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Isolation and partial characterization of bacteriophages infecting *Pseudomonas syringae* pv. *actinidiae*, causal agent of kiwifruit bacterial canker

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The phytopathogen *Pseudomonas syringae* pv. *actinidiae* (*Psa*) is the causal agent of bacterial canker of kiwifruit. In the last years, it has caused severe economic losses to *Actinidia* spp. cultivations, mainly in Italy and New Zealand. Conventional strategies adopted did not provide adequate control of infection. Phage therapy may be a realistic and safe answer to the urgent need for novel antibacterial agents aiming to control this bacterial pathogen. In this study, we described the isolation and characterization of two bacteriophages able to specifically infect *Psa*. ϕPSA1, a member of the *Siphoviridae* family, is a temperate phage with a narrow host range, a long latency, and a burst size of 178; ϕPSA2 is a lytic phage of *Podoviridae* family with a broader host range, a short latency, a burst size of 92 and a higher bactericidal activity as determined by the TOD value. The genomic sequence of ϕPSA1 has a length of 51,090 bp and a low sequence homology with the other siphophages, whereas ϕPSA2 has a length of 40 472 bp with a 98% homology with *Pseudomonas putida* bacteriophage gh-1. Of the two phages examined, ϕPSA2 may be considered as a candidate for phage therapy of kiwifruit disease, while ϕPSA1 seems specific toward the recent outbreak's isolates and could be useful for *Psa* typing.

Abbreviations: PSA – *Pseudomonas syringae* pv. *actinidiae*; **MOI** – multiplicity of infection; **PFU** – plaque forming unit; **TE** – tris–EDTA; **CFU** – colony forming unit; **TOD** – time of death; **OD_{600}** – at 600 nm wavelength

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Introduction

Pseudomonas syringae pv. *actinidiae* (*Psa*), the causal agent of bacterial canker of kiwifruit, is currently damaging both *Actinidia deliciosa* and *A. chinensis* worldwide with severe economic losses [1]. On these crops, a pandemic population of the pathogen, most probably originated in China [2, 3], incites different kinds of symptoms such as leaf spotting, twig wilting, flower necrosis, reddening of the lenticels, cankers along the leader and trunk as well as exudates oozing out from the canker. This *Psa* population differs from the one that caused relevant damages to the green-fleshed kiwifruit (i.e., *A. deliciosa*) in Japan and South Korea in the 1980–1990 period [4–7]. Control measures aiming to reduce the incidence, severity, and spreading of the disease, have been undertaken in all areas of cultivation. A common practice applied everywhere is the cutting and the subsequent destruction of the infected plants or plant parts to reduce the inoculum pressure of the pathogen. Different control strategies have followed in different countries. In New

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Zealand, the utilization of copper-based compounds during the year, antagonistic bacteria mainly during blooming time and streptomycin before blooming, have been suggested and have been largely applied by the farmers (Kiwifruit Vine Health, www.kvh.org.nz). However, despite such massive usage, the pathogen has continued to spread during a 2-year infection and currently it has been found on about 70% of the total kiwifruit orchards of the country (Kiwifruit Vine Health, www.kvh.org.nz). In Italy, where the agricultural use of antibiotics is not allowed, the copper-based compounds and some antagonistic bacteria have been, so far, the only temporarily registered products available to try to control the disease. This strategy, however, is not completely effective. Moreover, the massive use of copper and antibiotics can promote resistance(s) in the pathogen, a quite common phenomenon in plant pathogenic bacteria; resistance to both streptomycin and copper, indeed, has already been observed also for *Psa* and/or other phytopathogenic pseudomonads isolated in the same areas of kiwifruit cultivation [8–11]. These genetic traits, of course, can be exchanged through lateral gene transfer mechanisms among bacteria sharing the same environment [12, 13]. The utilization of a bacteriophage-mediated control can be a suitable alternative [14].

Bacteriophages specifically infect bacteria and, according to Rohwer [15], are the most abundant biological entities on earth. The infection of a sensitive bacterium by a lytic phage is followed by the rapid assembling of new viral progeny, which is spread in the environment as a consequence of activity of phage lytic enzymes endolysin and holin. In turn, the new phages can infect neighboring bacteria with the result of reducing and inhibiting the bacterial population. The lethal effect on a specific bacterial host was recognized with the discovery of bacteriophages by Frederick Twort and Félix d'Hérelle in the second decade of the last century [16]; d'Hérelle himself introduced the concept of "phage therapy," that is the use of bacteriophages for the treatment of bacterial infectious diseases [17]. The use of bacteriophages as biocontrol agents was hindered by the advent of antibiotics, but, in recent years, the continuous selection of bacteria resistant to antibiotics or other antimicrobial agents has led to a new emphasis on the phage therapy. This renewed interest is due to the high specificity of phages for target bacteria without altering beneficial microflora, to their non-toxic nature and ability to kill antibiotic resistant bacteria and, last but not least, to the possibility they offer of limiting the release of persistent/dangerous chemicals into the environment. Successful phage therapy has also been applied for the control of economically important plant diseases, such as bacterial spot of peach [18], fire blight of pear and apple [19–21], and treatments with bacteriophage are today commercially available (for review see Balogh *et al*. [22]).

In this study, we report the isolation and characterization of two bacteriophages specific for *Psa*, in order to assess their potential use for the treatment of bacterial canker of kiwifruit. The biological properties of the two bacteriophages, including lytic or lysogenic cycle, morphology, host range, thermal and pH stability, latency, and burst size have been analyzed. In literature, the characterization of bacteriophages has been described for several pathovars of *Pseudomonas syringae* such as *P. s*. pv. *phaseolicola* [23–25], *P. s*. pv. *syringae* [26], *P. s*. pv. *tomato* [27], and *P. s*. pv. *morsprunorum* [28] but not for *P. s*. pv. *actinidiae*; as far as we know, this is the first report on bacteriophages able to infect *Psa*.

