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Bacteriophages of *Ralstonia solanacearum*: Their Diversity and Utilization as Biocontrol Agents in Agriculture

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1. Introduction

Bacterial wilt is one of the most important crop diseases, and is caused by the soil-borne Gram-negative bacterium *Ralstonia solanacearum*. *R. solanacearum* was formerly classified as *Pseudomonas solanacearum* or *Bacterium solanacearum* (Smith, 1986; Yabuuchi et al., 1995). This bacterium has an unusually wide host range, infecting more than 200 species belonging to more than 50 botanical families, including economically important crops (Hayward, 1991; Hayward, 2000). *R. solanacearum* strains represent a heterogeneous group, 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. Race 1 is a poorly defined group with a very wide host range, and is endemic to tropical, subtropical, and warm areas. Strains of race 2 mainly infect bananas, and are found primarily in Southeast Asia and Central America. Race 3 strains are distributed worldwide, and are principally associated with potato. Strains of race 4 infect ginger in areas of Asia and Hawaii, and race 5 strains infect mulberries in China. Recently, a new classification system for *R. solanacearum* strains, based on phylogenetic information, has been proposed, where 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 & Prior, 2005).

In the field, *R. solanacearum* is easily disseminated via soil, contaminated irrigation water, surface water, farm equipment, and infected material (Janse, 1996). Bacterial cells can survive for many years in association with alternate hosts. Once identified as being infected, plants in cropping fields, gardens, or greenhouses must be destroyed, and soil and water draining systems that could potentially be contaminated with the bacteria must be treated with chemical bacteriocides. Soil fumigation with methyl bromide, vapam, or chloropicrin is of limited efficacy. Methyl bromide depletes the stratospheric ozone layer; therefore, the production and use of this gas was phased out in 2005, under the *Montreal Protocol and the Clean Air Act*. The limited effectiveness of the current integrated management strategies has

meant that bacterial wilt continues to be an economically serious problem for field-grown crops in many tropical, subtropical, and warm areas of the world (Hayward, 1991; Hayward, 2000). For example, bacterial wilt of potato has been estimated to affect 3.75 million acres in approximately 80 countries, with global damage estimates currently exceeding $950 million per year (Momol et al., 2006). At present, protection from losses by bacterial wilt is provided mainly by early detection and subsequent eradication by destroying the host. Development of effective disease management strategies and improvement in detection and monitoring tools are required.

Various kinds of bacteriophage with characteristic features have been isolated recently (Yamada et al., 2007), and have paved the way for new methods of biocontrol of bacterial wilt. These phages may be useful as tools for effective detection (diagnosis) of the pathogen in cropping ecosystems and in growing crops. They also have potential uses in eradication of the pathogen from contaminated soil or prevention of bacterial wilt in economically important crops. Like other methods of biological control, one advantage of phage biocontrol is the reduction in the use of chemical agents against pathogens. This prevents the problems of multiple environmental pollution, ecosystem disruption, and residual chemicals on the crops. Phage biocontrol in agricultural settings was extensively explored 40–50 years ago as a means of controlling plant pathogens (Okabe & Goto, 1992). Two major problems arose in those practical trials; (i) extracellular polysaccharides produced by pathogenic bacteria prevented phage adsorption, and (ii) there were various degrees of susceptibility among bacterial strains (Goto, 1992). Nevertheless, over recent decades, the use of phage biocontrol to restrict the growth of plant-based bacterial pathogens has been explored with increasing enthusiasm. Certain bacteriophages of *R. solanacearum* have already been isolated and some of their physical and physiological properties have been characterized. The virulent phage P4282, and its related phages, have a polyhedral head (69 nm in diameter) and a short tail (20 nm in length) (Tanaka et al., 1990), and contain a circular 39.3-kbp dsDNA genome (Ozawa et al., 2001). Another phage, PK-101, was isolated from soil, and has a linear 35-kbp dsDNA genome (Toyoda et al., 1991). Both of these phages show very narrow host ranges and infect only a few strains of *R. solanacearum*. Phage P4282, which infects strain M4S, was used to control bacterial wilt of tobacco plants in laboratory experiments and possible phage-mediated protection was observed (Tanaka et al., 1990). However, for practical use of phages as biocontrol agents against bacterial wilt, multiple phages with wide host-ranges and strong lytic activity are required.

