Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Isolation of *Ralstonia solanacearum*-infecting bacteriophages from tomato fields in Chiang Mai, Thailand, and their experimental use as biocontrol agents

A. Bhunchoth[1,2,3], N. Phironrit[1], C. Leksomboon[4], O. Chatchawankanphanich[1], S. Kotera[5], E. Narulita[5], T. Kawasaki[5], M. Fujie[5] and T. Yamada[5]

1 Plant Research Laboratory, National Center for Genetic Engineering and Biotechnology, NSTDA, Pathum Thani, Thailand
2 Center for Agricultural Biotechnology, Kasetsart University, Nakhon Pathom, Thailand
3 Center of Excellence on Agricultural Biotechnology: (AG-BIO/PERDO-CHE), Bangkok, Thailand
4 Department of Plant Pathology, Kasetsart University, Nakhon Pathom, Thailand
5 Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima, Japan

Keywords
bacterial wilt disease, bacteriophages, biocontrol, host range, *Ralstonia solanacearum*.

Correspondence
Takashi Yamada, Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan.
E-mail: tayamad@hiroshima-u.ac.jp

2014/2362: received 17 November 2014, revised 26 December 2014 and accepted 16 January 2015

doi:10.1111/jam.12763

Abstract

Aims: To isolate and characterize novel bacteriophages infecting the phytopathogen, *Ralstonia solanacearum*, and to evaluate them as resources with potential uses in the biocontrol of bacterial wilt.

Methods and Results: Fourteen phages infecting *R. solanacearum* were isolated from soil samples collected in Chiang Mai, Thailand. The phages showed different host ranges when tested against 59 *R. solanacearum* strains isolated from Thailand and Japan. These phages were characterized as nine podoviruses and five myoviruses based on their morphology. Podovirus J2 in combination with another podovirus (φRSB2) lysed host cells very efficiently in contaminated soil. J2 treatment prevented wilting of tomato plants infected with a highly virulent *R. solanacearum* strain.

Conclusions: Treatment with J2 effectively reduced the amount of the bacterial wilt pathogen in contaminated soil and prevented bacterial wilt of tomato in pot experiments. Myovirus J6 possessed jumbo phage features, giving a unique opportunity to study its utilization as a biocontrol agent.

Significance and Impact of the Study: As exemplified by J2, the phages isolated in this study represent valuable resources with potential uses in biocontrol of bacterial wilt. A rare jumbo phage J6 served as a valuable subject to understand and utilize this new group of phages.

Introduction

Ralstonia solanacearum is a soil-borne Gram-negative bacterium belonging to the Betaproteobacteria. It is the causative agent of bacterial wilt of many economically important crops (Yabuuchi *et al.* 1995; Hayward 2000; Denny 2006). This bacterium has an unusually wide host range, infecting more than 200 plant species in at least 50 botanical families, and there is great phenotypic and genotypic diversity among the different strains (Hayward 1991, 2000). *Ralstonia solanacearum* strains represent a heterogeneous group that can be subdivided into five races based on host range, and into five biovars based on physiological and biochemical characteristics (Hayward 2000). There is no general correlation between races and biovars, and the five races of *R. solanacearum* have different geographical distributions. Recently, a new classification system for *R. solanacearum* strains based on phylogenetic information has been proposed (Fegan and Prior 2005). In this system, the strains are sub-grouped into four phylotypes roughly corresponding to their geographic origin. 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 (Fegan and Prior 2005; Villa *et al.* 2005).

In the field, *R. solanacearum* spreads readily via soil, contaminated irrigation water, surface water, farm equipment and infected material (Janse 1996; Allen *et al.* 2001). Bacterial cells can survive for many years in association with alternate hosts (Prior *et al.* 1998). Once identified as infected, plants in cropping fields, gardens, or greenhouses must be destroyed, and soil and water drainage systems that may be contaminated with the bacterium must be treated with chemical bacteriocides. Fumigation of soil with vapam or chloropicrin is of limited efficacy (Allen *et al.* 2005). At present, protection from losses by bacterial wilt is achieved mainly by early detection and subsequent eradication by destroying the host.

Bacterial wilt caused by *R. solanacearum* is one of the most devastating diseases of many economically important crops in Thailand such as ginger, pepper, tomato, potato and *Curcuma alismatifolia* Gagnep. Most of the *R. solanacearum* strains found in Thailand have been classified into race 1 biovar 3 or 4 (Thammakijjawat *et al.* 1997; Thaveechai *et al.* 1997). However, the strain infecting potato crops in the highlands of northern Thailand has been identified as race 3 biovar 2 (Boonsuebsakul 1994). Because of the variability in *R. solanacearum* strains in Thailand, it is very difficult to control bacterial wilt. Efficient methods are required for early inspection of pathogens in the field to help farmers develop management strategies to control the disease. Certain cultivation practices and chemical control methods have been ineffective because of the wide host range of the pathogen, the high variability among pathogen strains and the persistence of the pathogen in the soil for long periods. There have been some reports on the use of antagonists for biological control to reduce the severity of disease epidemics (Kasigumpaiboon *et al.* 1992; Suwankeereekhan *et al.* 2004; Nguyen and Ranamukhaarachchi 2010; Nguyen *et al.* 2011). Six strains of bacteria – NA1, NA25, NA37, PH9, SU1 and CH4 – were isolated and were proven to reduce the severity of bacterial wilt of tomato (Kasigumpaiboon *et al.* 1992). In addition, when applied as a powder form, the *Pseudomonas fluorescens* strain CM-RO3 effectively controlled wilt disease in *C. alismatifolia* (Suwankeereekhan *et al.* 2004). Four other antagonists – *Bacillus megaterium*, *Candida ethanolica*, *Enterobacter cloaceae* and *Pichia guillermondi* – were tested for their ability to control bacterial wilt of tomato. All of them showed promising results in suppressing the pathogens and reducing disease severity (Nguyen and Ranamukhaarachchi 2010; Nguyen *et al.* 2011).

Recently, various kinds of bacteriophages that infect *R. solanacearum* have been isolated (Yamada *et al.* 2007) and proposed for use in the biocontrol of bacterial wilt. These phages may be useful as tools for effective detection (diagnosis) of the pathogen in cropping ecosystems and in cultivated crops. They also have potential uses in eradicating the pathogen from contaminated soil or preventing outbreaks of bacterial wilt in economically important crops. Similarly, phages infecting *R. solanacearum* have been isolated from the natural environment (Murugaiyan *et al.* 2010; Tan *et al.* 2010; Makari *et al.* 2013). Like other methods of biological control, an advantage of phage biocontrol is the reduction in the use of chemical control agents (Jones *et al.* 2008). This prevents the problems of environmental pollution, ecosystem disruption and residual chemicals on crops. However, for the practical use of phages as biocontrol agents against bacterial wilt, multiple phages with wide host ranges and strong lytic activity are required (Yamada 2012).