Materials and methods

Media

Nutrient broth medium (peptone (Oxoid Ltd., London, England), 5 g; beef extract (Oxoid) 3 g; NaCl 5 g per liter) was used as a broth or solidified with 1.5% agar (NA) to grow bacterial hosts. Soft agar for phage plaque-assays contained 0.8% agar. SM buffer (10 mM Tris–HCl, pH 7.5; 100 mM NaCl; 10 mM $MgSO_4$) was used for suspending and titrating the bacteriophages.

Phage isolation and purification

The test strain *Psa* CRA-FRU 8.43 (*Psa* 8.43) was used as indicator for phage isolation and propagation. The strain, responsible for the recent outbreak of bacterial canker on kiwifruit in Italy, was previously genomically assessed [6]. For indicator preparation, a single colony of *Psa* 8.43 was cultured in nutrient broth at 25 °C overnight. After centrifugation, the bacterial pellet was suspended in one tenth of the starting volume with SM buffer. Bacteriophages were obtained from leaves of *A. deliciosa* infected by *Psa*. The leaves were washed with SM buffer and the leaf-wash centrifuged for 2 min at 10,000*g* to remove the organic debris. After filtration through a 0.2 μm filter, aliquots of 100 μl were added to 200 μl of indicator and plated with the double-layer-agar technique [29]. Bacteriophages were also obtained by centrifuging of Rome municipal raw sewages for 2 min at 10,000*g* and then filtering the supernatant through a 0.2 μm filter. Samples of 100 μl of the filtrate were plated as above. For purification of a single bacteriophage, a single plaque was picked with a sterile glass Pasteur pipette and the phages were eluted for 1 h in 500 μl of SM buffer. After centrifugation (10,000*g*, 2 min) and dilution, the phage

suspension was plated to obtain isolated plaques. To ensure the purity of the phage isolate, three rounds of plaque purification and reinfection of exponentially growing *Psa* 8.43 were performed. To propagate phages about 1×10^6 phages were added to 200 μl of indicator and plated with soft agar. After overnight incubation at 25 °C, bacteriophages were recovered by adding 4 ml of SM buffer on top of the plates and keeping them for 4 h at room temperature with gently shaking. Soft agar and liquid were then scraped and centrifuged at 8000*g* for 10 min. A 50 μl volume of chloroform was added to the filtered lysate that was then stored at 4 °C. Serial dilutions in SM buffer were used to determine the phage concentration, with the double-layer-agar method. For long-term storage phage stocks were stored in SM buffer plus 25% glycerol at −70 °C.

Electron microscopy

Phage morphology was examined by transmission electron microscopy of negatively stained preparations. Phage stock aliquots were centrifuged at 70,000*g* for 1 h in Beckman SW 28 Ti rotor and suspended at about 10^{11} PFU ml^{-1} in SM buffer. A drop of each suspension was placed on carbon-coated 400 mesh grid and dried; afterwards, specimens were negatively stained with a drop of 1% phosphotungstic acid, pH 7.4 and examined with a Philips EM 208s electron microscope.

Determination of the lysogenization frequency of ϕPSA1

Cells of *Psa* 8.43 were incubated overnight at 25 °C. After centrifugation, the bacteria were suspended in SM buffer at about 1×10^9 CFU ml^{-1} and infected with ϕPSA1 at a multiplicity of infection (MOI) of 1 for 20 min at 25 °C. The infected bacteria were centrifuged 2 min at 8000*g* and the pellet suspended in 1 ml of SM buffer; in order to eliminate the non-adsorbed phages, both centrifugation and suspension steps were repeated three times more. Finally, the titer of the infected bacteria (infective centers) was determined by plaque formation after dilution and plating with the double-layer-agar method. Infected bacteria were also plated on NA plates to detect the lysogenic bacteria; the resultant colonies were replicated onto NA plates previously spread with the test strain. Lysogenic cells were identified as colonies able to produce plaque on the bacterial lawn. The lysogenization frequency was calculated as the ratio of lysogens/infective centers [30].

Phage adsorption

Cells of *Psa* 8.43 in exponential phase were centrifuged and suspended in SM buffer to the concentration of 1×10^9 CFU ml^{-1}. Two ml of bacteria were infected with 2×10^6 PFU ml^{-1} of phage suspension to give a MOI of 0.001 and incubated at 25 °C. At 5 min intervals, aliquots of 100 μl were added to 900 μl of SM buffer and centrifuged 2 min at 12,000*g*. The supernatants containing unadsorbed phages were filtered through 0.2 μm filter, diluted, and titrated. Adsorption was expressed as the percentual decrease of the phage titer in the supernatant, as compared to the T_0 one. Suspensions of phages without any cells were used as no-adsorption standard for calculations [31].

One-step growth experiments

For the one-step growth experiment, *Psa* 8.43 was incubated at 25 °C to the mid-exponential phase (OD_{600} 0.3–0.4 – about 2×10^8 CFU ml^{-1}). The pellet obtained from 1 ml of culture was suspended in the same volume of SM buffer and infected with 0.1 ml of 1×10^7 PFU ml^{-1} phage stock (MOI 0.01). The phages were allowed to adsorb on the bacteria for 10 min at 25 °C and the mixture centrifuged twice at 12,000*g* for 1 min to remove the non-adsorbed ones. The pellet was then suspended in 1 ml SM buffer, diluted 1×10^{-3} in 10 ml of nutrient broth, and incubated a 25 °C with shaking. Samples of 0.1 ml were taken at 5 or 10-min intervals and phage titer was determined immediately by the double-layer-agar technique. Assays were carried out in triplicate. The latent period is the time interval between the infection (not including 10 min phage adsorption and 5 min centrifugation for removal of non-adsorbed phages) and the beginning of the phage production. Burst size was calculated as the ratio of the final count of liberated phage particles to the initial count of infected bacterial cells during the latent period [29, 32].

Influence of pH on phage viability

Phages at approximately 10^8 PFU ml^{-1} were suspended in 1 ml SM buffer, previously adjusted with 1 M NaOH or 1 M HCl, to yield a pH range from 2.0 to 11.0. After 60 min of incubation at 25 °C and serial dilutions each treated sample was tested against *Psa* 8.43 in a double-layer-agar assay to check the viability of phage. The assays were carried out in triplicate and the results are reported as the mean of phage counts (PFU ml^{-1}) ± standard deviation.