2. Characterization and classification of bacteriophages infecting *R. solanacearum*

Recently, Yamada et al. (2007) isolated and characterized several different kinds of phage that specifically infect *R. solanacearum* strains belonging to different races and/or biovars. Two of the phages, ϕRSS1 and ϕRSM1, are filamentous, Ff-like phages (*Inoviridae*). As demonstrated for coliphage M13, filamentous phages are very useful as vectors to display proteins on the virion surface in the bacteriophage-display method (Smith, 1985; Smith, 1991). In the same way, ϕRSS1 and ϕRSM1 may be utilized to display a tag protein on the virion surface in diagnosis or monitoring applications (Kawasaki et al., 2007a). Another phage, ϕRSA1, is a P2-like head-tailed virus (*Myoviridae*), and has the widest host range; all but one of 18 strains tested of races 1, 3, or 4 and biovar 1, N2, 3, or 4 were susceptible to this

phage. ϕRSL1 is another myovirus having a very large genomic DNA of approximately 240 kbp and strongly lysed 17 of 18 different strains. Another lytic phage, ϕRSB1, shows a T7-like morphology (*Podoviridae*) and also has a wide-host-range (15 of 18 strains of races 1, 3, or 4 were susceptible). ϕRSB1 vigorously lyses host cells and forms very large clear plaques that extend for 10–15 cm on assay plates. The characteristics and host ranges of these phages are summarized in Table 1. Further surveys for phages infecting different strains of *R. solanacearum* detected many interesting examples, some of which were induced from lysogenic strains. Searching through the genomic databases for the sequences determined for these phages also revealed similar sequences integrated in various bacterial genomes (prophages). Such phages and prophages of *R. solanacearum* and related bacterial species are roughly grouped into six phage-types as follows: ϕRSS phages, ϕRSM phages, ϕRSB phages, ϕRSA phages, ϕRSL phages, ϕRSC phages , and others.

