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Biocontrol of *Ralstonia solanacearum* by Treatment with Lytic Bacteriophages[▽]†

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Ralstonia solanacearum **is a Gram-negative bacterium and the causative agent of bacterial wilt in many important crops. We treated *R. solanacearum* with three lytic phages: ϕRSA1, ϕRSB1, and ϕRSL1. Infection with ϕRSA1 and ϕRSB1, either alone or in combination with the other phages, resulted in a rapid decrease in the host bacterial cell density. Cells that were resistant to infection by these phages became evident approximately 30 h after phage addition to the culture. On the other hand, cells infected solely with ϕRSL1 in a batch culture were maintained at a lower cell density (1/3 of control) over a long period. Pretreatment of tomato seedlings with ϕRSL1 drastically limited penetration, growth, and movement of root-inoculated bacterial cells. All ϕRSL1-treated tomato plants showed no symptoms of wilting during the experimental period, whereas all untreated plants had wilted by 18 days postinfection. ϕRSL1 was shown to be relatively stable in soil, especially at higher temperatures (37 to 50°C). Active ϕRSL1 particles were recovered from the roots of treated plants and from soil 4 months postinfection. Based on these observations, we propose an alternative biocontrol method using a unique phage, such as ϕRSL1, instead of a phage cocktail with highly virulent phages. Using this method, ϕRSL1 killed some but not all bacterial cells. The coexistence of bacterial cells and the phage resulted in effective prevention of wilting.**

Bacterial wilt is an important crop disease, caused by the soilborne Gram-negative bacterium *Ralstonia solanacearum*. This bacterium has an unusually wide host range, infecting more than 200 species belonging to more than 50 botanical families, including economically important crops (9, 10). *R. solanacearum* strains represent a heterogeneous group subdivided into five races based on host range, five biovars based on physiological and biochemical characteristics (8), and four phylotypes roughly corresponding to geographic origins. Phylotype I includes strains originating primarily from Asia, phylotype II from America, phylotype III from Africa and surrounding islands in the Indian Ocean, and phylotype IV from Indonesia (4). In the field, *R. solanacearum* is easily disseminated via soil, contaminated irrigation water, surface water, farm equipment, and infected biological material (14). Bacterial cells can survive for many years in association with alternate hosts, and soil fumigation with methyl bromide, vapam, or chloropicrin is of limited efficacy. Because methyl bromide depletes the stratospheric ozone layer, the production and use of this gas was phased out in 2005 under the Montreal Protocol and the Clean Air Act. Due to the limited effectiveness of the current integrated management strategies, bacterial wilt continues to be an economically serious problem for field-grown crops in many tropical, subtropical, and warmer areas of the world (9, 10).

Like other methods of biological control, one advantage of phage therapy (also called phage biocontrol) is the reduction in the use of chemical agents against pathogens. This avoids problems associated with environmental pollution, ecosystem disruption and residual chemicals on crops. Phage therapy in agricultural settings was extensively explored 40 to 50 years ago as a means of controlling plant pathogens (3, 18). Two major problems arose in those trials: (i) extracellular polysaccharides produced by pathogenic bacteria prevented phage adsorption, and (ii) there were various degrees of susceptibility among bacterial strains (8). Nevertheless, over recent decades, the use of phage therapy to control the growth of plant-based bacterial pathogens has been explored with increased enthusiasm. To control *R. solanacearum*, two bacteriophages have already been isolated, and their physical and physiological properties have been characterized: phage P4282 (19, 21) and phage PK101 (22). Both of these phages demonstrate very narrow host ranges and infect only a few strains of *R. solanacearum*. Phage P4282, which infects *R. solanacearum* strain M4S, was used to control bacterial wilt in tobacco plants under laboratory conditions, and possible phage-mediated protection was observed (21). However, for the practical use of phages as biocontrol agents against bacterial wilt, it is believed that multiple phages with wide host ranges and strong lytic activity are required (22). Recently, Yamada et al. (23) isolated and characterized several different kinds of phage that specifically infected *R. solanacearum* strains belonging to different races and/or biovars. Phage ϕRSA1 is a P2-like head-tail virus (*Myoviridae*) with a very wide host range; all 15 strains tested from race 1, 3, or 4 and biovar 1, N2, 3, or 4 were susceptible to this phage (6). Phage ϕRSL1 is another myovirus containing ~231 kb in its genome; this phage was able to lyse 10 of 15 tested

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TABLE 1. Bacterial strains used in this study

R. solanacearum strain	Description			Source[a]
	Race	Biovar	Phylotype	
M4S	1	3	I	LTRC
Ps29	1	3	I	LTRC
MAFF 106611	1	4	I	NIAS
MAFF 730138	1	3	I	NIAS

[a] LTRC, Leaf Tobacco Research Center, Japan Tobacco, Inc. (23); NIAS, National Institute of Agrobiological Sciences, Japan (23).

strains (24). The most recently isolated phage, ϕRSB1, displayed a T7-like morphology (*Podoviridae*) and also had the widest host range, with 14 of 15 strains from race 1, 3, or 4 being susceptible (17). ϕRSB1 lyses host cells and forms very large clear plaques that are 10 to 15 mm in diameter on assay plates.

The three phages—ϕRSA1, ϕRSB1, and ϕRSL1—appear to be useful in the eradication of the bacterial wilt pathogen. To increase the antibacterial efficacy of these phages in biocontrol, a thorough understanding of phage ecology and complex phage-host interactions in various environments is necessary. Genomic information on these phages and their host bacteria will be useful for understanding the phage characteristics and the history and molecular mechanisms involved in the phage-bacterium interactions.