Effect of temperature on phage viability

Phages particles at about 10^8 PFU ml^{-1} were suspended in 1 ml SM buffer and incubated at 25 °C (control), 40, 50, and 60 °C for 10, 20, 40, and 60 min. The surviving phages were serially diluted and then counted with the double-layer-agar method on *Psa* 8.43. Each experiment was

performed three times and the mean of phage counts (PFU ml^{-1}) and standard deviation were determined.

Determination of bacteriophage host range

The bacterial strains used are listed in Table 1. Phage host range was determined by spot testing. Bacteria were grown in nutrient broth to OD_{600} 0.5, centrifuged, and suspended in the same volume of SM buffer. For each strain, 200 µl were plated with the double-layer-agar method. Once the overlay was gelled, 10 µl of diluted phage suspension at about 1×10^5 PFU ml^{-1} were spotted on the overlay. The plates were incubated at 25 °C and examined for plaques after 18–24 h. Bacterial sensitivity to a bacteriophage was established by lysis halo at the spot. According to the clarity of the spot, bacteria were differentiated into three categories: resistant, weakly sensitive, and sensitive. Bacterial strains resistant to ϕPSA1 were subsequently analyzed for the release of phage particles in order to discard the possibility that they were lysogenic for ϕPSA1. For this purpose, bacterial cells were treated with mitomycin C as described by Merabishvili *et al*. [33].

Time of death (TOD)

Psa 8.43 was incubated at 25 °C to OD_{600} 0.2 (approximately 1×10^8 CFU ml^{-1}) and infected in the presence of 10 mM $MgSO_4$ with 1×10^7 PFU ml^{-1} of phage suspension to give a MOI of 0.01. The infected bacteria and the not infected control were then incubated at 25 °C with shaking. The optical density (OD_{600}) of the samples was taken at intervals of 30 min or 2 h for 24 h.

The "time of death" (TOD) is the time required for reduction of the culture optical density from 0.2 to 0.1.

Bacteriophages genome sequencing and analysis

The DNA extraction was performed with the phage DNA isolation kit (Norgen Biotek Corp., Canada). DNA samples were processed using Nextera sample prep kit from Illumina (Illumina, Inc., CA, USA) and sequenced at paired-end 300 bp set-up on Illumina MiSeq (Illumina, Inc.) at IGA Technology Services (Udine, Italy). Raw FASTQ sequences were trimmed with erne-filter [34] using default parameters. Then sequencing adapters were removed with cutadapt [35] using default parameters. Erne-filter was used to remove possibly contaminated reads from the origin source, *Psa* 8.43. Finally, reads were assembled using CLC Genomics Workbench 6.5.1 with "Automatic word size" on and scaffolding turned off. The two putative assembled phage genome sequences were identified on the basis of the high coverage (phage ϕPSA1: mean coverage 24,377×; phage ϕPSA2: mean coverage 65,565×) and by performing a Blast similarity search in public databases. Translated ORFs were compared with known protein sequences using BLASTP, against the non-redundant protein GenBank database.

Nucleotide sequence accession numbers

The complete genomic sequences of ϕPSA1 and ϕPSA2 have been deposited in GenBank with the accession numbers KJ507100 and KJ507099, respectively.

Results

Phage isolation and enrichment

ϕPSA1 was the only phage isolated from infected leaves of *A. deliciosa* and formed clear plaques on the host strain with a diameter of 0.5–2 mm. ϕPSA2 was isolated from Rome municipal sewage, along with three other phages with different plaque phenotype. For this study, we chose ϕPSA2 for its clear and large size plaques (3–7 mm in diameter). Phages were purified by successive single plaque isolation. A high titer suspension ($1–3 \times 10^{10}$ PFU ml^{-1}) was obtained for both phages.

Virion morphology

The virion morphology was studied by transmission electron microscopy of negatively stained preparations.

Phage ϕPSA1 belong to the family *Siphoviridae* (Ackermann's viral morphological group B1) [36]. The virion has an isometric head approximately 60 nm in diameter and a long, flexible, non-contractile tail approximately 200 nm (Fig. 1A and B). ϕPSA2 has an isometric head with short, thin, non-contractile tail and thus is a member of the family *Podoviridae* (Ackermann's viral morphological group C1). Electron micrographs revealed heads approximately 60 nm in diameter; the tails were difficult to measure because they were partially obscured by negative staining (Fig. 1C and D).

Phage life cycle

Clear plaques are often indicative of the lytic nature of a phage while turbid plaques are characteristic of a temperate phage. On the basis of plaque phenotype, ϕPSA1 and ϕPSA2 were presumptively regarded as lytic. In order to test this hypothesis, we performed a spot test and characterized the bacterial colonies grown within the lysis area after a 3–5 days of incubation at 25 °C. In this way, we were able to isolate lysogens for ϕPSA1. These bacteria were immune to ϕPSA1 superinfection and produced phages after repeated cycles of single colony isolation. Therefore, ϕPSA1 is a temperate phage. We also measured the lysogenization frequency, on the

Table 1. Host range of the phages ϕPSA1 and ϕPSA2 on different hosts.

