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Genomic Features and Lytic Activity of the Bacteriophage PPPL-1 Effective against *Pseudomonas syringae* pv. *actinidiae*, a Cause of Bacterial Canker in Kiwifruit

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Bacterial canker in kiwifruit is caused by *Pseudomonas syringae* pv. *actinidiae* (*Psa*). In this study, the bacteriophage PPPL-1 effective against *Psa* was characterized. Belonging to the *Podoviridae* family, PPPL-1 was effective against most *Psa* strains as well as most *Pseudomonas syringae* pathovars. PPPL-1 carries a 41,149-bp genome with 49 protein coding sequences and is homologous to the previously reported phiPSA2 bacteriophage. The lytic activity of PPPL-1 was stable up to 40°C, within a range of pH 3-11 and under 365 nm UV light. These results indicate that the bacteriophage PPPL-1 might be useful to control *Psa* in the kiwifruit field.

Keywords: Bacterial canker, bacteriophage, genome analysis, kiwifruit, *Pseudomonas syringae* pv. *actinidiae*

Bacterial canker in kiwifruit trees is caused by *Pseudomonas syringae* pv. *actinidiae* (*Psa*), a Gram-negative bacterial pathogen. *Psa* was first reported from *Actinidia deliciosa* (green kiwifruit) in Japan in 1984 [1]. *Psa* infection has been reported in all kiwifruit-growing countries, including China in 1992 [2], South Korea in 1988 [3], Italy in 1992 [4], and New Zealand in 2010 [5]. Based on toxin production and genetic diversity, *Psa* can be divided into three main biovars: Psa1 and Psa2 have only been reported in Japan and South Korea, respectively, while the highly virulent Psa3 has recently been reported all over the world [6, 7]. Main symptoms of bacterial canker include die back or blight on young canes, red-rusty exudation on canes or trunks in winter, and yellowish halo on leaves in spring [8, 9].

Bacteriophages are viruses that infect specific host bacteria [10]. Most bacteriophages effective against plant-pathogenic bacteria belong to the *Caudovirales* order, composed of three families: *Myoviridae*, *Siphoviridae*, and *Podoviridae* [11]. Bacteriophages effective against *Psa* have been previously described. Frampton *et al*. [12] characterized 24 *Psa* bacteriophages isolated from soil, water, and leaf samples at the infected kiwifruit orchards. Among them, 22 bacteriophages belonged to *Myoviridae*, one to *Podoviridae* and one to *Siphoviridae*. They had a narrow host range [12]. Di Lallo *et al*. [13] reported two more bacteriophages, phiPSA1 and phiPSA2, isolated from leaves of *A. deliciosa* infected by *Psa*. phiPSA1, a temperate phage with a narrow host range, belonged to *Siphoviridae*, while phiPSA2, a lytic phage with a broad host range, belonged to *Podoviridae*. Genome analysis showed that phiPSA1 and phiPSA2 have 51,090-bp and 40,472-bp genomic DNA, respectively [13]. Genome information and certain characteristics of closely related bacteriophages, such as gh-1 effective against *P. putida* and philBB-PF7A and Phi-S1 effective against *P. fluorescens* also have been reported [14–16]. Previously, our group reported five bacteriophages effective against *Psa* isolated from soil in kiwifruit orchards in South Korea, of which two belonged to *Myoviridae* and three to *Podoviridae* [17]. In this study, another bacteriophage, PPPL-1, was isolated from soil of a kiwifruit orchard in Wando, South

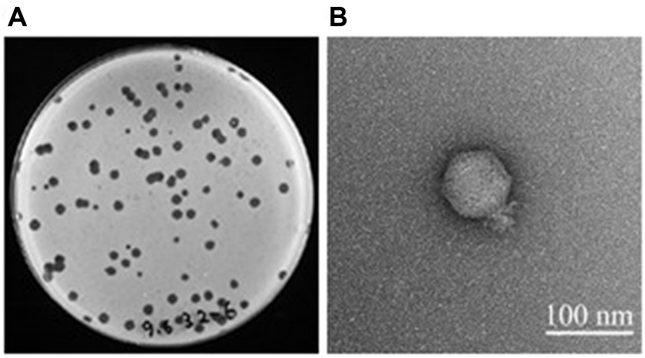

Fig. 1. Morphology of PPPL-1 observed using transmission electron microscopy.
PPPL-1 was isolated in soil from Wando, Korea and its lytic activity against *P. syringae* pv. *actinidiae* was shown in (**A**). Its morphology (**B**) was photographed at Korea Basic Science Institute after negative staining. PPPL-1 was found to belong to *Podoviridae*.