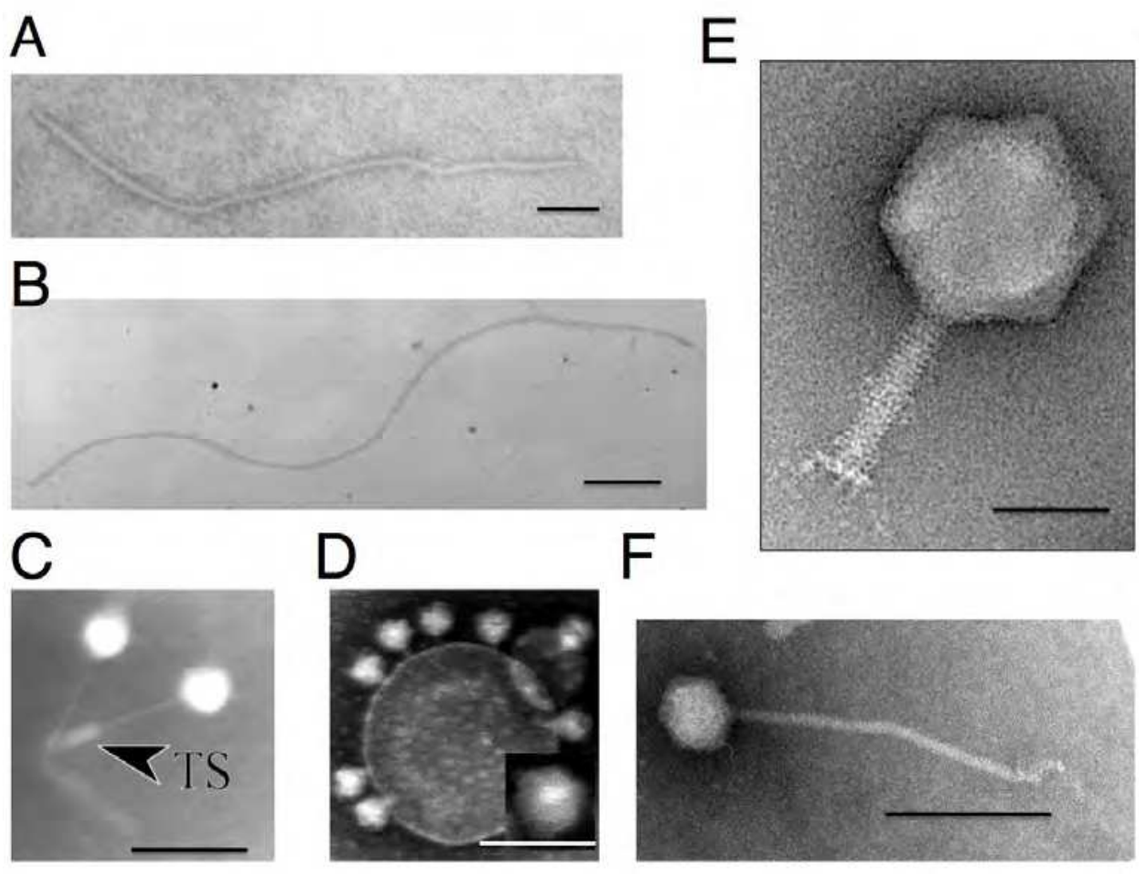


Fig. 1. Electron micrographs showing morphology of bacteriophages infecting *R. solanacearum*.

ϕRSS1 (A) and ϕRSM1 (B) are filamentous phages of *Inoviridae*. ϕRSA1 (C) and ϕRSL1 (E) are myoviruses (*Myoviridae*) with a contractile tail. ϕRSB1 is a podovirus (*Podoviridae*) with a head and a short tail (D). Several ϕRSB1 particles are seen attached to a membrane vesicle. ϕRSC1 is a λ like phage (*Siphoviridae*) with a non-contractile tail (F). TS, tail sheath. Bar represents 100 nm.

Ralstonia solanacearum		Phages					
Strain	Race, Biovar	ϕRSS1	ϕRSM1	ϕRSA1	ϕRSB1	ϕRSL1	ϕRSC1
C319	1, ND	+	−	+	+	+	+
M4S	1 . 3	−	+	+	+	+	+
Ps29	1 , 3	−	+	+	+	+	+
Ps65	1, ND	−	+	+	+	+	+
Ps72	1, ND	−	+	+	+	+	+
Ps74	1, ND	−	+	+	+	+	+
MAFF106603	1 , 3	+	−	+	+	+	+
MAFF106611	1 , 4	+	−	+	+	+	+
MAFF211270	1, N2	−	+	+	−	+	−
MAFF211271	3, N2	+	−	+	+	+	−
MAFF211272	4 , 4	−	−	+	+	−	−
MAFF301556	1 , 4	+	−	+	+	+	−
MAFF301558	3, N2	+	−	+	+	+	−
MAFF730135	1 , 4	+	−	+	+	+	−
MAFF730138	1 , 3	−	+	+	+	+	+
MAFF730139	1 , 4	+	−	+	+	+	+
RS1002	1 , 4	+	−	+	−	+	−
AA4017	1, ND	+	−	−	−	+	−

Phage susceptibility is shown as sensitive (+) or resistant (-). Some original data are updated (Yamada et al., 2007; Askora et al., 2009). ND, Not determined.