In the natural environment, various kinds of bacteriophages interact with bacterial hosts (Ashelford *et al.* 2003). Phages can play a key role in shaping bacterial population dynamics and can significantly alter both intra- and inter-specific competition among bacterial hosts (Abedon 2009). Phages affect microbial communities not only by lysing host cells but also by transferring genetic material and affecting lysogenic conversion. To investigate the potential effects of phages on the biological, physiological and pathological variants of *R. solanacearum*, we need information about phage ecology in crop fields. In this study, we conducted a large-scale survey of the distribution of phages in tomato fields in Chiang Mai, Thailand, and examined their use as biocontrol agents against bacterial wilt disease.

Materials and methods

Bacterial strains and culture conditions

The strains of *R. solanacearum* (37 from Thailand and 22 from Japan) used in this study, along with their hosts and taxonomic features, are listed in Table S1. The bacterial cells were cultured in CPG medium containing 0·1% (w/v) casamino acids, 1·0% (w/v) peptone and 0·5% (w/v) glucose (Horita and Tsuchiya 2002) at 28°C with shaking at 200–300 rev min^{-1}. For long-term storage, bacterial cultures were kept in sterile 20% (v/v) glycerol at −80°C.

Soil samples and plaque assay

Soil samples for phage isolation were collected from tomato crop fields in Chiang Mai, Thailand. In these areas, rice is the main crop, grown primarily in the rainy season (from May to October) and tomatoes are grown in the dry and cool season (from October to February) after the rice is harvested. Irrigation canal networks are

used to provide water in the dry season to sustain tomatoes. Sampling was performed in February, 2013 (the dry season). For each sample, 1 g soil was suspended in 2 ml distilled water and vigorously shaken for 1 h at room temperature to release bacteriophages. The mixture was filtered through a membrane filter (0·45-μm pore size; Steradisc, Kurabo Co. Ltd., Osaka, Japan), and then 100-μl aliquots of the suspension were used for a plaque-forming assay, with strains of *R. solanacearum* as the host, on CPG plates containing 1·5% agar overlaid with 0·75% CPG soft agar. In some cases, the soft agar concentration was reduced to 0·45% to isolate jumbo phages. The plates were incubated at 28°C for 1 or 2 days before plaque detection. The shape, size and number of plaques were recorded.

Purification and characterization of phages

Phages were propagated and purified from single-plaque isolates. Routinely, each phage was propagated using the strain in which it was originally detected as the host. An overnight culture of bacterial cells grown in CPG medium was diluted 100-fold with 100 ml fresh CPG medium in a 500-ml flask. When the cultures reached an OD_{600} of 0·5, a bacteriophage was added at a multiplicity of infection (MOI) of 0·001–1·0 (usually 0·01–0·1). After culturing for a further 12–24 h, the cells were removed by centrifugation at 8000 ***g*** for 15 min at 4°C in a R12A2 rotor in a Hitachi himac CR21E centrifuge. The supernatant was passed through a 0·45-μm membrane filter, and then the pellet was dissolved in SM buffer (50 mmol l^{-1} Tris-HCl at pH 7·5, 100 mmol l^{-1} NaCl, 10 mmol l^{-1} $MgSO_4$ and 0·01% gelatin). For further purification, the phage suspension was mixed with CsCl (9·4 g/20 ml) and subjected to ultracentrifugation in a P28S rotor in a Hitachi CP100β ultracentrifuge at 145 000 ***g*** for 18 h. In some cases, a sucrose gradient (linear 20%–60% in 10 mmol l^{-1} Tris-HCl containing 10 mmol l^{-1} $MgCl_2$, pH 7·4) instead of CsCl was used for ultracentrifugation to retain phage stability. The purified phages were stored at 4°C until use. The purified bacteriophage particles were stained with Na-phosphotungstate or uranyl acetate before observation with an JEOL JEM-1400 electron microscope according to Dykstra (1993). We used λ phage particles as an internal standard marker for size determination.

Optimal multiplicity of infection (MOI)

We determined the optimal MOI (the optimal ratio between phage particles and host bacterial cells to give the highest phage titer, Gasic *et al.* 2011) for phage J2, which had the widest host range of all the tested phages. For infection experiments, strains MAFF106603 and MAFF730138 were used because these strains were stable and seldom produced induced prophages. Bacterial cultures were infected with the phage at different MOI (0·01, 0·1, 1·0 and 5·0). After overnight incubation at 28°C with shaking at 200–300 rev min^{-1}, bacterial cultures were centrifuged (8000 ***g*** for 15 min at 4°C) and the supernatants were assayed to determine the phage titer. For time-course experiments on bacterial growth after infection with phages, the phages were added (MOI = 1·0, PFU/CFU) to cultures of strain MAFF106603 or MAFF730138 in CPG at the early exponential cell growth stage (OD_{600} = 0·3), and the cultures were grown at 28°C. We used φRSB2 (a T7-like podovirus, DDBJ accession no. AB597179; Yamada 2012) as the phage infection control.

Single-step growth experiment

Single-step growth experiments were performed as described previously (Yamada *et al.* 2010), with some modifications. Strains MAFF106603 and MAFF730138 were used as the host for the phage J2. Bacterial cells (0·1 U of OD_{600}) were harvested by centrifugation and resuspended in fresh CPG (approx. 1×10^8 CFU ml^{-1}) to a final culture volume of 10 ml. The phage was added at a MOI of 0·1 and allowed to adsorb for 10 min at 28°C. After centrifugation and resuspending in the initial volume of CPG with decimal dilution to a final volume of 10 ml, the cells were incubated at 28°C. Samples were taken at intervals (every 10 min up to 3·5 h) and the titers were determined by the double-layered agar plate method. For single-step growth experiments for phage J6, experimental procedures were essentially the same as those used for J2, but samples were taken at 30 min intervals.

Isolation and characterization of nucleic acids from phage particles

Standard molecular biology techniques for DNA isolation, digestion with restriction enzymes, construction of recombinant DNAs, and DNA sequencing were performed according to Sambrook and Russell (2001). Genomic DNA was isolated from the purified phage particles by phenol extraction. To determine the size of the genome (in the case of jumbo phages), the purified phage particles were embedded in 0·5% low-melting-point agarose (InCert agarose; FMC Corp., Philadelphia, PA). Then, after treatment with proteinase K (1 mg ml^{-1}; Merck Ltd., Tokyo, Japan) and 1% (w/v) Sarkosyl, the nucleic acids were subjected to pulsed-field gel electrophoresis with a CHEF MAPPER electrophoresis apparatus

(Bio-Rad, Hercules, CA) as described by Higashiyama and Yamada (1991). Shotgun sequencing of phage genomic DNA was performed at Hokkaido System Science Co., Ltd. (Sapporo, Japan) using a Roche GS Junior Sequence System. The draft assembly of the obtained sequences was assembled using GS De novo Assembler v2.6. The analyzed sequences corresponded to 174, and 236 times the final contig sizes of J2 (44 360 bp), and J6 (223 932 bp), respectively. Potential open reading frames (ORFs) larger than 150 bp (50 codons) were identified using Glimmer (Delcher *et al.* 1999) and GeneMark. Homology searches were performed using BLAST/RPS-BLAST (Altschul *et al.* 1997) against the UniProt sequence database (UniProt Consortium 2007) and the NCBI/CDD database (Wheeler *et al.* 2007), using an E-value lower than e^{-4} as a cutoff for notable similarity.