MATERIALS AND METHODS

Bacterial strains and phages. Strains of *R. solanacearum* were obtained from the culture collections as listed in Table 1 . The avirulent strain M4S was used for routine purposes (21). For plant inoculation, a few virulent strains, such as Ps29 and MAFF 730138, were used. Strain MAFF 106611 was used mainly for *in planta* detection of bacterial cells. The bacterial cells were cultured in CPG medium containing 0.1% Casamino Acids, 1% peptone, and 0.5% glucose (11) at 28°C with shaking at 200 to 300 rpm. Bacteriophages ϕRSA1, ϕRSB1, and ϕRSL1 (Table 2) were routinely propagated using strain M4S as the host. Bacterial cells in the stationary phase (16 to 24 h postinoculation) grown in CPG medium were diluted 100-fold with 100 ml of fresh CPG medium in a 500-ml flask. To collect sufficient phage particles, a total of 1 liter of bacterial culture was grown. When the optical density at 600 nm (OD_{600}) of the cultures reached 0.1 to 0.3, the phage was added at a dose of 0.5 PFU/host cell (0.5×10^8 PFU/ml) for ϕRSA1 and ϕRSB1 or 5.0 PFU/cell (5×10^8 PFU/ml) for ϕRSL1. After further growth for 6 to 24 h, the cells were removed by centrifugation in a Hitachi Himac CR21E centrifuge equipped with a R12A2 rotor (Hitachi Koki Co., Ltd., Tokyo, Japan) at 8,000 × *g* for 20 min at 4°C. To increase ϕRSA1 recovery, EGTA (final concentration, 1 mM) was added to the ϕRSA1-infected culture at 6 to 9 h postinfection (p.i.). The supernatant was passed through a 0.45-μm-pore-size membrane filter, and phage particles were precipitated by centrifugation in a Hitachi CP100β centrifuge with a P28S rotor at 40,000 × *g* for 1 h at 4°C and then suspended in SM buffer (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM $MgSO_4$, 0.01% gelatin). Purified phages were stored at 4°C until needed.

DNA manipulations. Standard molecular biological techniques for DNA isolation, digestion with restriction enzymes and other nucleases, and construction of recombinant DNAs were as described by Sambrook and Russell (20). Phage DNA was isolated from the purified phage particles by phenol extraction (2, 23).

***In planta* detection of *R. solanacearum* cells.** Seeds of the tomato (*Lycopersicon esculentum*) cultivar Oogata-Fukuju were obtained from Takii Co., Ltd. (Kyoto, Japan). For aseptic cultures, seeds were surface sterilized with sodium hypochlorite and cultured in a square dish (sterile square Schale no. 2; Eiken Chemical Co., Ltd., Tokyo, Japan) containing solid medium (0.15% Hyponex powder; Hyponex Japan Corp., Ltd., Osaka, Japan), 0.5% sucrose, and 1.5% agar adjusted to pH 5.8. Plants were grown in an incubator (Sanyo growth cabinet; Sanyo, Osaka, Japan) at 28°C under a 16-h light (300 μmol photons/s/m^2) and 8-h dark cycle. During the culture period, the dishes in the chamber were tilted to a 45° angle to encourage roots to grow on the surface of the medium. This made it easy to access the roots and monitor the inoculated bacterial cells under a stereomicroscope. To inoculate plants, bacterial cells (i.e., strain MAFF 106611 bearing pRSS12, a green fluorescent protein [GFP]-expressing plasmid with no effects on host virulence [5, 16]) were cultured in CPG medium and suspended in sterile distilled water at a density of 10^7 to 10^8 cells/ml. Tomato seedlings grown in culture dishes were cut at the tip of the taproot, 10 mm from the apex, with a razor blade and then pretreated with phages; 1.0 μl of phage preparation (10^9 PFU/ml) was added to the cut. For a mock control, 1.0 μl of distilled water was added. After 12 to 24 h from the phage treatment, 1.0 μl of bacterial suspension (10^7 to 10^8 cells/ml) was applied to the section. After inoculation, the plants in the dishes were cultured in the incubator until observation. Bacterial cells in the plants were observed by using an MZ16F fluorescence stereomicroscope (Leica Microsystems, Heidelberg, Germany) equipped with GFP2 and GFP3 filters and/or an Olympus BH2 fluorescence microscope (Olympus, Tokyo, Japan). Microscopic images were recorded with a charge-coupled device camera (VB-6010; Keyence, Osaka, Japan). For prolonged observation of plants, bacterium-treated plants were transferred from culture dishes to pots containing a mixture of peat moss and expanded vermiculite. Plants were grown under natural conditions.

TABLE 2. Bacteriophages used in this study

Bacteriophage	Family	Type	Genome size (bp)	Reference
ϕRSA1	*Myoviridae*	P2-like	38,760	6
ϕRSB1	*Podoviridae*	T7-like	43,077	17
ϕRSL1	*Myoviridae*	Jumbo phage	231,255	24

Detection of ϕRSL1 remaining in plants and soil. Two surviving plant cultures, 4 months after treatment, were subjected to assays to detect any remaining bacteriophages. Two parts of the stem (2.5 g at 10 cm above the soil surface and 0.7 to 0.8 g at 1 to 5 cm above the soil surface) were excised from the plant. For the root, 2 to 3 g (12 to 13 cm) was removed after washing the sample in running tap water. After the addition of 2 ml of CPG medium, the plant material was ground using a mortar and pestle at room temperature. After centrifugation at 4,200 × *g* for 5 min at 4°C, the supernatant was filtered through a 0.45-μm-pore-size membrane filter (Steradisc; Krabo Co., Okayama, Japan). The filtrate (100-μl aliquot) was subjected to plaque assays with strain Ps29 as the host on CPG plates containing 0.45% agar. A 2-g mass of pot soil was suspended in 5 ml of SM buffer, and then, after mixing and filtration through a membrane filter as described above, a 100-μl aliquot of the suspension was subjected to plaque assays.