Korea (Fig. 1A) and was further characterized.

For the classification of PPPL-1, its morphology was first examined. For this, the bacteriophage was propagated as described previously with minor modifications [18]. The host bacterium *Psa* KEB9 was cultured overnight at 26°C in a shaking incubator. The following day, it was re-cultured to the exponential phase (OD_{600}=0.5~0.6) and then mixed with bacteriophages. After overnight incubation, the supernatant was collected by centrifugation (10,000 rpm, 10 min, 4°C), filtered with a 0.22 μm pore size filter (Sartorius, Gottingen, Germany), and treated with 10% polyethylene glycol (PEG) 6,000 and 1 M NaCl overnight at 4°C. After centrifugation, the bacteriophage pellet was re-suspended in SM buffer [50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 10 mM $MgSO_4$]. Finally, the bacteriophage was purified by cesium chloride (CsCl) density gradient using ultracentrifugation

Table 1. Host range of PPPL-1 against 18 strains of *P. syringae* pv. *actinidiae* and other *Pseudomonas syringae* pathovars.

Species & Pathovars (pv.)	Strain	Biovar	Lytic activity*	Species & Pathovars (pv.)	Strain	Lytic activity
P. syringae				pv. *dysoxyli*	KACC12842	++
pv. *actinidiae*	KBE9	2	++	pv. *eriobotryae*	KACC10395	++
	KGY4	2	-	pv. *garcae*	KACC 10398	-
	PJC7	2	++	pv. *glycinea*	ATCC8727	-
	YCS3	2	++	pv. *helianthi*	KACC11618	++
	JJ18	2	++	pv. *japonica*	KACC11638	++
	JYS5	2	++	pv. *lapsa*	KACC12216	++
	SYS1	3	++	pv. *maculicola*	KACC11617	-
	SYS2	3	++		LMG5071	++
	SYS3	3	-	pv. *mellea*	KACC12844	++
	SYS4	3	++	pv. *mori*	KACC10390	-
	KACC10584	2	++	pv. *morsprumorum*	KACC10397	++
	KACC10587	2	+	pv. *myricae*	KACC12845	++
	KACC10592	2	++	pv. *panici*	KACC 11619	++
	KACC10593	2	++	pv. *papulans*	LMG5077	-
	KACC10595	2	++		LMG5571	-
	KACC16847	2	++	pv. *passiflorae*	KACC12846	-
	KACC16848	2	++	pv. *phaseolicola*	NPS3121	++
	KACC16849	2	++	pv. *pisi*	KACC11620	++
pv. *antirrhini*	KACC10392		-	pv. *syringae*	DSM10604	++
pv. *aptata*	KACC12132		++	pv. *tabaci*	DSM1856	-
pv. *atrofaciens*	KACC11626		++		KACC10388	++
pv. *atropurpurea*	DSM50255		++	pv. *tagetis*	KACC10389	++
pv. *berberidis*	KACC12850		++	pv. *theae*	LMG5092	++
pv. *ciccaronei*	KACC12841		++	pv. *tomato*	DC3000	-
pv. *coronafaciens*	KACC12133		++		DSM50315	++
pv. *delphinii*	KACC10394		++	pv. *ulmi*	KACC11633	-

*Based on dotting assay. ++, clear plaques; +, opaque plaques; -, no plaques.

(25,000 rpm, 2 h, 4°C). The negatively stained bacteriophage was observed using transmission electron microscopy at 120 kV. Based on its morphology, the bacteriophage PPPL-1 belongs to *Podoviridae* (Fig. 1B). Total length and head size of PPPL-1 were estimated to be 100 nm and 70 nm, respectively.