Decontamination of pathogen-contaminated soil with lytic bacteriophages

To assess the effects of the phages on pathogen-contaminated soils, a natural field soil (collected from the botanical garden at the Graduate School of Science, Hiroshima University) supplemented with *R. solanacearum* ($0{\cdot}5 \times 10^7$ CFU g^{-1} soil) was treated with J2 alone or in combination with φRSB2. Lysates of J2 and RSB2, cultured with strain MAFF106603 and MAFF730138, respectively, as the hosts in 100 ml CPG, were filtered through a membrane filter (0·45 μm pore size, Steradisc) before use. Cells of *R. solanacearum* MAFF211514 grown in CPG were harvested at the exponential growth phase by centrifugation (8000 ***g*** for 15 min at 4°C), and then washed and resuspended in distilled water (approx. 10^8 CFU ml^{-1}). Then, 50 ml of the cell suspension was added to autoclaved soil (300 g) in a flask and mixed well. The phages in 5 ml DW were added to the contaminated soil (each 40 g portion in a 50 ml centrifuge tube) as follows: (i) J2 at MOI 10, (ii) φRSB2 at MOI 10, (iii) J2 and φRSB2 each at MOI 10 and (iv) 5 ml of distilled water as the control. The soil mixtures were incubated at room temperature and samples were collected at various times to monitor the number of *R. solanacearum* cells. The cell number was counted after spreading on CPG plates. These experiments were performed three times.

In planta virulence assay of *Ralstonia solanacearum*

Cells of *R. solanacearum* (strain MAFF211514, highly virulent to tomato cultivars) were grown in CPG medium for 1–2 days at 28°C. After centrifugation, the cells were resuspended in distilled water at a cell density of 5×10^8 cells ml^{-1} (OD_{600} = 1·0). Then, 5 ml of the cell suspension was inoculated into the soil of 4-week-old tomato plants (*Solanum lycopersicum* L. cultivar 'Oogata-fukuju', with 4–6 leaves) whose roots had been cut with a spatula. The final concentration of *R. solanacearum* was approx. 1×10^6 CFU g^{-1} soil. For the phage treatments, the phage suspension (5 ml, containing 2×10^{10} PFU ml^{-1}) was applied to the plants 1 day before bacterial challenge. As a control, distilled water was added in the same manner. Each experiment included four plants and was repeated three times. Plants were cultivated in a Sanyo Growth Cabinet at 25°C (16 h light/8 h dark) for 2–4 weeks before detailed examination. Symptoms of wilting were graded from 1 to 5 as described by Winstead and Kelman (1952).

Results

Isolation of bacteriophages from tomato field soil samples collected in Chiang Mai, Thailand

In February 2013, 43 soil samples were collected from geographically separated tomato fields in Chiang Mai, Thailand. The samples were assayed for the presence of lytic bacteriophages against various Thai and Japanese strains of *R. solanacearum* as the host. The *R. solanacearum* strains included those belonging to races 1, 3 and 4 and biovars 3, 4 and N2 (Table S1). Approximately 30% of the tested soil samples (15 samples) produced lytic plaques on assay plates. Single plaques were isolated from each assay plate for further purification, amplification and characterization. In general, one distinct phage plaque giving reproducible plaques on assay plates was selected from each soil sample, but in a few cases, more than two morphologically different plaques were selected for further analyses. After enrichment of phages using appropriate host strains, eight phages isolated using Thai host strains and six phages isolated using Japanese strains were selected for further characterization (Table S2).

Morphological characteristics of phage particles

Transmission electron microscopic observations revealed that phages C3, C6, C8, C10, C11, C12, J2, J3 and J5 showed characteristic features of the family Podoviridae (Ackermann 2003; Hendrix and Casjens 2006a), namely an icosahedral head (45–60 nm in diameter) with a short tail (10–20 nm long) (Fig. 1a,b). Four phages (C5, C7, J1 and J4) showed features of the family Myoviridae (P2-like morphology) (Ackermann 2003; Hendrix and Casjens 2006b) such as an icosahedral head (50–60 nm in diameter) with a long contractile tail (100–150 nm long, 15–20 nm wide) (Fig. 1). The particles of the phages C5, C7, J1 and J4 resembled those of the *R. solanacearum* phage φRSA1 (Yamada *et al.* 2007). The particles of phage J6 were very large with a head (115 nm in

diameter) and a long contractile tail (180-nm long and 25-nm wide) (Fig. 1). This jumbo myovirus (Hendrix 2009) formed discernible plaques on 0·45% top agar but not on ordinary 0·75% soft agar.

Host range of phages isolated from Chiang Mai

The phages were tested for their ability to infect 59 *R. solanacearum* strains (37 Thai isolates and 22 Japanese isolates). In each assay, the plaque formation efficiency and plaque morphology were carefully checked. The identity of the input and output phages was confirmed by restriction analysis of genomic DNA. As shown in Table S2, three Thai strains showed the widest host ranges; C11 (infecting 17/37 Thai isolates), C12 (22/37) and J2 (22/37). The phage J6 formed very small plaques (approx. 0·1 mm in diameter) because of its jumbo phage nature (Hendrix 2009), but formed larger clear plaques (1–2 mm) when the top agar concentration was decreased to 0·45%. J6 formed plaques with 12 of the 37 Thai strains (33%) (Table S2). When Japanese strains were used as the hosts, phages C11, C12 and J2 again