Table 1. Host specificity of bacteriophages infecting *R. solanacearum*

Ralstonia solanacearum		Phages						
Strain	Race, Biovar	ϕRSS1	ϕRSS0	ϕRSS2	ϕRSS4	ϕRSM1	ϕRSM3	ϕRSM13
C319	1, ND	+	−	−	+	−	+	+
M4S	1 . 3	−	−	+	−	+	−	−
Ps29	1 , 3	−	−	+	−	+	−	−
Ps65	1, ND	−	−	+	−	+	−	−
Ps72	1, ND	−	−	+	−	+	+	+
Ps74	1, ND	−	−	+	−	+	+	+
MAFF106603	1 , 3	+	+	−	+	−	+	+
MAFF106611	1 , 4	+	+	−	+	−	+	+
MAFF211270	1, N2	−	−	+	−	+	+	+
MAFF211271	3, N2	+	−	−	+	−	+	+
MAFF211272	4 , 4	−	−	−	−	−	+	+
MAFF301556	1 , 4	+	−	−	+	−	+	+
MAFF301558	3, N2	+	−	−	+	−	+	+
MAFF730135	1 , 4	+	−	ND	+	−	ND	ND
MAFF730138	1 , 3	−	−	+	−	+	−	−
MAFF730139	1 , 4	+	+	−	+	−	+	+
RS1002	1 , 4	+	+	ND	+	−	ND	ND
AA4017	1, ND	+	−	ND	+	−	ND	ND

Phage susceptibility is shown as sensitive (+) or resistant (-). Some original data are updated (Yamada et al., 2007; Askora et al., 2009). ND, Not determined.

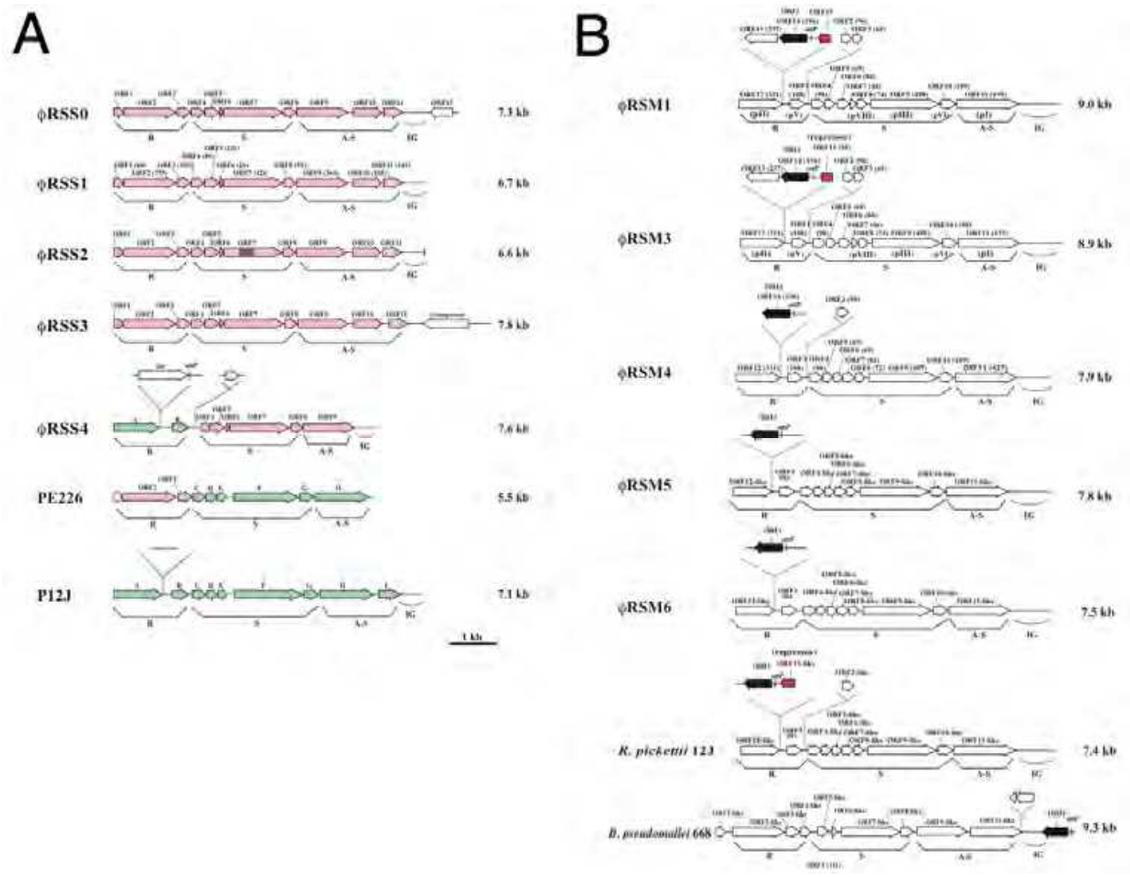
Table 2. Host specificity of different phages of ϕRSS and ϕRSM groups