Species and pathovars	Strain[a]	Host-cultivar	Origin	Year of isolation	Infectivity of phage[b]	
					ϕPSA1	ϕPSA2
P. syringae pv. *actinidiae*	KW30	*A. deliciosa* – Hayward	Japan	1984	–	+
	NCPPB3739	*A. deliciosa* – Hayward	Japan	1984	–	+
	SUPP1747 A19	*A. deliciosa* – Hayward	Japan	1986	–	+
	SUPP765 Kw712[c]	*A. deliciosa* – Hayward	Japan	1987	+/–	+
	NCPPB3871	*A. deliciosa* – Hayward	Italy, Lazio	1992	–	+
	KN.2	*A. deliciosa*	Korea	1997	–	+
	SUPP2211	*A. deliciosa* – Hayward	Japan	2002	–	+
	CRA-FRU8.43	*A. chinensis* – Hort16A	Italy, Lazio, Latina	2008	+	+
	SFR-BO4252 A,1	*A. chinensis* – CK2	Italy, Emila Romagna	2009	+	+
	CRA-FRU10.09	*A. chinensis* – Hort16A	Italy, Lazio	2009	+	+
	CRA-FRU10.10	*A. chinensis* – Hort16A	Italy, Lazio	2009	+	+
	CRA-FRU10.11	*A. chinensis* – Hort16A	Italy, Lazio	2009	+	+
	CRA-FRU10.12	*A. chinensis* – Hort16A	Italy, Lazio	2009	+	+
	CRA-FRU10.14	*A. chinensis* – JinTao	Italy, Lazio	2009	+	+
	CRA-FRU8.55	*A. deliciosa* – Hayward	Italy, Lazio, Latina	2009	+	+
	CRA-FRU8.69	*A. deliciosa* – Hayward	Italy, Lazio, Latina	2009	+	+
	CRA-FRU8.79	*A. deliciosa* – Hayward	Italy, Lazio, Latina	2009	+	+
	CRA-FRU14.10	*A. chinensis*	Italy, Calabria	2010	+	+
	SFR-BO8615,1	*A. deliciosa* – Hayward	Italy, Emilia Romagna	2010	+	+
	SFR-BO10574,A1	*A. chinensis* – Belen	Italy, Emilia Romagna	2010	+	+
	SFR-TO1616-291a	*A. deliciosa* – Hayward	Italy, Piemonte	2010	+	+
	SFR-TO1616-231Aa	*A. chinensis*	Italy, Piemonte	2010	+	+
	SFR-VR4175	*A. chinensis*	Italy, Veneto	2010	+	+
	ANSES37.26	*A. deliciosa* – Summerkiwi	France	2011	–	+
	ANSES37.51	*A. chinensis* – Hort16a	France	2011	+	+
	ANSES40.01	*A. chinensis* – Oscar Gold	France	2011	+	+
	ANSES37.37	*A. deliciosa*	France	2011	+	+
	CRA-FRU12.50	*A. chinensis* – JinTao	Italy, Campania	2011	+	+
	SFR-BO8112,1	*A. deliciosa* – Hayward	Italy, Emilia Romagna	2011	+	+
	SFR-BO7457,1	*A. chinensis* – JinTao	Italy, Emilia Romagna	2011	+	+
	SFR-TO23b	*A. chinensis*	Italy, Piemonte	2011	+	+
	ICMP18801	*A. chinensis* – New Gold	New Zealand	2011	+	+
	ICMP18802[d]	*A. chinensis* – Hort 16A	New Zealand	2011	–	+/–
	ICMP18883[d]	*A. deliciosa*	New Zealand	2011	–	+
	ICMP18884	*A. deliciosa*	New Zealand	2011	+	+
	CRA-FRU14.08	*A. deliciosa*	Portugal	2011	+	+
	IVIA 3729.2	*A. deliciosa*	Spain	2011	+	+
P. syringae pv. *theae*	NCPPB2598	*Thea sinensis*	Japan	1970	–	+
P. avellanae	NCPPB3487	*Corylus avellana*	Greece	1973	–	+
	CRA-FRU111	*Corylus avellana*	Italy	1998	–	+
	CRA-FRU6.44	*Corylus avellana*	Italy	2007	–	+
P. syringae pv. *morsprunorum*	RIPF25	*Prunus avium*	Poland	2007	–	+
	RIPF38	*Prunus avium*	Poland	2007	–	+
P. syringae pv. *morsprunorum*	RIPF25	*Prunus avium*	Poland	2007	–	+
	RIPF38	*Prunus avium*	Poland	2007	–	+
P. syringae pv. *phaseolicola*	NCPPB4478	*Phaseolus vulgaris*	Ethiopia	1985	–	–
P. viridiflava	CRA-FRU 10.33	*A. deliciosa* – Hayward	Italy	n.k.	–	–
	CRA-FRU 10.34	*A. deliciosa* – Hayward	Italy	n.k.	–	–
P. mediterranea	UNICT 3c	*Solanum lycopersicum*	Italy, Sicily	1993	–	–
P. corrugata	UNICT 4.3t	*Solanum lycopersicum*	Italy, Sicily	1993	–	–
P. fluorescens	ATCC 13525				–	–

[a]The strain prefix is indicative of its origin according to the following scheme: SFR, KN2, and CRA-FRU are from Culture Collection of Centro di Ricerca per la Frutticoltura; ANSES is from ANSES Culture Collection, France; KW and SUPP are from Culture Collection of the Shizuoka University; NCPPB is from National Collection of Plant Pathogenic Bacteria; ICMP is from International Collection of Microorganisms from Plant; RIPF is from Research Institute of Pomology and Floriculture, Skierniewice; UNICT is from Culture Collection Catania.

[b]–, resistant; +, sensitive; +/–, weakly sensitive; n.k., not known.

[c]Copper and streptomycin resistant.

[d]Low virulent.