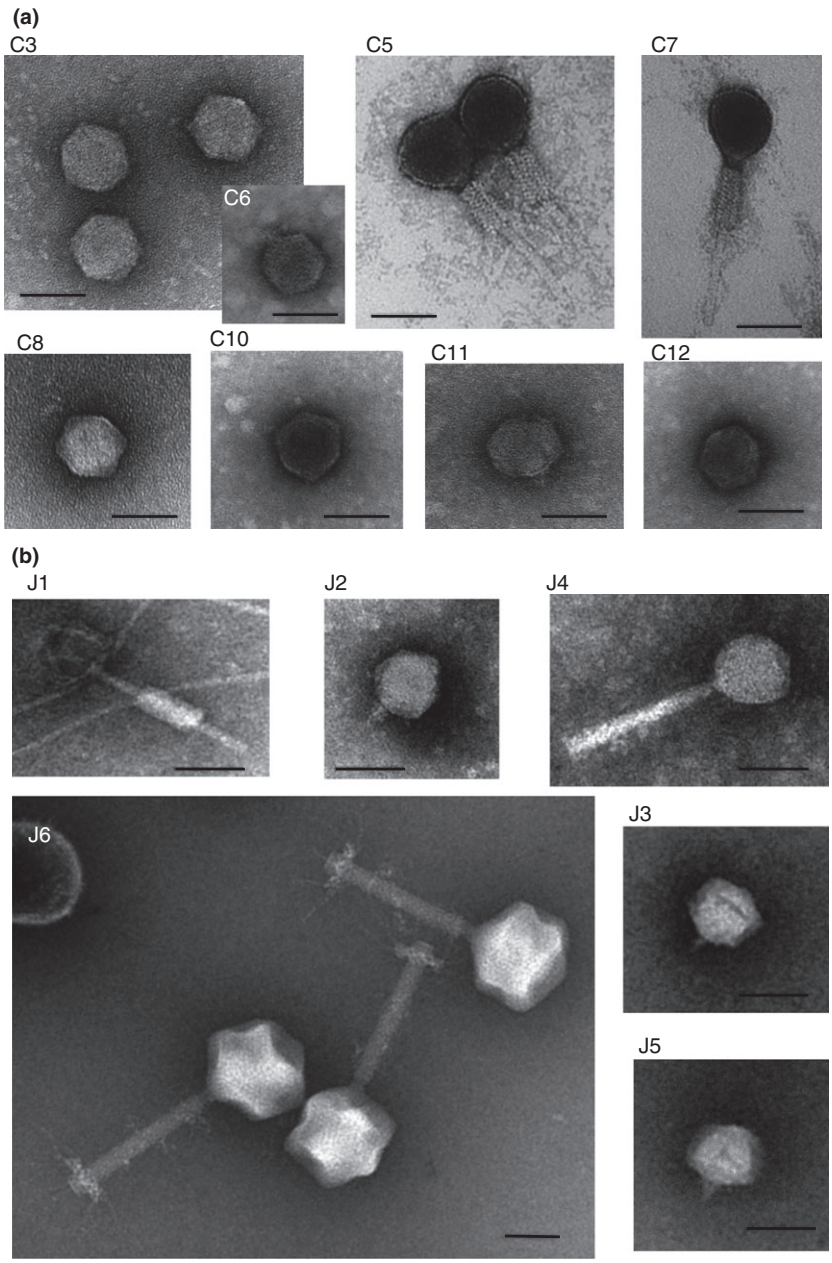

Figure 1 Electron micrographs of bacteriophages isolated from tomato fields in Chiang Mai. (a) Bacteriophages isolated with Thai *Ralstonia solanacearum* strains as hosts. (b) Bacteriophages isolated with Japanese *R. solanacearum* strains as hosts. Phage particles were negatively stained with phosphotungstate. Bar = 50 nm.

showed the widest host ranges (infecting 19/22, 18/22 and 18/22 Japanese strains, respectively). J6 showed the second widest host range (15/22). Therefore, C11, C12 and J2 infected more than 60% of the strains tested. Phages C3 and C6 did not infect any of the tested Japanese strains.

Genomic characterization of phages isolated from Chiang Mai

Genomic DNA was isolated from particles of 14 phages and digested with restriction enzymes. Figures 2a,b show the agarose gel separation patterns of phage DNA fragments produced by *Hinc*II digestion. For 13 of the 14 phages, there were many bands with sizes that summed up to 40–50 kb in total. For phage J6, there were more than 20 bands that summed up to more than 200 kbp, indicating a very large DNA genome. Analysis of the J6 DNA genome by pulsed-field gel electrophoresis revealed a single band of approx. 230 kbp (data not shown), confirming the large size of its genome. As shown in Fig. 2, *Hinc*II digestion produced similar fragmentation patterns of DNA in some cases. For example, C5 and C7 showed similar fragmentation patterns, as did C11 and J2. The top band of J2 was derived from terminal regions in a circular replicative form, and sometimes disappeared like in C11. However, the band patterns of these pairs of phages differed from each other when they were treated with other restriction enzymes such as *Kpn*I (data not shown). To compare these Thai phages with those isolated in various countries whose genomic sequences are available in the databases, the genomic sequences of J2 and J6 were determined and bioinformatically analyzed. The genomic sequence of J2 (44 360 bp) has been deposited into GenBank (accession no. AB920995). It contained 46 genes (Fig. S1), typically arranged in the three functional modules (Class I, Class II and Class III) of T7-type podoviruses (Dunn and Studier 1983). Many of the genes in the J2 genome show high similarity to those in the genomes of the *R. solanacearum* phages, φRSB1 (accession no. AB451219, 67·1% gene identity), φRSB2 (accession no. AB597179, 46·5% gene identity) and *φ*RSB3 (accession no. AB854109, 65·1% gene identity) (E. Narulita *et al.*, manuscript in preparation). All of these *φ*RSB phages were isolated from soils collected in Japan (Yamada 2012). The genomic sequence of J6 (223 932 bp) contained 224 putative ORFs most of which did not show any significant similarity with genes in the databases (data not shown).

Growth characteristics of phages J2 and J6

In our analyses, phages C11, C12 and J2 were identified as lytic podoviruses. These phages showed the widest host ranges of all the tested phages, infecting both Thai and Japanese strains of *R. solanacearum* (Table S2). The three phages showed similar particle morphology, plaque morphology, infection efficiency (phage titers) and DNA

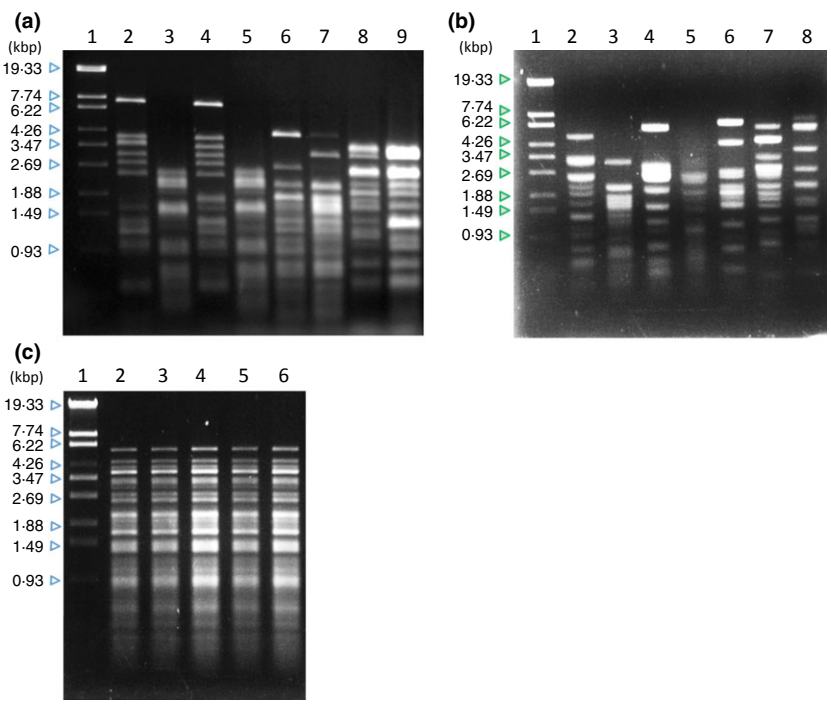

Figure 2 Digestion of genomic DNA of bacteriophages with *Hinc*II. (a) Eight phages isolated with Thai *Ralstonia solanacearum* strains as hosts: Lanes 1–9; molecular size markers (λDNA digested with *Sty*I), C3, C5, C6, C7, C8, C10, C12 and C11. (b) Five phages isolated with Japanese *Ralstonia solanacearum* strains as hosts compared with RSA1 (Fujiwara *et al.* 2008) and RSB1 (Kawasaki *et al.* 2009). Lanes 1–8; molecular size markers (λDNA digested with *Sty*I), J2, J3, J1, J5, J4, RSA1 and RSB1. (c) Jumbo phage J6. Lanes 1–6; molecular size markers (λDNA digested with *Sty*I), and five individual plaques of J6 picked from different assay plates. J6 always produced numerous *Hinc*II-digested bands in a stable pattern.