Treatment of tomato plants in soil with phages and inoculation with *R. solanacearum* cells. Tomato seeds (cv. Oogata-Fukuju) were planted in Jiffy 7 peat pellets (42 mm in diameter; Sakata Seed Co., Ltd., Kanagawa, Japan), which were soaked with a phage solution (1.3×10^{10} PFU/pot). For controls, pellets were soaked with tap water. After cultivation for 1 month, the plants (20 to 23 cm in height) were treated again with the phage solution (1.3×10^{10} PFU/pot); the pellet was soaked with the phage solution. Two days later, the plants were cut at the root tips with scissors and dipped in a bacterial suspension containing strain MAFF 106611 cells at 10^8 cells/ml for 30 s. The bacterium-treated plants were transferred to pots (9 cm in diameter) containing peat moss and expanded vermiculite and grown in an incubator (Sanyo) at 28°C under a 16-h light and 8-h dark cycle. Symptoms of wilting were graded from 0 to 5 as follows: 0, no symptoms; 1, only one petiole is wilting; 2, two to three petioles were wilting; 3, all but two to three petioles were wilting; 4, all petioles were wilting; and 5, the plant died.

Effect of temperature on the stability of ϕRSL1. Phage preparations of ϕRSA1 (10^9 PFU/ml), ϕRSB1 (10^9 PFU/ml), and ϕRSL1 (10^9 PFU/ml) in SM buffer (0.5 ml) were incubated in sealed tubes at 4, 11, 28, 37, and 50°C for various periods before the plaque assay with strain Ps29 as the host. A 10-ml volume of each phage solution was added to 100 g of autoclaved field soil. The phage-soil mixture was divided into five equal amounts and separately filled into 15-ml tubes. After sealing, the tubes were incubated at 4, 11, 28, 37, and 50°C for various periods before the plaque assay was carried out. These experiments were performed twice.

RESULTS

Treatment of *R. solanacearum* cells with three different phages. *R. solanacearum* M4S cells were treated with three

lytic phages—ϕRSA1, ϕRSB1, and ϕRSL1—alone or in combination. The optimal phage doses per bacterial cell to give the highest phage yield were determined to be 0.5, 0.5, and 5.0 for ϕRSA1, ϕRSB1, and ϕRSL1, respectively. Figure 1 shows the effect of phage infection on bacterial growth. ϕRSA1 and ϕRSB1 infection, irrespective of whether they were used solely or mixed with other phages, were able to readily lyse growing cultures. However, at approximately 30 h p.i., resistant cells started to grow and reached the stationary state at 70 h p.i. The results were the same at different phage doses (PFU/bacterial cell) for each of the phages. Recovering cells demonstrated a general resistance to both ϕRSA1 and ϕRSB1, irrespective of the initial phage species they were infected with (Table 3). The growth recovery pattern at 30 h p.i. was not affected by the addition of other phage species at 40 h p.i. (data not shown). In contrast, cells infected solely with ϕRSL1 grew slowly until 40 to 50 h p.i., instead of quickly lysing, and were maintained at a low and steady level until 60 h p.i. (Fig. 1). This steady-state growth pattern may indicate an equilibrium between cell growth and lysis by the phage or resistant bacterial cells that are less robust in their growth (1). Infection of ϕRSL1 in combination with ϕRSA1 and/or ϕRSB1, however, resulted in cell lysis and recovering growth patterns similar to that seen in bacterial cells infected solely with ϕRSA1, ϕRSB1, or a mixture. Our previous results have shown that these three phages are virulent and form clear plaques on culture plates (23). Both ϕRSA1 and ϕRSB1 lyse the host cells at 2 to 3 h p.i. (6, 17), whereas ϕRSL1 takes longer for lysis to occur (3 to 4 h) (24). Therefore, one possible explanation for the observation in the mixed infection with ϕRSL1 is that ϕRSA1 and/or ϕRSB1 predominantly infected and lysed the host cells, and over time the titers of these phages were greatly increased compared to that of ϕRSL1, resulting in the lysis-recovery pattern of cell growth (Fig. 1). We confirmed this through plaque assays with the culture fluid after 100 h p.i. In the case of the ϕRSL1 and ϕRSA1 mixed infection, 74% of plaques ($\sim 10^8$) formed on the plates were due to ϕRSA1, easily distinguishable by plaque morphology. For the culture coinfected with ϕRSL1 and ϕRSB1, 92% of plaques (2×10^8) were due to ϕRSB1. ϕRSB1 was also predominant (>90% of 9×10^8 plaques) when combined with ϕRSA1. Phage-resistant cells from cultures infected with ϕRSA1 and ϕRSB1 were susceptible to ϕRSL1, as shown in Table 3. Therefore, ϕRSL1 presumably has a different host recognition way from the other phages. These results obtained with strain M4S as a host were reproducible with other *R. solanacearum* strains such as Ps29 and MAFF 730138.

From these results, we concluded that for control *R. solanacearum* cells, a cocktail of ϕRSA1, ϕRSB1, and ϕRSL1 may not be suitable due to the presence of phage-resistant cells. Instead, ϕRSL1 infection somehow maintains the cell population at lower levels and limits the growth of resistant cells recovering from ϕRSA1 and/or ϕRSB1 infection. Therefore, we attempted to use ϕRSL1 as an agent to control the growth of *R. solanacearum* cells and bacterial wilt disease.