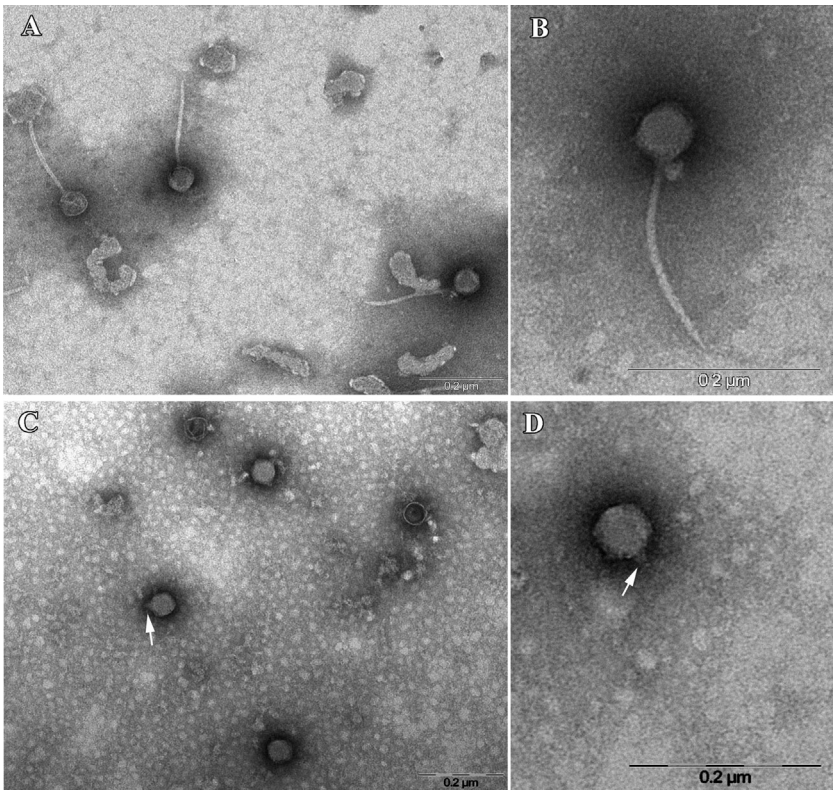

Figure 1. Negative staining electron micrographs showing (A) several viral particles of ϕPSA1 with a morphology typical of the *Siphoviridae* family; (B) a ϕPSA1 particle with the tip at the distal end of the tail; (C) Several "full" and "empty" ϕPSA2 viral particles with the typical morphology of the *Podoviridae* family (arrow shows the short tail); (D) a single ϕPSA2 particle consisting of a capsid and a short tail (arrow).

recipient strain *Psa* 8.43 at a MOI of 1.0, and found it to be equal to 28 ± 6%. No lysogens were found in spot tests with phage ϕPSA2 confirming its lytic nature.

Phage adsorption and one-step growth

The adsorption rate of ϕPSA1 and ϕPSA2 on *Psa* 8.43 was similar (data not shown). Approximately, 30% of phages was adsorbed on the host cell after 5 min, rising very slowly to 40–50% after 30 min postinfection (Fig. 2).

The one-step growth curve for ϕPSA1 and ϕPSA2 was determined in nutrient broth at 25 °C (Fig. 3). From the triphasic curves obtained, a latent period of 100 min, a rise period of 50 min and a burst size of 178 ± 23 were calculated for ϕPSA1. ϕPSA2 is characterized by a short latency of 15 min, a rise period of 15 min and a burst size of 92 ± 21. Determination of burst size was based on the ratio of the mean phage particles liberated at plateau to the mean yield of infected bacteria at the latency phase.

TOD of the bacterial population

The phage ability to kill bacteria results from many features, such as the adsorption rate, the length of the latent period, the burst size, and phage decay rate. All of these may be evaluated as such but, aiming at a more direct method to assess the phage ability to kill its host, we introduced a new parameter, including all the above

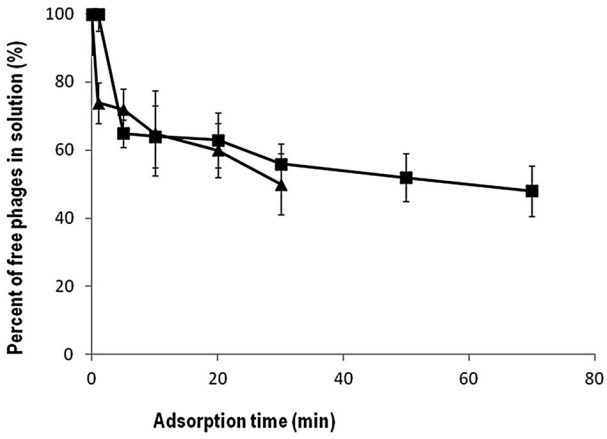

Figure 2. Adsorption of bacteriophages ϕPSA1 (■) and ϕPSA2 (▲) to *Psa* 8.43. Percent unadsorbed phage is the ratio of PFU in the supernatant to the initial PFU. The initial PFU was determined by titering an equivalent dilution of the phage in the absence of host cells. Data are shown as mean and SD.

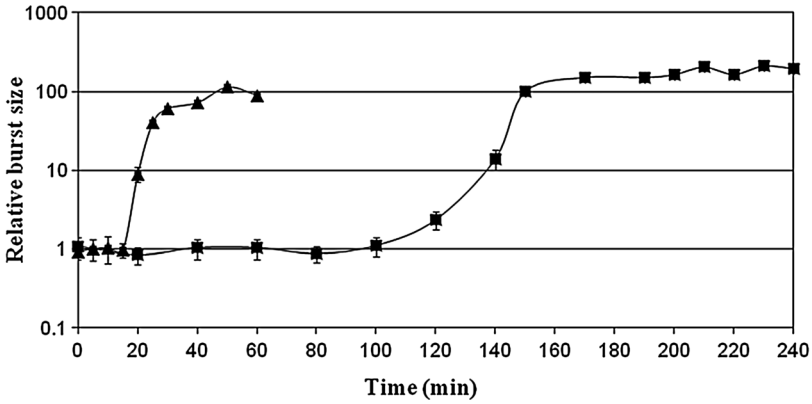

Figure 3. One-step growth curves of bacteriophages ϕPSA1 (■) and ϕPSA2 (▲) on *Psa* 8.43. The PFU/infected cell ratio at different time is shown. Data are shown as mean and SD.

listed parameters: the "TOD" of the bacterial population. TOD is the time that a bacteriophage requires to reduce the optical density (A_{600}) of a bacterial population, infected at MOI of 0.01, from 0.2 to 0.1. As shown in Fig. 4, the TOD of ϕPSA2 is approximately 155 min. The TOD of ϕPSA1 could not be determined because the optical density of the bacterial culture never dropped below its starting value. The turbidity was monitored up to 24 h; at that time ϕPSA2 had drastically decreased the culture turbidity as compared to the control, while the turbidity of the culture challenged with ϕPSA1 lowered only slightly.