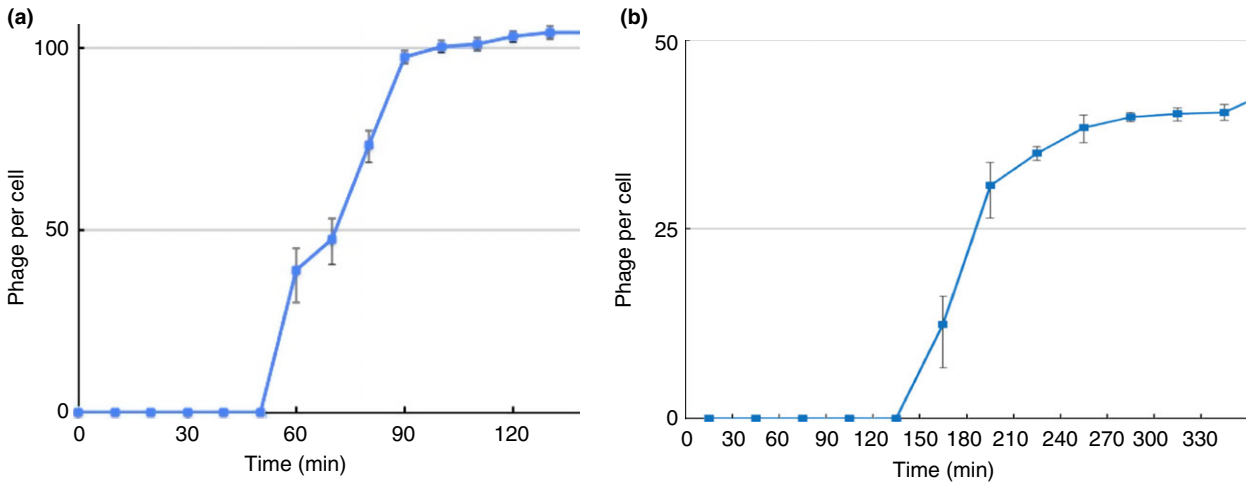

Figure 3 Single-step growth curves of phages J2 (a) and J6 (b) growing in *Ralstonia solanacearum* MAFF106603 and MAFF730138 as the host, respectively. Values shown are PFU per infected cell in cultures at different times post infection. Samples were taken at intervals (every 10 min for J2 and every 30 min for J6), immediately diluted and then titers were determined by the double-layered agar plate method. Error bars indicate the standard deviation calculated from three independent experiments.

restriction patterns (Fig. 2). We further characterized phage J2, as a representative of these three phages, to evaluate its infection dynamics. When the *R. solanacearum* strain MAFF106603 was infected with J2 at MOI of 0·01, 0·1, 1·0 and 5·0, the phage titers obtained were 1·0 (±0·3, $n = 3$) × 10^{12}, 2·4 (±0·5, $n = 3$) × 10^{12}, 6·0 (±0·3, $n = 3$)) × 10^{10} and 1·5 (±0·1, $n = 3$) × 10^{10} PFU ml^{-1}, respectively, indicating that MOI 0·1 was optimal for this phage. In the case of MAFF730138, the optimal MOI was also 0·1 but the phage titers were always lower (1/5 ~ 1/10) compared with MAFF106603 as the host (data not shown). In the case of phage J6, which also showed a wide host range, the infection cycle was longer, and the phage titers were comparatively lower. The maximum titer of J6 was 1·0 (±0·2, $n = 3$) × 10^{11}, obtained at MOI 0·5 with MAFF730138 as the host strain. The titer was less (<1/10) with MAFF106603 as the host. Therefore, infection efficiency of these phages varied considerably depending on the host strains. To characterize infection cycles of phages J2 and J6, we conducted single-step growth experiments. When strain MAFF106603 was used as the host, the phage J2 gave an approx. 60-min latent period followed by a 60-min rise period. A one-round cycle of infection took approx. 120 min and the average burst size was 100–110 PFU per infected cell. The use of strain MAFF730138 as a host gave essentially the same infection cycle but with a lower burst size (30 ~ 50 PFU per cell, data not shown). A single-step growth curve was also constructed for the jumbo phage J6 with MAFF730138 as the host. As shown in Fig. 3b, a one-round cycle of infection took approx. 4–4·5 h with a latent period of 120 min, and the average burst size was approx. 40 PFU per infected cell.

Decontamination of pathogen-contaminated soil with lytic bacteriophages

The podoviruses isolated in this study showed strong lytic activity and wide host ranges (Table S2). Therefore, these phages have potential uses in the decontamination of pathogen-infected field soils. In an experimental system, natural field soil supplemented with high levels of *R. solanacearum* MAFF211514 (approx. 10^8 CFU g^{-1} soil) was treated with J2 alone or in combination with φRSB2 (MOI = 10). After incubation at room temperature, soil samples were collected and bacterial cells were counted after spreading on CPG plates. As shown in Fig. 4, these phages (J2 alone or in combination with φRSB2) drastically reduced the bacterial cell density (to $<10^{-2}$-fold compared with the control). Especially in the combination of J2 and φRSB2, the cell density was approx. 10^{-3}-fold compared with the control even after 168 h post infection.

Prevention of bacterial wilt by treatment with phage J2

Lytic phages with a wide host range may be used directly to prevent bacterial wilt caused by *R. solanacearum* in the soil. To explore this possibility, we used J2 to treat tomato plants challenged by the highly virulent strain MAFF211514. One-month-old tomato plants (20–23 cm high) were treated with J2 and then inoculated with MAFF211514 cells as described in Materials and methods. Wilting symptoms were recorded every 2 days. As shown in Fig. 5, J2 treatment was able to prevent wilting of tomato plants infected with MAFF211514. Plants that did