***In planta* inhibition of *R. solanacearum* growth and movement by treatment with ϕRSL1.** The effect of ϕRSL1 infection to stably limit the growth of *R. solanacearum* cells *in vitro* leads us to examine bacterial growth and movement *in planta* after treatment with ϕRSL1. We monitored real-time bacterial dynamics in inoculated tomato plants grown on solid agar medium using a previously described method (5, 16). Seven-day-old seedlings of the tomato cultivar Oogata-Fukuju were first treated with ϕRSL1 (1.0 μl containing 10^6 PFU) at a cut made in the tip of the taproot, 10 mm from the apex. At various times after treatment, bacterial cells were inoculated at the cut and observed (Fig. 2A). Every experiment was performed in triplicate with 10 individual plants. Figure 2B shows the time course of bacterial growth and movement in a tomato taproot without ϕRSL1 treatment (control). After bacterial inoculation, GFP fluorescence intensity increased with time, and GFP-labeled bacterial cells moved upward through xylem vessels until 48 h p.i. At 72 h p.i., GFP fluorescence was apparent outside the taproot, suggesting cell movement, and growth occurred outside the taproot. Slimy colonies of cells covered the entire taproot by 96 h p.i. At this stage, the hypocotyls and young leaves were wilting (Fig. 2B).

Remarkably different phenomena were observed when tomato plants were treated with ϕRSL1 preceding bacterial inoculation. As shown in Fig. 2C, up until 96 h p.i. no bacterial growth and movement into plant bodies were apparent; faint GFP fluorescence was retained at the inoculation point. During this period, lateral shoots frequently formed as in healthy plants; this was never observed in control plants that were bacterially challenged but not phage treated. At 120 h p.i., GFP fluorescence intensity increased slightly, and GFP-labeled cells were observed to be moving upward along the taproot. However, the growth and movement was quite limited; at 192 h p.i., the GFP fluorescence pattern remained unchanged. The GFP fluorescence was unclear again at 216 h p.i. The cotyledons, leaves, and the meristem looked healthy with no symptoms of wilting (Fig. 2C). When tomato seedlings were treated in the same way with ϕRSA1 or ϕRSB1, bacterial penetration and growth in xylem vessels were obvious at 48 h p.i. (data not shown). These results indicated that bacterial growth and movement were drastically limited in tomato plants by pretreatment with ϕRSL1. Thus, pretreatment of tomato seedlings with ϕRSL1 may limit infection by *R. solanacearum* or prevent bacterial wilt disease.

Prolonged cultivation in soil of tomato plants treated with ϕRSL1 after inoculation with *R. solanacearum*. Six tomato plants were treated with ϕRSL1 after inoculation with *R. solanacearum* (Fig. 2), transferred from culture dishes to pots, and then cultivated in a natural environment for prolonged observation. At 49 days posttransfer (1.5 months p.i.), four plants still grew well without any symptoms of wilting. Two plants died soon after transfer (2 to 3 days); we considered that this was caused by physical/physiological damage such as water shock or root damage and not by bacterial infection because few bacterial cells were detected in plant organs.

Persistence and stability of ϕRSL1 particles in plants and soil. The stems, leaves, and roots of plants and culture soil from two pot cultures described above were subjected to phage titer assays 4 months later. One plant, 52 cm in height, was treated with ϕRSL1 only (control plant 1), and the other plant, 62 cm in height, was treated with ϕRSL1 followed by the pathogen (plant 2), and harvested 4 months p.i. The data shown in Table 4 indicated that no ϕRSL1 phage was detected from the stems or leaves of either plant. However, a considerably large number of phages were retained in the roots of both