Effect of pH and temperature on phage infectivity

The possible bias of different pH values on the infectivity of the two phages was assessed. No reduction in infectivity was observed after 1 h of incubation in pH ranging from 5.0 to 9.0 (Fig. 5). ϕPSA1 lost its infectivity at pH 2, retaining a 7% of infection ability at pH 3.0. The infectivity decreased, to a lesser extent, at pH 10.0 and 11.0 (64 and 32% of infectivity retained, respectively). ϕPSA2 shows the same trend as ϕPSA1 but it is more sensitive to low pH values with a complete loss of infectivity at both pH 2.0 and 3.0. On the other hand, the decline observed at pH 10.0 and 11.0 is slighter with 78 and 71% of phages still infectious after 1 h incubation, respectively. The effect of the temperature on phage viability was carried out at 40, 50, and 60 °C (Fig. 6). The results show that ϕPSA1 and ϕPSA2 are viable after being exposed to 40 °C for 60 min and both retain almost 80% of viability after 60 min at 50 °C. The treatment at 60 °C reduced rapidly phage infectivity; after 10 min the surviving phages were 0.6% for ϕPSA1 and 0.3% for ϕPSA2. No lysis plaques were obtained for both phages after 40 min exposure to 60 °C, suggesting a loss of phage viability in these conditions.

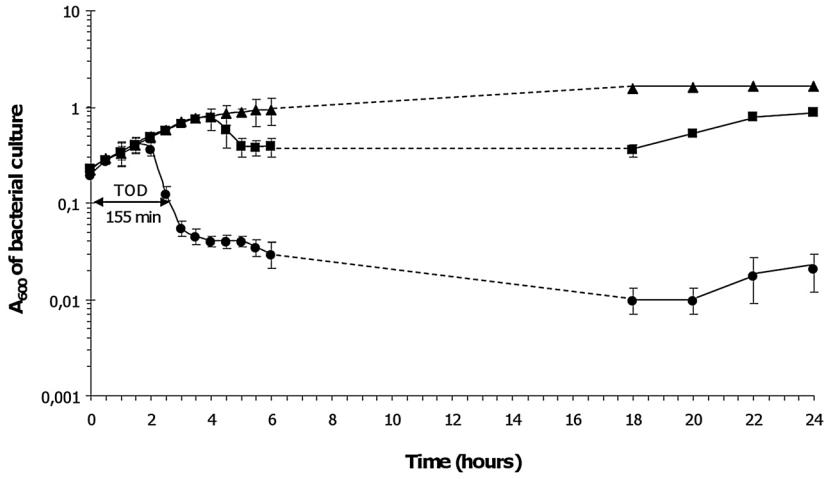

Figure 4. Lytic activity of ϕPSA1 and ϕPSA2, and TOD determination on *Psa* 8.43. A_{600} values of *Psa* cultures infected with ϕPSA1 (■), ϕPSA2 (●) and uninfected control (▲) are shown. Data are shown as mean and SD.

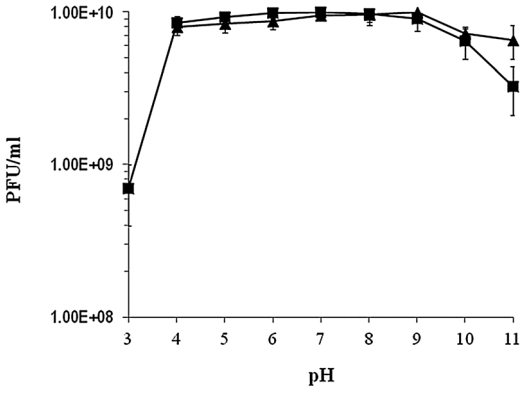

Figure 5. pH stability test of ϕPSA1 (▲) and ϕPSA2 (▲). Phage was exposed to different pHs for 1 h. Data are shown as mean and SD.

Phage host range

The host range of ϕPSA1 and ϕPSA2 was evaluated on a number of phytopathogenic *Pseudomonas* spp. and *P. syringae* pathovars (Table 1). For *Psa* 37 strains were tested; seven of them were responsible for the bacterial canker outbreaks on *A. deliciosa* during the last two decades of the XX century in Japan, Italy, and Korea. The remaining strains were responsible for the most recent outbreak, observed between 2008 and 2011, on both *A. chinensis* and *A. deliciosa* in Italy, New Zealand, and other countries. Besides the pathovar *actinidiae*, we tested *P. s.* pv. *theae* and *P. avellanae*, belonging to the same genomospecies 8. We also investigated less related strains as *P. s.* pv *syringae* (genomospecies 1), *P. s.* pv. *phaseolicola*, and *P. savastanoi* pv. *fraxinii* (genomospecies 2), *P. s.* pv. *tomato*, and *P. s.* pv. *morsprunorum* (genomospecies 3), *P. viridiflava* (genomospecies 6), as well as *P. corrugata*, *P. mediterranea*, and *P. fluorescens*. The results indicated that ϕPSA1 has a narrow host range, being unable to infect pseudomonads other than *P. s.* pv. *actinidiae*. Interestingly, with the exception of one *Psa* isolate from France and two from New Zealand, only the strains responsible for the recent bacterial canker outbreaks were susceptible to ϕPSA1 infection, supporting the observations of Marcelletti *et al.* [6] that two genetically distinct populations of *Psa* have caused past and recent epidemics.

Unlike ϕPSA1, ϕPSA2 exhibits a broader host range; besides all the *Psa* strains tested, indeed, also other pathovars of genomospecies 8 and 3 were susceptible to this phage. *P. savastanoi* pv. *fraxinii* was weakly sensitive (Table 1).