2.1 ϕRSS phages of the *Inoviridae*

ϕRSS1 was isolated from a soil sample collected from tomato crop fields. It infected 10 of 18 strains tested and gave relatively small and turbid plaques on assay plates. ϕRSS1 particles

have a flexible filamentous shape of 1100 ± 100 nm in length and 10 ± 0.5 nm in width (Fig. 1), giving a morphology resembling coliphage fd (Buchen-Osmod, 2003; ICTVdB). Infection with ϕRSS1 phage does not cause host cell lysis, but establishes a persistent association between the host and phage, releasing phage particles from the growing and dividing host cells. The ϕRSS1 particles contain single-stranded (ss)DNA as the genome. Therefore, ϕRSS1 belongs to Ff-like phages or inoviruses. In general, the genome of Ff-phages is organized in a module structure, in which functionally related genes are grouped (Hill et al., 1991; Rasched & Oberer, 1986). Three functional modules are always present: the replication module, the structural module, and the assembly and secretion module. The replication module, contains the genes encoding rolling-circle DNA replication and single-strand DNA (ssDNA) binding proteins; gII, gV, and gX (Model & Russel, 1988). The structural module contains genes for the major (gVIII) and minor coat proteins (gIII, gVI, gVII, and gIX), and gene gIII encodes the host recognition or adsorption protein pIII (Armstrong et al., 1981). The assembly and secretion module contains the genes (gI, and gIV) for morphogenesis and extrusion of the phage particles (Marvin,1998). Gene gIV encodes protein pIV, an aqueous channel (secretin) in the outer membrane, through which phage particles exit from the host cells. Some phages encode their own secretins, whereas others use host products (Davis et al., 2000). The genome of ϕRSS1 is 6,662 nt long (DDBJ accession No. AB259124), with a GC content of 62.6 %, which is comparable to that of *R. solanacearum* GMI1000 (66.97 %, Saranoubat et al., 2002). There are 11 open reading frames (ORFs), located on the same strand (Fig. 2A). Frequently, the termination codon of the preceding gene overlaps with the initiation codon of the following gene. The coding sequence occupied by these ORFs accounts for 91.6% of the total ϕRSS1 sequence. A survey of the databases for amino acid sequences of ϕRSS1 ORFs leads to the gene organization as shown in Fig. 2A. The ϕRSS1 genes fit well with the general arrangement of Ff-like phages. Homology searches revealed that the dsDNA plasmid pJTPS1, found in a strain *R. solanacearum* (accession no. AB015669), has significant nucleotide sequence similarity to ϕRSS1 DNA. The size of pJTPS1 is 6,633 bp; 29 bp smaller than that of ϕRSS1. Nucleotide sequence identity between the two DNAs was 95%. Compared with pJTPS1, two major different regions in the ϕRSS1 DNA are evident: One region (ϕRSS1 positions 2,674-3,014) corresponds to ORF7, putatively encoding pIII, which is a minor coat protein at one end of the phage particle required to recognize and adsorb host cells (Marvin, 1998, Model and Russel, 1988). The other extended change was found at positions 6,632-6,657 corresponding to the IG (intergenic region), which is highly conserved in other Ff-like phages (Model and Russel, 1988). This region may be involved in the rolling circle DNA replication mechanism, producing phage genomic ssDNA molecules. These results suggest that pJTPS1 may have been derived from a ϕRSS1-like phage, followed by changes in the phage DNA. We tested the phage nature of pJTPS1 in strain M4S, where pJTPS1 was initially identified as a circular dsDNA plasmid (Negishi, et al., 1993). We could not detect dsDNA plasmid in strain M4S, but found a ϕRSS1-related sequence integrated into the M4S genome. According to the pJTPS1 sequence, the corresponding sequence was amplified by PCR and circularized by self-ligation. When the resulting DNA was introduced in M4S cells, plaques appeared on assay plates. The nucleotide sequence of the phage coincided with pJTPS1 (Kawasaki et al., 2007a). The plaque size was relatively small and the frequency was relatively low, indicating that pJTPS1 is a ccc form of a ϕRSS1-related phage (designated as ϕRSS2) and is integrated in strain M4S. There is no immunity in the phage