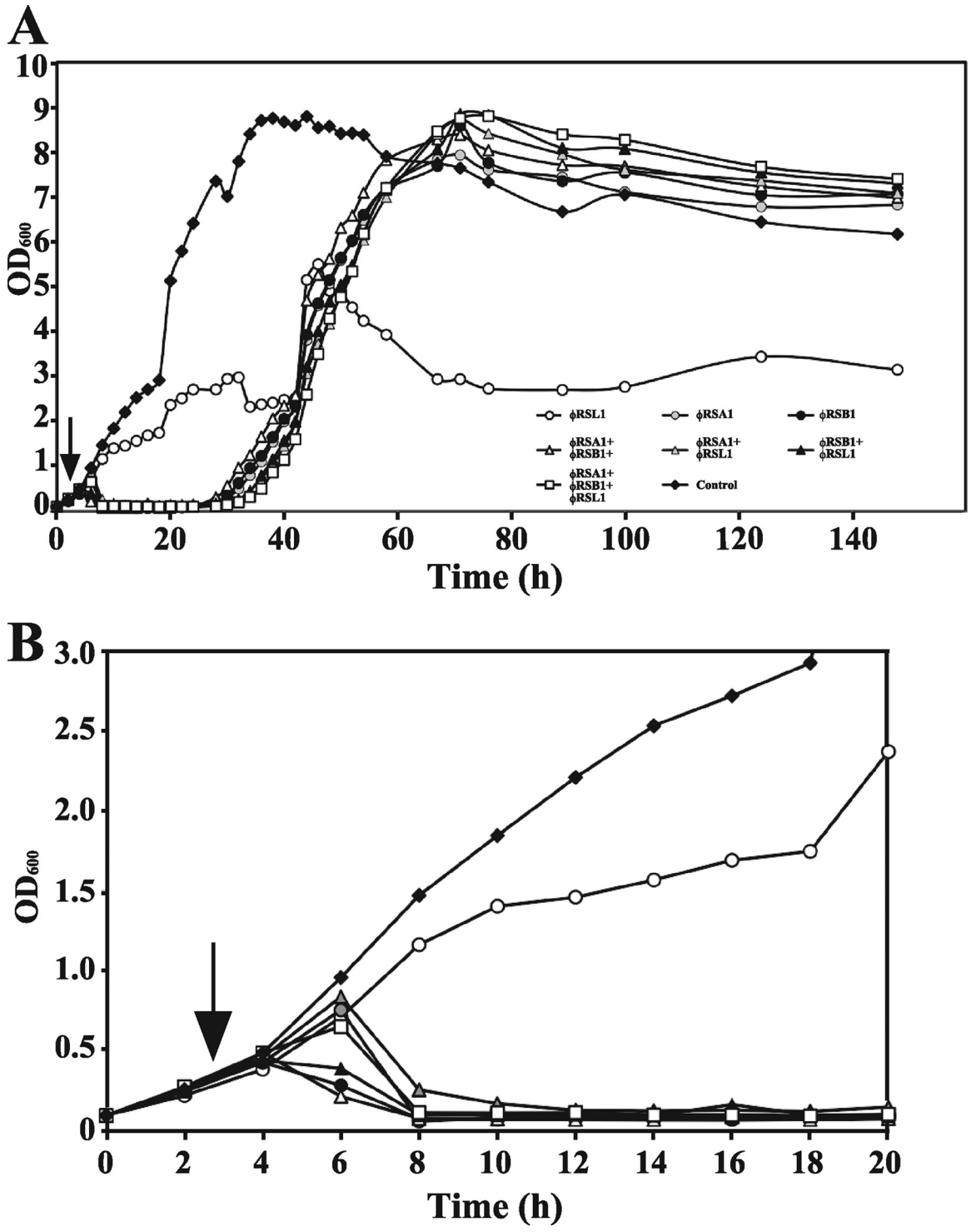

FIG. 1. Time course of bacterial growth after infection with bacteriophages. The first 20 h region is enlarged in panel B (the symbols are the same as shown in panel A). Cells of *R. solanacearum* strain M4S (OD_{600} = 0.3 corresponding to ~10^8 cells/ml; vertical arrow) were infected solely or by mixing with three phages: ϕRSA1, dose = 0.5×10^8 PFU/ml; ϕRSB1, dose = 0.5×10^8 PFU/ml; and ϕRSL1, dose = 5.0×10^8 PFU/ml. At about 30 h p.i., resistant cells started to grow when treated with ϕRSA1 and/or ϕRSB1, either alone or as part of a phage mixture. Cells solely infected with ϕRSL1 were kept at a low cell density. Similar results were obtained with different *R. solanacearum* strains (data not shown).

plants. The plants inoculated with bacterial cells gave titers 10 times higher than those without inoculation. Similar results were obtained in the *in vitro* experiments shown in Fig. 2). The soils from both cultures also yielded phage plaques. Plaques appearing on the assay plates always displayed homologous morphology, resembling ϕRSL1 plaques (23). The genomic DNA isolated from random plaques coincided with ϕRSL1 DNA by restriction digestion patterns (data not shown). These

TABLE 3. Phage resistance of *R. solanacearum* cells in a culture treated with ϕRSA1 or ϕRSB1

Single colony[a]	Phage resistance[b]		
	ϕRSA1	ϕRSB1	ϕRSL1
A^r-1	+	+	−
A^r-2	+	+	−
A^r-3	+	+	−
B^r-1	+	+	−
B^r-2	+	+	−
B^r-3	+	+	−

[a] Data for the single colonies isolated from ϕRSA1- or ϕRSB1-treated cultures at 100 h p.i. shown in Fig. 1 are presented. A^r-1, A^r-2, and A^r-3 and B^r-1, B^r-2, and B^r-3 were obtained from cultures treated with ϕRSA1 and ϕRSB1, respectively.
[b] Phage resistance is indicated as resistant (+) or sensitive (–).

TABLE 4. Persistence and stability of ϕRSL1 particles in plants and soil

Plant or soil[a]	Wt (g)	Mean phage titer[b] (PFU/g) ± SD
Plant 1		
Stems and leaves (10 cm)	2.50	ND
Stems (1 to 5 cm parts above ground)	0.75	ND
Roots (13 cm)	2.50	$1.8 \times 10^3 \pm 2.0 \times 10^2$
Soil (rhizosphere)	2.00	$2.7 \times 10^1 \pm 8.5 \times 10^0$
Plant 2		
Stems and leaves (10 cm)	2.50	ND
Stems (1 to 5 cm parts above ground)	0.80	ND
Roots (13 cm)	3.10	$1.9 \times 10^4 \pm 2.5 \times 10^3$
Soil (rhizosphere)	2.00	$5.5 \times 10^2 \pm 1.5 \times 10^2$
Plant 3		
Roots	0.51	$5.7 \times 10^5 \pm 3.0 \times 10^4$
Soil (rhizosphere)	2.00	$7.1 \times 10^5 \pm 1.8 \times 10^5$

[a] Plant 1, a control plant treated with ϕRSL1 only (*in vitro* treated and transferred to soil); plant 2, a plant treated with ϕRSL1, followed by the pathogen (*in vitro* treated and transferred to soil); plant 3, a ϕRSL1-treated plant with no wilting symptom 18 days after the pathogen challenge (in soil experiments).
[b] Assays were repeated three times. ND, not detected.

results indicated that ϕRSL1 phages were stably retained in plants roots, as well as in soils, with a lower concentration.

