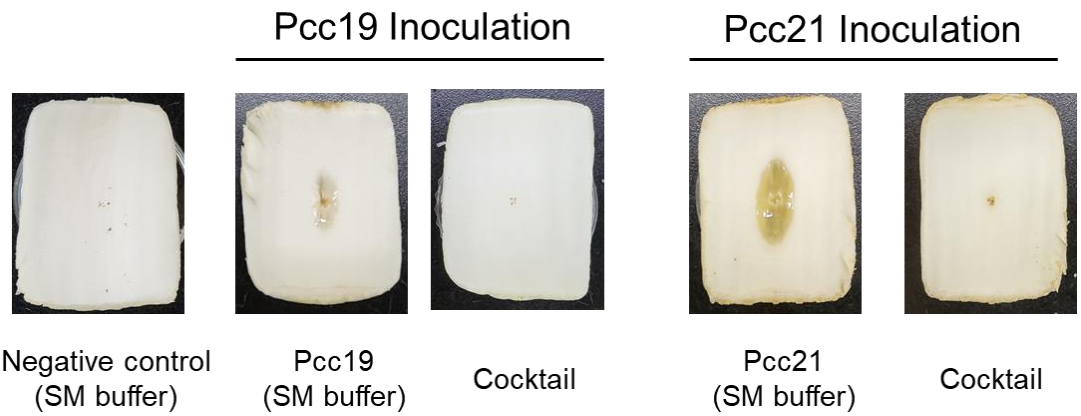
53

54 **Fig S4.** Transmission electron micrographs of three phages. Bars, 200 nm (POP12); 100 nm

55 (POP15); and 50 nm (POP17).

56

57



58

59 **Figure S5**. The symptoms of soft rot were monitored after 24 h of Pcc19 and Pcc21

60 inoculations. An inoculated sample with SM buffer was used as a negative control. Pcc19 or

61 Pcc21 inoculated samples with SM buffer were used as a non-phage control.

62

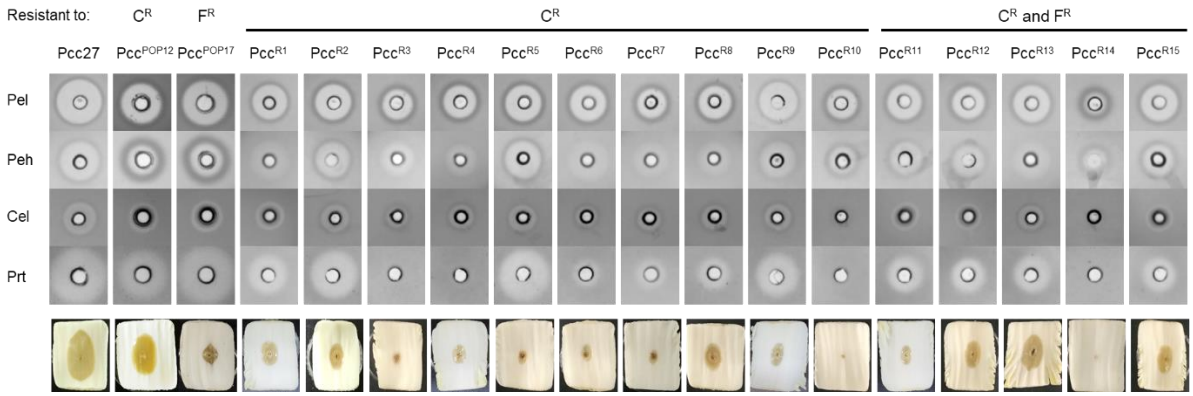


Fig S6. Attenuated virulence of phage-resistant mutants evaluated by extracellular enzyme assay. The diameter of haloes around the wells was measured to represent the enzyme activities (details in the *Material and Methods*). Artificially inoculated napa cabbage with Pcc27 and phage-resistant mutants (Pcc^{R1} to Pcc^{R15}) were monitored after 36 h of incubation. Pel (pectate lyase); Peh (polygalacturonase); Cel (cellulase); Prt (protease). One representative result of triplicated experiments is shown. C^R, resistant to CA-recognizing phage; F^R, resistant to flagella-recognizing phage.

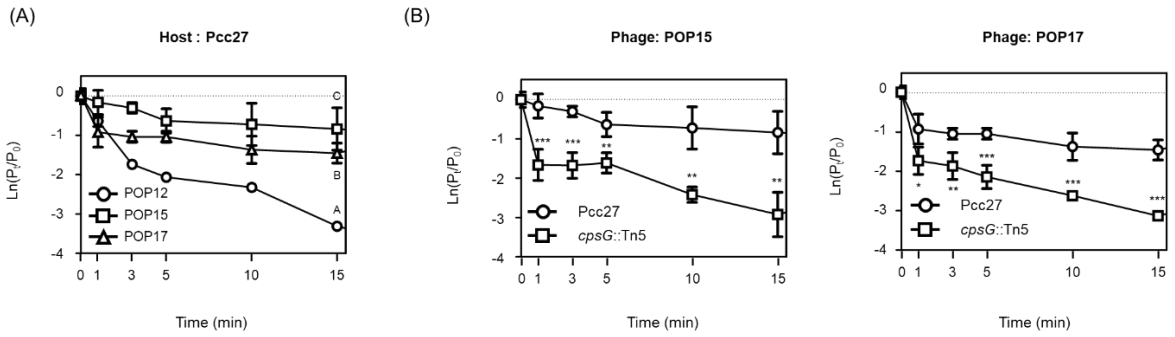


Fig S7. (A) Comparison of the adsorption of three phages (e.g., POP12, POP15, and POP17) to the WT Pcc27 strain. Statistical analysis for the last point of each graph was performed by a one-way analysis of variance with Tukey's multiple comparison tests. The significant differences among the experimental groups are marked with letters. (B) Decreased adsorption efficiencies of the flagella-dependent phages (e.g., POP15 and POP17) to the WT Pcc27 strain than to the Pcc27 lacking CA. Statistical analysis for each point of graphs was performed by unpaired t-tests. Each data point is presented as the natural logarithm of the ratio of end to initial phage titer [ln (P_t/P_0)]. The significant differences between the experimental groups are marked with asterisks.*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Reference

1. Kim H, Kim M, Bai J, Lim J-A, Heu S, Ryu S. 2019. Colanic Acid Is a Novel Phage Receptor of *Pectobacterium carotovorum* subsp. *carotovorum* Phage POP72. Frontiers in microbiology 10.