infection. Interestingly, the pJTPS1 phage (ϕRSS2) infected ϕRSM1-sensitive strains, including M4S (see below) and did not infect ϕRSS1-sensitive strains, including strain C319. This may be because of the specific difference in a region of pIII host recognition protein between ϕRSS1 and ϕRSS2.



According to the *E. coli* M13-model (Model and Russel, 1988; Marvin, 1998), ORFs identified on the phage genome are grouped in the functional modules for replication (R), structure (S), and assembly-secretion (A-S). IG, large intergenic region. Among ϕRSS1-related phages (A), ϕRSS0, ϕRSS2 and ϕRSS3 were derived from prophages of strains C319, M4S, and MAFF106611, respectively. P12J and PE226 are phages of *Ralstonia pickettii* (accession no. AY374414) and Korean strains of *R. solanacearum* (Murugaiyan et al., 2010). Of ϕRSM1-related phages (B), ϕRSM3, ϕRSM4, ϕRSM5, and ϕRSM6 are prophages of strains MAFF730139, UW551 (race 3, biovar 2; Gabriel et al., 2006), IPO1609 (race 3, biovae 2; Remenant et al., 2010), and CMR15 (phylotype III, Remenant et al., 2010), respectively. A similar prophage found in *Ralstonia pickettii* 12J is also shown.

Fig. 2. Genomic organization of ϕRSS1-related phages (A) and ϕRSM1-related phages (B).

Genomic Southern blot hybridization showed frequent examples of ϕRSS1-related sequences integrated in the genomes of various *R. solanacearum* strains (Yamada et al., 2007). As seen above, ϕRSS2 is integrated in the genome of strain M4S. ϕRSS1 was also found to exist as a lysogenic phage in strain C319 (Kawasaki et al., 2007). By analyzing the host genomic sequences flanking the ϕRSS1 region, its integration site was determined to correspond to ϕRSS1 position 6,629 in the IG region, 34 nt upstream from ORF1. However, the nucleotide

sequence around this ϕRSS1 showed no significant homology to the core sequences involved in the XerC/D recombination system (Kawasaki et al., 2007a). Tasaka et al. (unpublished data) further characterized the nucleotide sequences in the neighborhood of the ϕRSS1-integration site in strain C319. It was revealed that ϕRSS1 is a truncated form of a larger phage (designated as ϕRSS0) of 7,288 nt in size, 626 nt larger than ϕRSS1 (Fig. 2A). The 626 nt ϕRSS0 sequence missing from ϕRSS1 DNA contains two nucleotide sequence elements that repeat at both *att*L and *att*R, the latter of which has the sequence 5′-TATTT AACAT AAGAT AAAT, corresponding to *dif* of *R. solanacearum* (Carnoy and Roten, 2009). Thus, ϕRSS0 is integrated at a *dif* site, similarly to CTXϕ of *Vibrio cholerae*, which uses the host XerC/D recombination system (Huber and Waldor, 2002). Interestingly, one ϕRSS0 ORF (ORF13; 156 amino acid residues), located within the region missing in the ϕRSS1 DNA, shows sequence homology to DNA-binding phage regulator (accession no. B5SCX5, E-value 1e-29). This may function as a phage repressor for immunity, because C319 (ϕRSS0 lysogen) is resistant to second infection by ϕRSS0 (Table 2). C319 is susceptible to ϕRSS1, thus ϕRSS1 (without ORF13) may be an escaped superinfective phage.