Prevention of bacterial wilt by treatment with ϕRSL1. The effect of ϕRSL1 infection to stably limit the growth of *R. solanacearum* cells *in vitro* and *in planta* led us to examine whether ϕRSL1 treatment of tomato plants under soil conditions prevents wilting by inoculated *R. solanacearum* cells. One-month-old tomato plants (20 to 23 cm in height) pretreated with ϕRSL1 were inoculated with *R. solanacearum* cells as described in Materials and Methods. Wilting symptoms were recorded every 2 days. The results shown in Fig. 3 indicated an efficient prevention of wilting by treatment with ϕRSL1. Plants without ϕRSL1 started to show wilting symptoms 4 days p.i. and all 11 plants showed wilting symptoms 18 days p.i. (Fig. 3A and C). In contrast, all 11 ϕRSL1-treated plants showed no symptoms of wilting during the experimental period (Fig. 3B and C). The ϕRSL1 phages were stably retained in plant roots as well as in soils at this stage, as shown in Table 4. Three random tomato cultures treated with ϕRSL1 were subjected to phage titer assay as described above. Because all three cultures showed almost the same values, only one example (plant 3) is included in Table 4. These results suggest the effectiveness of ϕRSL1 in the practical

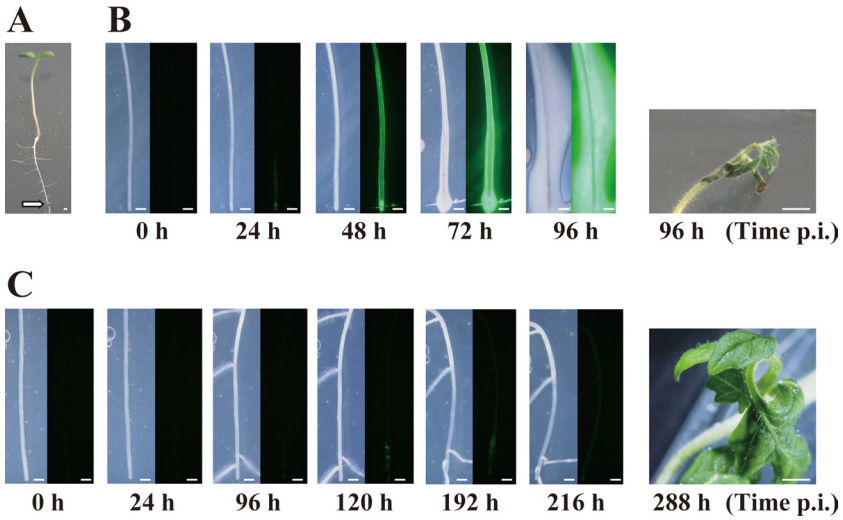

FIG. 2. *In planta* inhibition of *R. solanacearum* growth and movement by treatment with ϕRSL1. (A) Tomato seedlings grown in culture dishes (7 days old) were cut at the tip of the taproot (arrow) and treated with 1 μl of ϕRSL1 (10^6 PFU). After 12 to 24 h, bacterial cells (~10^5 cells) of strain MAFF 106611 harboring pRSS12 were inoculated at the cut. (B) Without phage treatment (control), bacterial penetration into the taproot, successive upward movement, and growth in the tissues was evident at 24 h p.i. (left panel, bright-field image; right panel, dark-field image). GFP fluorescence was apparent outside the taproot at 72 h p.i. The hypocotyl and young leaves were wilted at 96 h p.i. No lateral shoot formation was observed throughout the period. (C) With phage treatment, no bacterial growth and movement in plant bodies was apparent until 96 h p.i. Faint GFP fluorescence was retained at the inoculation point. During this period, lateral shoots frequently formed; even though at 120 h p.i. some GFP-labeled cells were visible, their growth and movement were quite limited. The cotyledon, leaves, and the meristem appeared healthy with no symptoms of wilting at 288 h p.i. The 30 different plants tested displayed similar results. Scale bar, 0.5 mm.

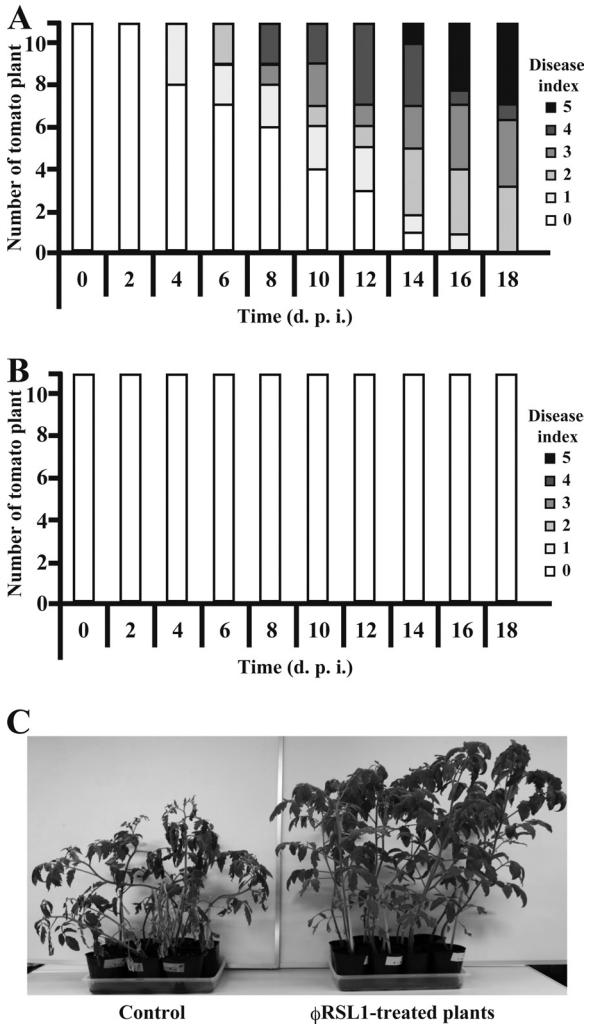

FIG. 3. Prevention of bacterial wilt by treatment with ϕRSL1. One-month-old tomato plants (20 to 23 cm in height) pretreated with tap water (A, control) or ϕRSL1 (B) were inoculated with *R. solanacearum* cells as described in Materials and Methods. Wilting symptoms were graded from 0 to 5 as follows: 0, no symptoms; 1, only one petiole was wilting; 2, two to three petioles were wilting; 3, all but two to three petioles were wilting; 4, all petioles were wilting; and 5, the plant died. (C) Tomato plants observed at 18 days p.i.

application as a biocontrol agent against bacterial wilt. Plants can be treated with ϕRSL1 during the early stages of growth. When tomato plants were pretreated with a phage mixture containing two phages (ϕRSA1 and ϕRSB1) or three phages (ϕRSA1, ϕRSB1, and ϕRSL1) and challenged by *R. solanacearum* cells in the same way, as described above, wilting symptoms appeared by 4 days p.i., and all of them wilted after 14 days p.i. (see Fig. S1 in the supplemental material). The wilting patterns were generally similar to those observed in control plants without phage treatment. These results were consistent with the growth of resistant cells observed in the *in vitro* and *in planta* experiments in which a phage mixture was used.