The host range of PPPL-1 was determined by a dotting assay against 18 *Psa* strains, including biovars 2 and 3, 31 other *P. syringae* pathovars, and 10 other bacterial species. Of 18 *Psa* strains, 10 were obtained from Sunchon National University, South Korea, and 8 were provided from the Korean Agriculture Culture Collection (KACC) (Table 1). *Psa* strains were grown in tryptic soy broth, and other bacterial strains were grown in King's broth at 26°C. PPPL-1 was effective against 16 out of 18 *Psa* strains (Table 1). Interestingly, PPPL-1 was effective against at least 20 more *P. syringae* pathovars, including *P. syringae* pv. *aptata*, pv. *atrofaciens*, pv. *atropurpurea*, pv. *berberidis*, pv. *ciccaronei*, pv. *coronafaciens*, pv. *dysoxyli*, pv. *eriobotryae*, pv. *helianthi*, pv. *japonica*, pv. *lapsa*, pv. *mellea*, pv. *morsprumorum*, pv. *myricae*, pv. *panici*, pv. *phaseolicola*, pv. *pisi*, pv. *syringae*, pv. *tagetis*, and pv. *theae* (Table 1). However, it had no effect on other bacterial species such as *P. fluorescens*, *Acidovorax citrulli*, *A. valerianellae*, and *Ralstonia solanacearum* (Table S1). These results indicate that PPPL-1 is specific to *P. syringae* species. In general, specificity of bacteriophages is determined by interactions between the bacteriophage tail and its receptor(s) in the bacterial cell surface [19]. In Gram-negative bacteria, receptors mostly exist in flagella, pili, or capsules. Long thin helical flagella, rod-shaped filamentous pili, and flexible capsules act as binding agents between bacteria and bacteriophages [20]. Because PPPL-1 is specific to the species level of *P. syringae*, the receptor could be one of these structural parts common to *P. syringae* species.