Another form of ϕRSS1-type prophage was found in strain MAFF106601 (Tasaka et al., unpublished data). This prophage (designated as ϕRSS3) has an extended size (8,193 nt) caused by an insertion of an IS1405 element (transposase) within the IG (positions 6,250-6,251 of ϕRSS1). The map of ϕRSS3 is compared with other related phages in Fig. 2A. This phage was active when its genomic DNA was amplified from the MAFF106601 genome by PCR and introduced into MAFF106603 as a host. It is interesting to note that ϕRSS3 may exploit the transposase to move or transmit itself among genomes. A chimeric form of a ϕRSS1-type phage is seen in the structure of ϕRSS4, a prophage in strain MAFF211271 (Kawasaki et al., unpublished data). This phage (7,618 nt in size) is integrated at the host gene for arginine tRNA (CGG) as *att*B and it contains a 58 nt sequence corresponding to the 3′-portion of arginine tRNA (CGG) as *att*P. An ORF of 363 aa residues is closely associated with *att*P and this unit (*att*P/Int) is almost identical to that of ϕRSA1 (Fujiwara et al., 2008), a P2-type myovirus as described below. The map of ϕRSS4 is shown in Fig. 2A. As can be seen, an approximately 2.2 kb region (corresponding to the structure module) containing ORF4 to ORF8 and an ORF of assembly-secretion module (ORF9) are almost identical between ϕRSS1 and ϕRSS4, but most of the replication module in ϕRSS4 is replaced with that of phage P12J infecting *Ralstonia pickettii* (accession no. AY374414). The replication module of ϕRSS4 is inserted with an ORF (ORF2) and attP; ORF 2 shows amino acid sequence similarity to *Burkholderia pseudomallei* bacteriophage integrase (accession no. Q63PM9, E-value e-100). A similar, but significantly smaller, phage, PE226, infecting *R. solanacearum* strains was recently reported (Murugaiyan et al., 2010). The ϕRSS4 structure suggests that ϕRSS phages can exchange each of the Ff-phage modules (Model and Russel, 1988) among related phages and evolve to a new characteristic phage.

2.2 ϕRSM phages of the *Inoviridae*

ϕRSM1 was isolated from a soil sample and can form small plaques on assay plates with limited strains (7 of 18 strains tested) as a host. ϕRSM1 particles show a long fibrous shape of 1400 ± 300 μm in length and 10 ± 0.7 nm in width by electron microscopy (Fig. 1), giving a shape similar to coliphage M13 (Buchen-Osmond, 2003; ICTVdB). The infection cycle of ϕRSM1 phage resembles that of ϕRSS1. The genome of ϕRSM1 is 9,004 nt long (DDBJ