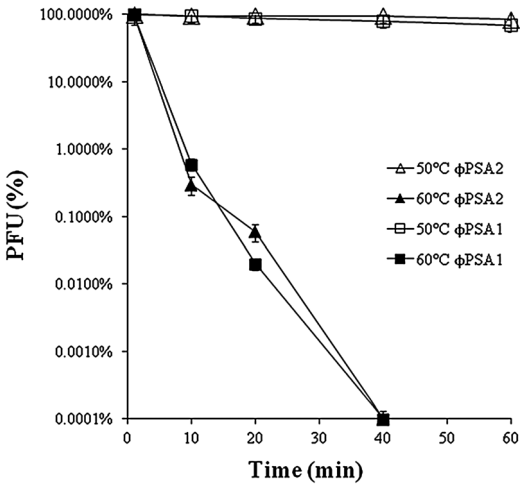

Figure 6. Thermal stability of ϕPSA1 and ϕPSA2. Phages particles were incubated at 40, 50, and 60 °C for 10, 20, 40, and 60 min (thermal stability at 40 °C, not shown). Data are shown as mean and SD.

Genome analysis

ϕPSA1 has a 51,090 bp linear dsDNA genome with 52 putative ORFs. No significant homology was found with other siphophages while high homology at protein level was determined with several putative prophages of *P. syringae* pathovars. Recurrent is the homology with the *P. syringae* pv. *syringae* putative prophage PSSB64-02: for example, the protein identity for repressor, anti-repressor, integrase, and tail tape-measure protein is in order 98, 94, 94, and 66%. The overall genomic GC content is 58.5% and corresponds to the GC content of Psa [6]. This evidence suggests a good adaptation of this phage to its host. The gene repertoire confirms that ϕPSA1 is a temperate phage.

The genome of ϕPSA2 is linear dsDNA of 40 472 bp with 47 putative ORFs and is flanked by 216 bp long direct terminal repeats (DTRs). The GC content value is 57.4%, slightly lower than its host bacterium (58.5%). The genome organization and protein sequence identity indicate that ϕPSA2 is a member of the "T7-like viruses" genus. Its most notable feature is a high homology with the *Pseudomonas putida* phage gh-1 [37]. At DNA sequence level, the identity is about 98% over almost the entire length of the gh-1 genome and most of the proteins and enzymes involved in replication, maturation, and phage assembly show an amino acid identity between 98 and 100% (Table 2). We identified 10 potential ϕPSA2 promoters in the same position and with identical sequence to gh-1 promoters; this suggests that the regulation of mRNA synthesis could be equal in the two phages. Instead, significant differences are present in the tail fiber protein, coded by gene *17*, where identity is just 59%; this is the viral receptor-recognition protein and the observed differences reflect the different host range. Another striking difference between ϕPSA2 and gh-1 phage is the presence in ϕPSA2 of a region containing six ORFs between the left DTR and the gene for phage RNA polymerase. This region, missing in gh-1, is present in the

Table 2. Putative genes of ϕPSA2 and their protein homologies to T7, gh-1, and other bacteriophages.

ORF[a]	From[b]	To[b]	Size (aa)	Putative function	% Identity to T7	% Identity to gh-1[c]	% Identity to other phages[d]
orf1	937	1191	84	Hypothetical protein			
orf2	1191	1388	65	Hypothetical protein			44.8 to ϕS1_02 [YP_007869879.1]
orf3	1756	2235	159	Hypothetical protein			56.4 to ϕIBB-PF7A p43 [YP_004306363.1]
orf4	2266	2802	178	Hypothetical protein			24.9 to ϕIBB-PF7A p02 [YP_004306317.1]
orf5	2799	3503	234	Hypothetical protein			45.6 to ϕIBB-PF7A p03 [YP_004306318.1]
orf6	3609	4148	179	Hypothetical protein			28.0 to ϕIBB-PF7A p05 [YP_004306320.1]
1	4277	6934	885	RNA polymerase	57.3	99.9	
1.1	6948	7085	45	Hypothetical protein	28.9	100	
orf9	7082	7354	90	Hypothetical protein		83.3 to *orf1*	
orf10	7354	7740	128	Hypothetical protein		93.8 to *orf2*	
1.3	7752	8816	354	ATP-dependent DNA ligase	39.9	98.3	
orf12	8820	9098	92	Hypothetical protein			
1.6	9291	9548	85	Hypothetical protein	33.0	20.0 to *orf3A*	
orf14	9545	10192	215	Hypothetical protein		100 to *orf3B*	
2	10189	10356	55	Host RNA-polymerase inhibitor	35.4	100	
orf16	10353	10718	121	Hypothetical protein		98.3 to *orf4*	
2.5	10772	11473	233	ssDNA-binding protein	50.8	100	
3	11473	11916	147	Endonuclease I	60.0	100	
3.5	11919	12359	146	Lysozyme	52.0	100	
orf20	12429	12968	179	Hypothetical protein		96.2 to *orf5*	
4A	12937	14646	569	DNA primase/helicase	56.0	96.7	
orf22	14665	14868	67	Hypothetical protein		95.5 to *orf6*	
orf23	14932	15441	169	Hypothetical protein		100 to *orf7*	
5	15452	17599	715	DNA polymerase	55.8	98.7	
orf25	17610	18005	131	Hypothetical protein		37.9 to *orf8*	
5.7	17998	18207	69	Hypothetical protein	47.8	100	
6	18204	19148	314	Exonuclease	37.0	100	
6.5	19217	19459	80	Hypothetical protein	42.4	100	
6.7	19456	19734	92	Involved in virion morphogenesis	29.9	100	
7	19731	20174	147	Hypothetical protein	20.5	99.3	
7.3	20146	20448	100	Tail assembly protein	47.3	99.0	
8	20463	22094	543	Head–tail connector protein	68.2	100	
9	22163	23038	291	Capsid assembly protein	45.2	99.7	
10A	23138	24181	347	Major capsid protein	72.0	100	
11	24245	24832	195	Tail tubular protein A	62.2	100	
12	24842	27268	808	Tail tubular protein B	56.1	99.0	
13	27327	27761	144	Internal virion protein A	28.9	98.6	
14	27772	28359	195	Internal virion protein B	33.5	97.9	

(*Continued*)

Table 2. (*Continued*)