Effects of temperature on the stability of ϕRSL1 in soil. We further studied the effects of temperature on the stability of ϕRSL1 compared to ϕRSA1 and ϕRSB1. Phage preparations were kept at different temperatures in the range 4 to 50°C, in the presence or absence of soil. As shown in Fig. 4A, in the absence of soil, all three phages were essentially stable below 28°C, whereas ϕRSL1 exhibited greater stability compared to other phages at higher temperatures (37 and 50°C). After a 15-day incubation, ca. 10% ϕRSL1 survived at 50°C. No phages were detected after a 3-day incubation with ϕRSA1 and after a 9-day incubation with ϕRSB1. Similar stability patterns were observed under different soil temperatures. At low temperatures, the titers of some phages, especially ϕRSA1, were decreased probably due to nonspecific adsorption to soil particles. ϕRSL1 also exhibited the highest stability in soil (Fig. 4B).

DISCUSSION

Alternative phage biocontrol using ϕRSL1. For phage biocontrol, only virulent phages are used, thereby avoiding the problem of lysogeny. Phage cocktails are recommended to prevent the problem of resistance. These cocktails ideally contain several phages with different host specificities, replication mechanisms, and/or infection cycles (7, 15). In the present study, when host *R. solanacearum* cells were quickly lysed by treatment with ϕRSA1 or ϕRSB1, resistant cells presumably preexisting in the population at a very low frequency were increased at 30 h p.i. Given that the majority of the cell population contains susceptible cells, the elimination of these cells may allow minor cells to predominate to the next generation. Since such recovering cells were somehow resistant to both ϕRSA1 and ϕRSB1 (Table 3), treatment with a mixture of these phages resulted in the same death and recovery patterns as bacterial cells treated with a single phage (Fig. 1). The resistance mechanisms used by these cells are currently unknown, and the host specificity was different between ϕRSA1 (a P2-like myovirus) and ϕRSB1 (a T7-like podovirus) (23). In our preliminary observations, several phage-resistant mutants, induced by transposon mutagenesis, displayed differences in resistance to ϕRSA1 and ϕRSB1. Therefore, cells recovering from the phage treatment may include resistant cells caused by different mechanisms and with different characteristics. A cocktail containing three phages—ϕRSA1, ϕRSB1, and ϕRSL1—also failed to stably prevent bacterial growth (Fig. 1). Although ϕRSL1 could lyse some recovering cells after ϕRSA1 and/or ϕRSB1 treatment (Table 3), in mixed phage treatments, cells may have been quickly lysed by ϕRSA1 or ϕRSB1, thereby interrupting the replication of ϕRSL1 and reducing the ϕRSL1 titer. ϕRSL1 may not be able to effect the lysis of cultures if its density is too low. Compared to ϕRSA1 and ϕRSB1, RSL1 takes longer for lysis to occur (3 to 4 h) with a latent period of 2.5 h (24). This infection cycle is rather longer than the doubling time of host cells (3 h) under routine culture conditions. Therefore, a higher dose may be required to efficiently infect and lyse the host cells. This was supported by the observation that little ϕRSL1 was detected in the culture treated with three phages at 100 h p.i.

Consequently, the strategy of using a phage cocktail in the biocontrol of *R. solanacearum* cells did not work in the present study. Instead, treatment with ϕRSL1 alone did not rapidly kill cells but kept the cell density at a low level (Fig. 1). *In planta* monitoring of bacterial cells showed that treatment with