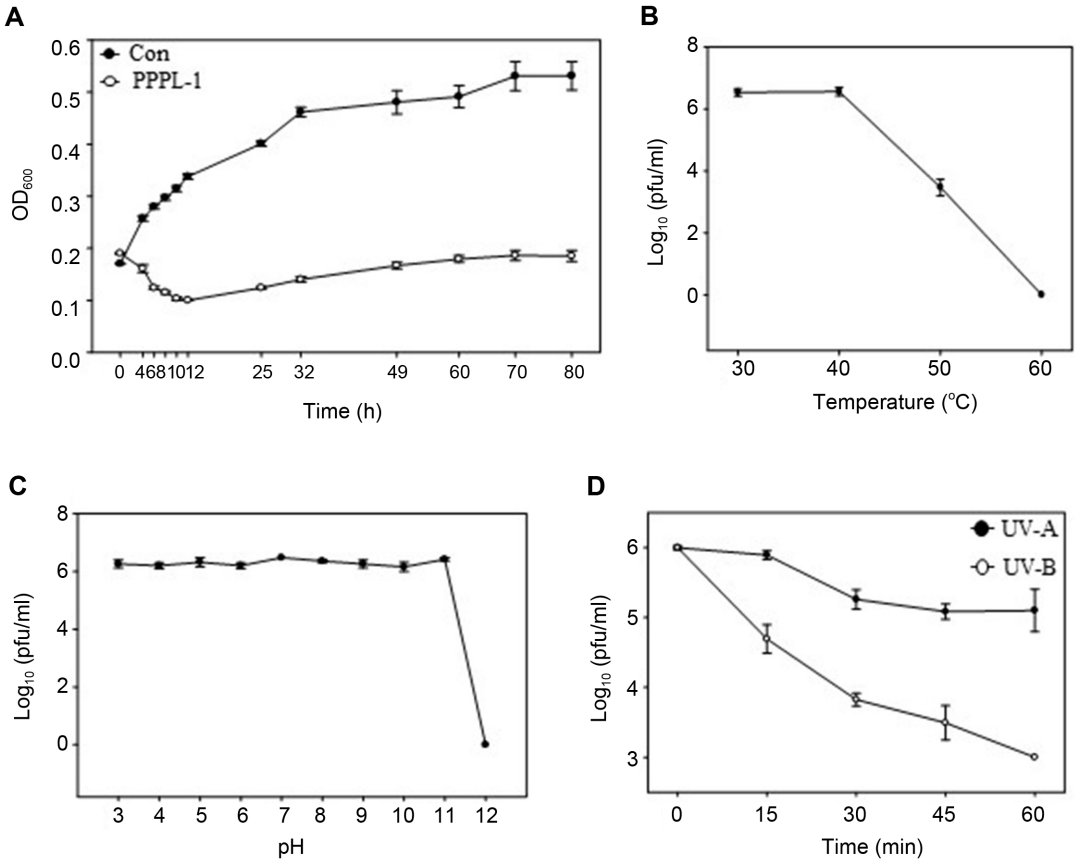

Fig. 2. Lytic activity of PPPL-1 against *Pseudomonas syringae* pv. *actinidiae* strain KBE9 under various conditions.

(**A**) The bacteriophage (MOI = 0.01) was added to a bacterial suspension (10^8 CFU/ml) in the early exponential phase, and OD_{600} was measured at the designated time points. Con, bacterial growth without bacteriophage in SM buffer. (**B**) Stability of PPPL-1 to temperatures. (**C**) Stability of PPPL-1 to pH values. (**D**) Stability of PPPL-1 under 365 nm or 306 nm UV lights. After treatment, titers of living bacteriophages (pfu/ml) were determined using plaque assay. Error bars indicate standard errors.

To determine genes that PPPL-1 carries, its whole genome was sequenced. Total genomic DNA was isolated with the phage DNA isolation kit (Norgen, Thorold, ON, Canada). Genome sequencing was performed with a 454 GS FLX system. GS FLX data processing was performed using the Roche GS FLX software v 2.9. Complete genome sequence was assembled by GS De Novo Assembler software v 2.9. Open reading frames (ORFs) and the functions of ORFs were predicted by BLAST using the GenBank database. The bacteriophage PPPL-1 (GenBank accession number KU064779) carries 41,779-bp genomic DNA with 49 ORFs (Table S2). Its G+C content is 57%. It carries a type II holin and a phage lambda Rz-like lysis protein, indicative of a lytic bacteriophage. Genome comparison using Easyfig software showed that PPPL-1 is most homologous to phiPSA2 (GenBank Accession No. KJ507099) (Fig. S1). It is also homologous to other *Pseudomonas* bacteriophages, such as gh-1 (GenBank Accession No. AF493143), Phi-S1 (GenBank Accession No. JX173487), and philBB-PF7A (GenBank Accession No. GU583987).

Stability of lytic activity against target bacteria is important for phage therapy. To examine the length of lytic activity of PPPL-1 in vitro with a target bacterium, a bacteriophage aliquot was added to a bacterial solution at the exponential phase (KBE9 strain, 10^8 CFU/ml) to a multiplicity of infection (MOI) of 0.01. The OD_{600} was measured for 80 h using a TECAN microplate reader (TECAN, Männedorf, Switzerland). The bacterial culture was inoculated with the same volume of SM buffer as the negative control. The bacterial density was gradually reduced nearly to 0.1 for 12 h and then was slowly increased up to 80 h (Fig. 2A). Based on these results, PPPL-1 can maintain its lytic activity against *Psa* strain KBE9 stably. This pattern is very similar to those of previously reported bacteriophages (KHUφ34, KHUφ38, and KHUφ44) [17].

For use of bacteriophages as biocontrol agents, their lytic activity must be stable under various environmental conditions like temperature, UV light, and soil pH. Therefore, stability under these conditions was examined by incubating bacteriophages under the indicated conditions for 1 h in vitro: 30–60°C, pH 3–12, and 365 nm (UV-A) or 306 nm (UV-B) UV light. PPPL-1 was stable up to 40°C, but its stability decreased above 40°C, and it was inactivated at 60°C (Fig. 2B). It was stable in the range of pH 3 to 11 (Fig. 2C). PPPL-1 was overall stable under UV-A (365 nm) light, but its stability was rapidly decreased under UV-B (306 nm) light (Fig. 2D). These results indicate that the lytic activity of PPPL-1 is somewhat stable under the environmental conditions of kiwifruit orchards.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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