ORF[a]	From[b]	To[b]	Size (aa)	Putative function	% Identity to T7	% Identity to gh-1[c]	% Identity to other phages[d]
15	28352	30568	738	Internal virion protein C	32.9	99.9	
16	30581	34759	1392	Internal virion protein D	41.5	99.1	
17	34822	36651	609	Tail fiber protein	34.3	57.3	
orf42	36691	37047	118	Hypothetical protein		52.1 to *orf9*	
17.5	37047	37262	71	Type II holin	37.5	93.0	
18	37259	37516	85	DNA packaging protein, small subunit	48.3	100	
18.5	37516	37965	149	Phage lambda Rz-like lysis protein	32.5	98.0	
19	37965	39713	582	DNA packaging protein B	60.9	100	
19.5	39892	40065	57	Hypothetical protein	29.8	100	

[a]T7 nomenclature was used for assigning gene names in ϕPSA2 when homology was found; the remaining putative ORFs were numbered according to their position starting from the left end of the genome.
[b]Nucleotide positions include initiation and termination codon. All starting codons are ATG except in gene *7* and *8* (GTG).
[c]Phage gh-1 gene names were reported where different from ϕPSA2.
[d]Other phages were considered where non-T7 and non-gh-1 homologs were found. In brackets is the GenBank accession number.

other members of T7-like phages and has probably been lost by gh-1 after the recent separation from the last common ancestor.

Discussion

The phytopathogen *Psa* has caused and is still causing severe economic losses and nowadays is a serious limiting factor for cultivating kiwifruit in many countries. The control strategies adopted, including preventive measures and chemical treatments, have not proved resolutive in the eradication of the disease. Innovative approaches, involving the use inducers of plant systemic acquired resistance, chitin-based products, and silver nanoparticles, are still under evaluation [38]. On the other hand, there is a growing interest in the use of bacteriophages for the prevention and treatment of bacterial infectious diseases mainly in response to the emergence of multidrug-resistant bacteria [39, 40]. Due to the lack of efficient bactericides, phage therapy is a promising approach also for the control of the phytopathogenic bacteria that infect many economically important crops [22, 41]. In the current study, two bacteriophages, selected for their ability to infect and kill *Psa*, were taken into account for their potential use as biocontrol agents.

Bacteriophage ϕPSA1 was isolated from *Psa*-infected leaves of green fleshed kiwifruit. This phage, a member of the *Siphoviridae* family, contains a dsDNA of 51,090 bp. The genomic structure of ϕPSA1 compared with GenBank shows homologies with several *P. syringae* putative prophages but no significant homology with other sequenced siphophages. One-step experiments showed a relatively high burst size of 178 indicating that phage replicates efficiently in *Psa* but at the expense of a long latency period (about 2 h). Moreover, ϕPSA1 is a temperate phage and about 28% of the infected cells do not lyses but become lysogenic bacteria and therefore immune to superinfection. These properties result in a low capacity to kill the bacterial cells as it is also evident from the determination of the "TOD" of the infected bacterial population, whose optical density never dropped below its initial value. Finally, ϕPSA1 has a narrow host range with a limited ability to infect *Psa* (not all *Psa* strains tested were susceptible) and is unable to infect other pseudomonads. On the whole, these features represent a serious limitation for potential use of ϕPSA1 in phage therapy because temperate phages should be avoided for the problems related to lysogeny [42–44] and a phage with narrow host range may not infect all (or the majority of) pathogenic strains involved in the disease. ϕPSA1, however, may provide a useful tool for *Psa* strain typing because it seems to infect preferentially the strains responsible for the recent outbreaks, pointing out significant genetic differences between the current bacterial population and the one that caused outbreaks in Japan, South Korea, and Italy 25–30 years ago [6]. Two strains, ICMP18802 and ICMP18883, isolated in New Zealand in 2011, were resistant to ϕPSA1 unlike other recently isolated *Psa* strains: these strains are less virulent and genetically different from the population of the

current epidemics in Italy and New Zealand [7]. No information is available for the strain ANSES37.26, isolated in France in 2011 and resistant to ϕPSA1. In conclusion, the phage appears specific for the new population of highly virulent strains, of which *Psa* CRA-FRU 8.43 is the type-strain, isolated at the beginning of the recent epidemic on *A. chinensis* cv. Hort16A in Italy, and now spreading in southern Europe, New Zealand, Chile, and South Korea. The properties at the base of the different sensitivity of *Psa* populations to ϕPSA1 are yet to be investigated.

Bacteriophage ϕPSA2, isolated from raw sewages, is a member of the "T7-like viruses" and highly homologous to the *P. putida* phage gh-1; it has a dsDNA genome of 40,472 bp and replicates faster than ϕPSA1 due to its short latency period (about 15 min). Differently from ϕPSA1, ϕPSA2 is a strictly lytic phage, efficiently lysing the host strain *Psa* 8.43 with a TOD of 155 min. ϕPSA2 infected cultures at MOI of 0.01 were still clear after 24 h of incubation, suggesting a low occurrence of resistant bacteria. The lytic nature of phage is an essential prerequisite for successful phage biocontrol and ϕPSA2 satisfies this condition. Another favorable aspect for a candidate to phage therapy is a broad host range in order to control the majority of the pathogenic strains responsible for a given disease. With regard to this aspect, ϕPSA2 was able to infect all *Psa* strains tested. Equally sensitive were also *P. avellanae* (three strains tested), and *P. s*. pv. *theae* (one strain tested) which, together with *Psa*, belong to the genomospecies 8 [45]. Closely related to genomospecies 8 is genomospecies 3 [46] and sensitive to ϕPSA2 were also *P. s*. pv. *morsprunorum* (two strains tested) and *P. s*. pv *tomato* (one out of two strains tested) of this genomospecies. Sensitivity to the phage was observed, but to a lesser extent, for *P. savastanoi* pv. *fraxinii* of genomospecies 2. From these results it is evident that ϕPSA2 has a broad host range with strong lytic potential against *Psa* and other pathovars. It can be concluded that ϕPSA2 may be considered as a candidate for phage therapy and the current characterization may provide a starting point for further exploration of its potential in enhancing biological control of bacterial canker of kiwifruit.

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Conflict of interest

The authors declare that they have no competing interests.

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