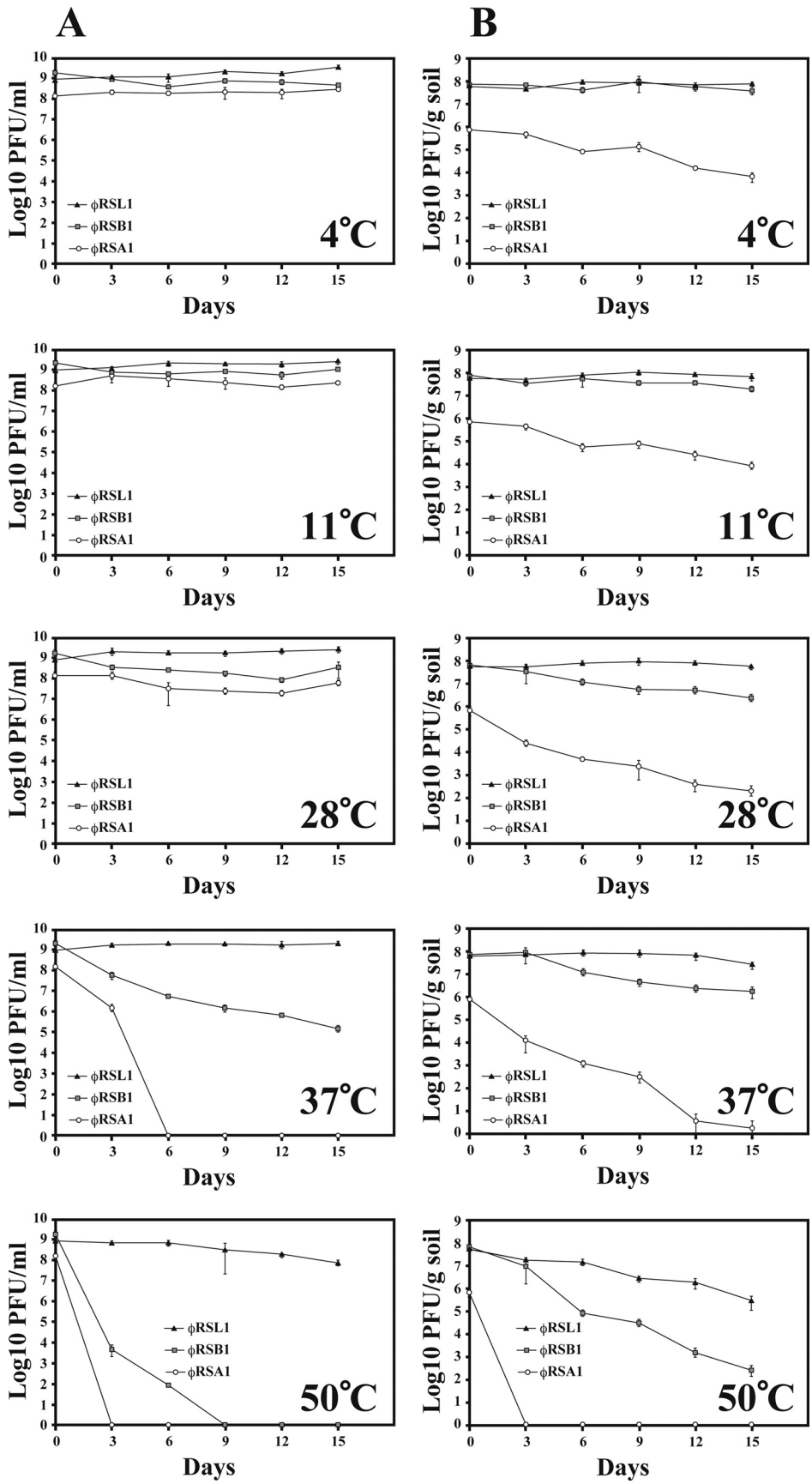

FIG. 4. Effect of temperature on the stability of ϕRSL1 compared to ϕRSA1 and ϕRSB1. Phage preparations were kept at a range of temperatures (4 to 50°C) for various periods without (A) or with (B) soil. ϕRSL1 displayed significant stability at higher temperatures (37 and 50°C). Error bars indicate the standard error ($n = 3$).

ϕRSL1 resulted in a blockage of the growth and movement of bacterial cells in the tomato root. Treated plants survived for as long as 4 months. ϕRSA1 or ϕRSB1 were not able to induce similar plant-protecting effects. Based on these observations, we propose an alternative phage biocontrol method, using a unique phage such as ϕRSL1, instead of a phage cocktail containing highly lytic phages. With this method, bacterial cells are not killed altogether but a sustainable state of phage-bacterium coexistence is maintained, like a carrier state or pseudolysogeny (1). To apply this method practically, variants of ϕRSL1-type phages would be required that have different host ranges covering most strains of *R. solanacearum*.

Coexistence of *R. solanacearum* cells and ϕRSL1. ϕRSL1 is a unique phage with a very large genome of 231 kb containing 343 open reading frames. A lysogenic cycle or episomal replication of ϕRSL1 has not been elucidated; genomic Southern blot analysis of many field-isolated strains has not identified any significant hybridizing signals with ϕRSL1 DNA as a probe (23; data not shown). After infection with ϕRSL1, cell density was maintained stably at low levels, as shown in Fig. 1, suggesting an equilibrium between cell growth and lysis. The establishment of equilibrium may be explained by a long latent period and small burst size of a phage. Our previous study revealed that ϕRSL1 takes longer for lysis to occur (3 to 4 h) with a latent period of 2.5 h and a burst size of 80 to 90 PFU per infected cell (24). Other possible explanations for the cell-phage balance may come from some specific regulation of phage infectivity or a low frequency of resistant cells and the production of growth-inhibitory factors due to phage infection. We are interested in the expression of many unique genes, including those for lysis genes encoded in the ϕRSL1 genomic DNA (24). Further characterization of the expression and function of these genes may reveal the mechanism for establishment of equilibrium. It is also interesting that in some cases, lysogenic filamentous phages were observed to be induced in the remaining host cells after infection with ϕRSL1, which may also contribute to the equilibrium.

Stability of ϕRSL1 in soil and its practical application. Phages are utilized for controlling plant pathogens either in the rhizosphere or phyllosphere. The direct application of phages to the phyllosphere is subject to serious phage stability problems (15). Field and laboratory studies have demonstrated that phages are inactivated rapidly by exposure to sunlight, high temperatures, extremes in pH, oxidative conditions, and flowing water (12, 13). In the case of bacterial wilt, phages for biocontrol can be applied to the rhizosphere. Sunlight, the most destructive environmental factor, and oxidative inactivation are not as relevant in this case. ϕRSL1 was shown to be relatively stable in soil, especially at higher temperatures (Fig. 4). In fact, active ϕRSL1 particles were consistently recovered from the roots of treated plants and soils at 4 months p.i. (Table 4). This ϕRSL1 stability (Fig. 4) in soil appears to be another advantage of ϕRSL1 as a biocontrol agent. Moreover, bulk production at high concentrations of ϕRSL1 particles ($\sim 10^{12}$ PFU/ml) is possible by centrifugation at 15,800 $\times$ *g* for 20 min. Prolonged disease control may be possible if ϕRSL1 is applied to plants at the seedling stage.

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