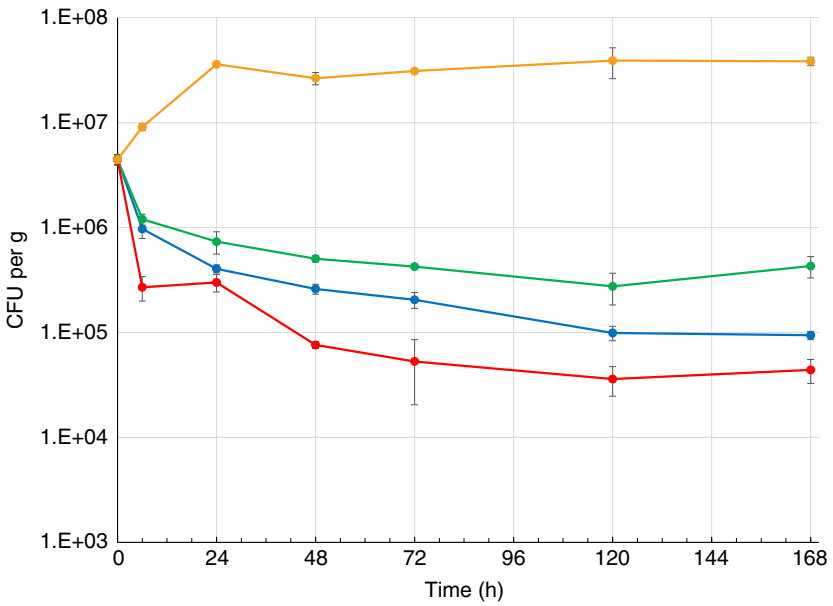

Figure 4 Decontamination of pathogen-contaminated soil with lytic bacteriophages. Natural field soil added with *Ralstonia solanacearum* MAFF211514, a highly virulent strain ($0{\cdot}5 \times 10^7$ CFU g^{-1} soil) was treated with J2 alone or in combination with φRSB2. After incubation at room temperature, soil samples were collected and bacterial cells were counted after spreading on CPG plates. J2 phage in combination with φRSB2 drastically reduced the bacterial cell density under soil conditions. Error bars indicate the standard deviation calculated from three independent experiments. Lines: blue, φRSB2; green, φRSJ2; red, φRSB2/φRSJ2; orange, control.

not receive the phage treatment started to show wilting symptoms at 8 days post inoculation (dpi) and all plants showed severe wilting symptoms at 10 dpi (Fig. 5b). Almost the same results were obtained for plants when bacterial cells were added preceding the phage treatment (data not shown). In contrast, half of the J2-treated plants did not wilt at all during the experimental period (Fig. 5a). Essentially the same results were reproducibly obtained in three independent experiments.

Discussion

Widespread distribution of bacteriophages in tomato fields in Chiang Mai (Sampling points, geographical distribution, plaque detection, host strains)

In this study, a total of 14 phages were isolated from soil samples collected in Chiang Mai, Thailand. These phages were characterized as nine podoviruses and five myoviruses based on their morphology. The particles of the nine podoviruses showed similar dimensions (Fig. 1) and resembled those of three podoviruses previously isolated in Japan; *φ*RSB1, *φ*RSB2 and *φ*RSB3 (Kawasaki *et al.* 2009; Yamada 2012). The phages were isolated from independent sampling locations, but the same soil sample sometimes yielded different phages, for example, C3 and C6 were obtained from soil sample CM20.

In spite of their morphological similarities, the nine podoviruses showed largely divergent genomic sequences, as revealed by restriction digestion analysis (Fig. 2). The myoviruses C5, C7, J1 and J4 showed P2-like morphology, and their particle dimensions were almost the same as those of particles of φRSA1 isolated in Japan (Yamada *et al.* 2007; Fujiwara *et al.* 2008). The particles of C5, C7, J1 and J4 (Fig. 1) had a contracted tail; the neck parts were unstable and easily broke off from the particle, resulting in the same racket-frame structure as that observed for φRSA1 (Yamada *et al.* 2007). The C5, C7 and J1 phages were isolated from Field 1 but J4 was isolated from Field 4. Phages C5 and C7 seemed to be very closely related to each other according to the genomic restriction patterns (Fig. 2). The particles of phage J6 (isolated from Field 3) were very large, comparable to those of the jumbo phage *φ*RSL1 (Yamada *et al.* 2010). The head of J6 (115 nm) was slightly smaller than that of *φ*RSL1 (123 nm), while its tail (180 nm) was longer than that of *φ*RSL1 (115 nm). The genome of J6 was approx. 230 kbp, comparable to the DNA genome of *φ*RSL1 (240 kbp). Since jumbo phages with genomic DNA larger than 200 kbp have rarely been isolated from soil bacteria, these two different jumbo phages of *R. solanacearum* found in Thailand and Japan will be useful materials to study the origin and evolution of jumbo phages, and their biological and environmental effects.

No filamentous phages (inoviruses) were detected in this study, in contrast to previous studies on soil samples collected in Japan (Yamada *et al.* 2007; Yamada 2012) and Korea (Murugaiyan *et al.* 2010), in which various types of inovirus were detected. It is unclear whether this is because the soil conditions in Thailand (submerged during a rainy season) differ from those in Japan and Korea, or because the Thai bacterial strains have been specifically adapted to Thai conditions.

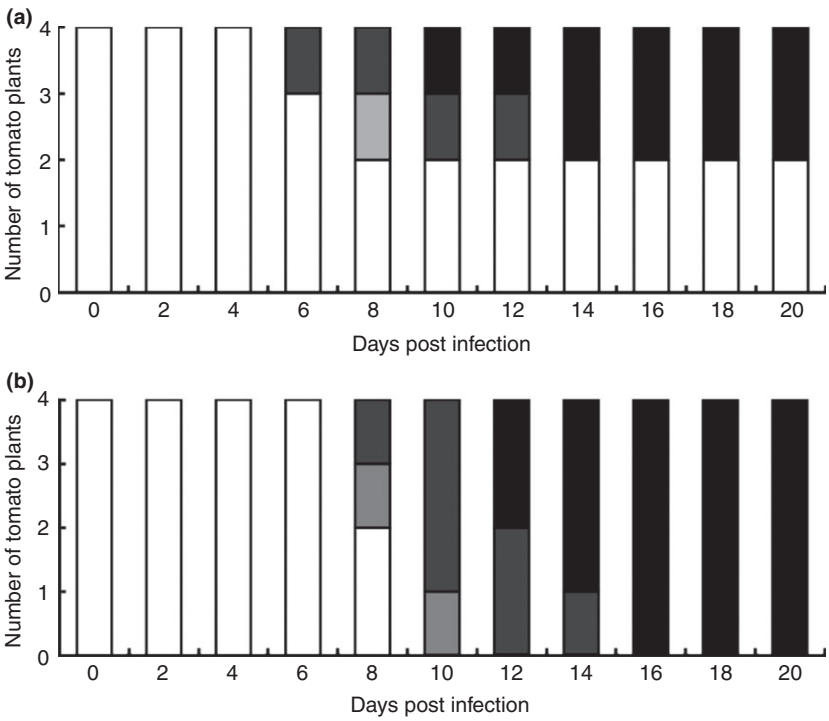

Figure 5 Prevention of bacterial wilt by treatment with phage J2. One-month-old tomato plants pretreated with phage J2 (a) or distilled water (b, control) were inoculated with *Ralstonia solanacearum* cells as described in Materials and methods. Wilting symptoms were graded from 0 to 5 (from white to black) according to Winstead and Kelman (1952). Four plants were included in each experiment. Three independents experiments essentially showed the similar results.

Host range of phages isolated from Chiang Mai

Most phages isolated in this study infected Thai and Japanese strains of *R. solanacearum*. However, because the two phages C3 and C6 could not infect Japanese strains, they may be useful for typing and characterizing geographically divergent strains of *R. solanacearum*. Three other podoviruses (C11, C12 and J2) and the jumbo phage J6 showed wide host ranges, infecting Thai and Japanese *R. solanacearum* strains. As shown in Table S1, most strains of *R. solanacearum* distributed in Thailand and Japan are of races 1 and 3 and of biovar 3, 4 and N2 (also of phylotypes I and IV).

Generally, podoviruses such as the *Escherichia coli* phage T7 are highly lytic and do not have a lysogenic cycle (Dunn and Studier 1983). These attributes make them good candidates as biocontrol tools for bacterial diseases. Because approx. 70% of the *R. solanacearum* strains tested, including Thai and Japanese strains, were susceptible to either of C11, C12, or J2 (Table S2), a mixture of these phages (a phage cocktail) may be a better biocontrol agent. In this context, phage J2, as a representative, was further characterized to determine its efficiency of bacterial infection. The typical infection cycle of phage J2 took approx. 120 min for one round, with a burst size of 100–110 PFU per cell. Therefore, these phages (combined with other phages) may be useful for preventing bacterial growth over a long periods. These results indicate that these highly lytic podoviruses with a wide host range (C11, C12 and J2) could have potential uses as anti-pathogen agents against bacterial wilt, especially for decontaminating field soils or irrigation water.

The myoviruses J1 and J4 showed relatively wide host ranges for the Japanese *R. solanacearum* strains, infecting more than 50% of these strains. However, these P2-like phages often have an integrase gene and a lysogenic cycle. The lysogenic host cells, once established, usually show immunity to the phage, and then the phage is no longer able to kill the host. Therefore, these phages are likely not good biocontrol candidates.

Plant biocontrol experiments

In the plant challenge experiments, pretreatment of plants with J2 prevented wilting after subsequent inoculation with the pathogen (Fig. 5). Therefore, these phages are valuable resources with various practical uses. For example, plant seedlings may be treated with these phages before planting in the fields to prevent bacterial wilt. If bacterial wilt occurred in the field, uninfected plants in the neighbourhood may be treated with these phages to stop expansion of the disease. The phage resources should be enlarged by collecting many more new phages from the natural environment to challenge a wide variety of host strains, especially to strains that are resistant to phages like C3 and C6 (Table S2).

The jumbo phage J6 may also function as effective tool for bacterial wilt control, in a different manner from that

demonstrated for φRSL1 (Fujiwara *et al.* 2011). The infection cycle of φRSL1 (4–5 h) is longer than the doubling time of host cells (2–3 h). In the presence of φRSL1, the cell-killing effects of this phage result in an equilibrium state between growing and dying cells (at low cell densities). Keeping the cell density levels below the virulence threshold is very effective to prevent bacterial wilt. As demonstrated in this work, the infection cycle of J6 was very long (4–5 h, with a latent period of approx. 2·5 h), although the burst size of this phage was relatively low, approx. 40 PFU per cell (Fig. 3b).

The host range and infection mechanism of J6 may be different from those of φRSL1, yet these phages appear to have very similar infection kinetics. This kind of phage could be useful for sustainable biocontrol methods, namely long-lasting, pathogen-specific, and eco-friendly methods.

Acknowledgements

This study was supported by the Strategic Japanese-Thai Research Cooperative Program on Biotechnology (JST/BIOTEC-SICP). The authors thank Yuya Sugimura and Taiki Yasuda for plant challenge assays. They also thank Likhit Maneesinthu for the guidance to tomato fields in Chiang Mai, Thailand.

Conflict of Interest

The authors have no conflict of interest to declare.

References

Abedon, S.T. (2009) Phage evolution and ecology. *Adv Appl Microbiol* **67**, 1–45.

Ackermann, H.W. (2003) Bacteriophage observations and evolution. *Res Microbiol* **154**, 245–251.

Allen, C., Kelman, A. and French, E.R. (2001) Brown rot of potatoes. In *Compendium of Potato Diseases* eds. Stevenson, W.R., Loria, R., Franc, G.D. and Weingartner, D.P., pp 11–13. St. Paul, MN, USA: American Phytopathology Society Press.

Allen, C., Prior, P. and Hayward, A.C. (2005) *Bacterial Wilt Disease and the Ralstonia solanacearum* Species Complex. St. Paul, MN, USA: American Phytopathology Society Press.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Ashelford, K.E., Day, M.J. and Fry, J.C. (2003) Elevated abundance of bacteriophage infecting bacteria in soil. *Appl Environ Microbiol* **69**, 285–289.

Boonsuebsakul, W. (1994) Study on the biovar of *Pseudomonas solanacearum* causing bacterial wilt of potato in Thailand. *Annu Rep Plant Pathol and Microbiol Div, Dept Agric, Bangkok, Thailand*. 162.

Delcher, A.L., Harmon, D., Kasif, S., White, O. and Salzberg, S.L. (1999) Improved microbial gene identification with GLIMMER. *Nucleic Acids Res* **27**, 4636–4641.

Denny, T.P. (2006) Plant pathogenic *Ralstonia* species. In *Plant-Associated Bacteria* ed. Gnanamanickam, S.S. pp. 573–644. Amsterdam, Netherlands: Springer Verlag.

Dunn, J.J. and Studier, F.W. (1983) Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *J Mol Biol* **166**, 477–535.

Dykstra, M.J. (1993) *A Manual of Applied Technique for Biological Electron Microscopy*. New York, NY, USA: Plenum Press.

Fegan, M. and Prior, P. (2005) How complex is the *Ralstonia solanacearum* species complex? In *Bacterial Wilt: the Disease and the Ralstonia solanacearum* Species Complex. eds Allen, C., Prior, P. and Hayward, A.C. pp. 449–461. St. Paul, MN, USA: American Phytopathology Society Press.

Fujiwara, A., Kawasaki, T., Usami, S., Fujie, M. and Yamada, T. (2008) Genomic characterization of *Ralstonia solanacearum* phage ϕRSA1 and its related prophage (ϕRSX) in strain GMI1000. *J Bacteriol* **190**, 143–156.

Fujiwara, A., Fujisawa, M., Hamasaki, R., Kawasaki, T., Fujie, M. and Yamada, T. (2011) Biocontrol of *Ralstonia solanacearum* by treatment with lytic bacteriophages. *Appl Environ Microbiol* **77**, 4155–4162.

Gasic, K., Ivanovic, M.M., Ignjatov, M., Calic, A. and Obradovic, A. (2011) Isolation and characterization of *Xanthomonas euvesicatoria* bacteriophages. *J Plant Pathol* **93**, 415–423.

Hayward, A.C. (1991) Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annu Rev Phytopathol* **29**, 65–87.

Hayward, A.C. (2000) *Ralstonia solanacearum*. In *Encyclopedia of Microbiology*, vol 4. ed. Lederberg, J. pp. 32–42. San Diego, CA, USA: Academic Press.

Hendrix, R.W. (2009) Jumbo bacteriophages. *Curr Top Microbiol Immunol* **328**, 229–240.

Hendrix, R.W. and Casjens, S.R. (2006a) Index of viruses-Podoviridae. In *ICTVdB-The Universal Virus Database*, ver 4 ed. Buchen-Osmond, C. New York, NY, USA: Columbia University.

Hendrix, R.W. and Casjens, S.R. (2006b) Index of viruses-Myoviridae. In *ICTVdB-The Universal Virus Database*, ver 4. ed. Buchen-Osmond, C. New York, NY, USA: Columbia University.

Higashiyama, T. and Yamada, T. (1991) Electrophoretic karyotyping and chromosomal gene mapping of *Chlorella*. *Nucleic Acids Res* **19**, 6191–6195.

Horita, M. and Tsuchiya, K. (2002) Causal agent of bacterial wilt disease *Ralstonia solanacearum*. In *MAFF Microorganism Genetic Resources Mannual No.12* ed. National Institute of Agricultural Sciences. pp. 5–8.

Tsukuba, Japan: National Institute of Agricultural Sciences.

Janse, J. (1996) Potato brown rot in western Europe-history, present occurrence and some remarks on possible origin, epidemiology and control strategies. *Bull OEPP/EPPO* **26**, 679–695.

Jones, J.B., Jackson, L.E., Balogh, B., Obradovic, A., Iriarte, F.B. and Momol, M.T. (2008) Bacteriophages for plant disease control. *Annu Rev Phytopathol* **45**, 245–262.

Kasigumpaiboon, U., Kositratana, W., Thaveechai, N. and Makesongsri, L. (1992) Effect of antagonistic bacteria on the protection of tomato from bacterial wilt disease. In *Proceedings of the 30th Kasetsart University Annual Conference: Plants*. pp. 321–328. Bangkok, Thailand: Kasetsart University.

Kawasaki, T., Shimizu, M., Satsuma, H., Fujiwara, A., Fujie, M., Usami, S. and Yamada, T. (2009) Genomic characterization of *Ralstonia solanacearum* phage φRSB1, a T7-like wide-host-range phage. *J Bacteriol* **191**, 422–427.

Makari, H.K., Palaniswamy, M. and Angayarkanni, J. (2013) Isolation of lytic bacteriophage against *Ralstonia solanacearum* causing wilting symptoms in ginger (*Zingiber officinale*) and potato (*Solanum tuberosum*) plants. *Int Res J Biol Sci* **2**, 78–84.

Murugaiyan, S., Bae, J.Y., Wu, J., Lee, S.D., Um, H.Y., Choi, H.K., Chung, E., Lee, J.H. *et al.* (2010) Characterization of filamentous bacteriophage PE226 infecting *Ralstonia solanacearum* strains. *J Appl Microbiol* **110**, 296–303.

Nguyen, M.T. and Ranamukhaarachchi, S.L. (2010) Soil-borne antagonists for biological control of bacterial wilt disease caused by *Ralstonia solanacearum* in tomato and pepper. *J Plant Pathol* **92**, 395–406.

Nguyen, M.T., Ranamukhaarachchi, S.L. and Hannaway, D.B. (2011) Efficacy of antagonist strains of *Bacillus megaterium*, *Enterobacter cloaceae*, *Pichia guillermondi* and *Candida ethanolica* against bacterial wilt disease of tomato. *J Phytopathol* **3**, 1–10.

Prior, P., Allen, C. and Elphinstone, J. (1998) *Bacterial Wilt Disease: Molecular and Ecological Aspects*. Berlin, Germany: Springer Verlag.

Sambrook, J. and Russell, D.W. (2001) *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, New York, USA: Cold Spring Harbor Laboratory Press.

Suwankeereekhan, J., Thaveechai, N., Chamswarng, C. and Wannakairoj, S. (2004) Integrated treatments to control of bacterial wilt of patumma caused by *Ralstonia solanacearum*. In *Proceedings of the 42th Kasetsart University Annual Conference: Plants*. pp. 429–436. Bangkok, Thailand: Kasetsart University.

Tan, G.H., Nordin, M.S. and Napsiah, A.B. (2010) Effect of phage infection on pathogenic activity of *Ralstonia solanacearum* in tomato. *J Tropic Agric Food Sci* **38**, 123–130.

Thammakijjawat, P., Thaveechai, N., Paradornuwat, A., Wannakairoj, S. and Suthirawut, S. (1997) Control of bacterial wilt of patumman by soil amendments. *Agric Sci J* **30**, 35–49.

Thaveechai, N., Kositratana, W., Phuntumart, V., Leksoomboon, C. and Khongplean, P. (1997) Management of bacterial wilt of tomato. In: *AVRDC. Collaborative Vegetable Research in Southeast Asia: Proceeding of the AVNET-II Final Workshop*. pp. 397–407. Bangkok, Thailand.

UniProt Consortium (2007) The universal protein resource (UniProt). *Nucleic Acids Res* **35**, D193–D197.

Villa, J.E., Tsuchiya, K., Horita, M., Natural, M., Opina, N. and Hakumachi, M. (2005) Phylogenetic relationships of *Ralstonia solanacearum* species complex strains from Asia and other continuents based on 16S rDNA, endoglucanase, and *hrpB* gene sequences. *J Gen Plant Pathol* **71**, 39–46.

Wheeler, D.L., Barrett, T., Benson, D.A., Bryant, S.H., Canese, K., Chetvrnin, V., Church, D.M., DiCuccio, M. *et al.* (2007) Database resources of the National Center for Biotechnology Information. *Nucleic Acids Res* **35**, D5–D12.

Winstead, N.N. and Kelman, A. (1952) Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. *Phytopathology* **42**, 628–634.

Yabuuchi, E., Kosako, V., Yano, I., Hotta, H. and Nishiuchi, Y. (1995) Transfer of two *Burkholderia* and an *Alcaligenes* species to *Ralstonia* gen. nov.: proposal of *Ralstonia picketii* (Ralston, Palleroni and Doudoroff 1973) comb. nov., *Ralstonia solanacearum* (Smith 1896) comb. nov. and *Ralstonia eutropha* (Davis 1969) comb. nov. *Microbiol Immunol* **39**, 897–904.

Yamada, T. (2012) Bacteriophages of *Ralstonia solanacearum*: their diversity and utilization as biocontrol agents in agriculture. In *Bacteriophages* ed. Kurtboke, I., pp 113–139. Rijeka, Croatia: InTech-Open Access Publisher.

Yamada, T., Kawasaki, T., Nagata, S., Fujiwara, A., Usami, S. and Fujie, M. (2007) New bacteriophages that infect the phytopathogen *Ralstonia solanacearum*. *Microbiology* **153**, 2630–2639.

Yamada, T., Satoh, S., Ishikawa, H., Fujiwara, A., Kawasaki, T., Fujie, M. and Ogata, H. (2010) A jumbo phage infecting the phytopathogen *Ralstonia solanacearum* defines a new lineage of the Myoviridae family. *Virology* **398**, 135–147.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Predicted ORFs found in the genomic sequence of phage J2.

Table S1 Strains of *R. solanacearum* used in this study.

Table S2 Host range of bacteriophages isolated in Chiang Mai.