accession No. AB259123) with a GC content of 59.9%, which is lower than that of *R. solanacearum* GMI1000 (66.97%). There are 12 putative ORFs located on the same strand and two on the opposite strand. Compared with the conserved gene arrangement of Ff-like phages, the ϕRSM1 genes can be drawn as Fig. 2B (Kawasaki et al., 2007a). Here, ORF13 and ORF14 (reversely oriented) are inserted between ORF11, corresponding to pII as a replication protein, and ORF1, corresponding to an ssDNA-binding protein like pV, in the putative replication module. ORF13 and ORF14 show amino acid sequence similarity to a proline-rich transmembrane protein and a resolvase/DNA invertase–like recombinase, respectively. There are two additional ORFs (ORF2 and ORF3) between the replication and structural modules. The functions of these ORF-encoded proteins are not known. In genomic Southern blot hybridization, two different types of ϕRSM1-related prophage sequences were detected in *R. solanacearum* strains. Strains of type A include MAFF211270 and produce ϕRSM1 itself, and strains of type B (giving different restriction patterns) are resistant to ϕRSM1 infection, but serve as hosts for ϕRSS1 with different nature and host range as described above (Kawasaki et al., 2007a). By determining the nucleotide sequences of junction regions of the ϕRSM1-prophage in the MAFF211270 chromosomal DNA, an *att*P/*att*B core sequence was identified as 5′-TGGCGGAGAGGGT-3′, corresponding to positions 8,544-8,556 of ϕRSM1 DNA, located between ORF14 and ORF1. Its nucleotide sequence is identical to the 3′-end of the host *R. solanacearum* gene for serine tRNA(UCG) in the reverse orientation. By PCR with appropriate primers containing these *att* sequences, a ϕRSM1-like prophage (type B) in strain MAFF730139 (designated ϕRSM3) was obtained (Askora et al., 2009). Compared with the ϕRSM1 genome, the ϕRSM3 prophage sequence (8,929 bp) is 75 bp shorter. The sequences show 93% nucleotide identity and major differences are found within two regions; positions 400-600 and positions 2,500-3,000 in the ϕRSM1 sequence. The former region corresponds to ORF2, which is inserted between the replication module (R) and the structural module (S), and the latter falls into the possible D2 domain of ORF9 (pIII), as described below. All 14 ORFs identified along the ϕRSM3 sequence show high amino acid sequence homology (more than 90% amino acid identity) with their counterparts on the ϕRSM1 genomic DNA, except for ORF2 (no similarity) and ORF9 (79% identity). It is interesting that the amino acid sequence of ORF14 (putative DNA invertase/recombinase) is 100% identical in the two phages. The gene arrangements are compared in Fig. 2B. During database searches for homologous sequences, we found that an approximately 8 kbp region of the *R. solanacearum* UW551 genome (accession no. DDBJ/EMBL/GenBank AAKL00000000; RS-UW551-Contig0570-70-86K) at positions 3,039-10,984 shows significant DNA sequence homology with ϕRSM1 and ϕRSM3. The prophage of UW551 (designated ϕRSM4, Askora et al., 2009) contains 7,929 bp flanked by *att* sequences, as in ϕRSM1, comprising 13-bp of the 3′-end of serine tRNA (UCG). The 7,929-bp ϕRSM4-prophage sequence shows 72% nucleotide sequence identity with ϕRSM1 DNA. There are three deletions in ϕRSM4 compared with the ϕRSM1 gene arrangement; ORF3, ORF13, and part (positions 5,370-5,800) of the intergenic region (IG) are missing. Other ORFs identified in the ϕRSM4 sequence show variable amino acid sequence similarity to their counterparts in ϕRSM1: no similarity in ORF2, moderate similarity in ORF4 (57% amino acid identity), ORF5 (77%), and ORF9 (73%), and high similarity in ORF1 (93%), ORF6 (98%), ORF7 (98%), ORF8 (93%), ORF10 (86%), ORF11 (89%), ORF12 (89%), and ORF14 (81%). The gene arrangement of ϕRSM4 prophage DNA is also included in the comparison shown in Fig. 2B.

One of the major differences in the predicted genes between ϕRSM1 and ϕRSM3 is confined within the middle part of ORF9, corresponding to pIII, the host recognition and adsorption minor coat protein. Interestingly, this internal region of ϕRSM3 (also 67% identical in ϕRSM4) shows a significant similarity (79%) to the corresponding region of ϕRSS1, which shows a different host range from ϕRSM1 (Yamada et al., 2007). These results predict that ϕRSM3 and ϕRSM4 share a common host range, which differs from that of ϕRSM1, and suggest that the host range determined by the pIII-D2 domain may be exchangeable among phages. This was experimentally confirmed. The circularized ϕRSM3 PCR product was introduced into MAFF211272 and resulting phage with the expected DNA restriction pattern was subjected to host-range assay. The results are summarized in Table 2. The host range of ϕRSM3 is incompatible with that of ϕRSM1; namely ϕRSM1-susceptible strains are resistant to ϕRSM3 and vice versa, except for strains Ps74 and MAFF211270, which are susceptible to both phages. ϕRSM3 has a wider host range, and all 15 strains are susceptible to either ϕRSM1 or ϕRSM3. The host range of ϕRSM3 partially overlaps with that of ϕRSS1 (Yamada et al., 2007), but ϕRSS1 recognizes only ten strains (Table 2), suggesting the involvement of some additional factors in ϕRSS1 host recognition. To confirm the host recognition by the D2 domain of pIII in ϕRSM1 and ϕRSM3, this domain of ϕRSM1 was replaced with the corresponding region of ϕRSM3. The resulting phage (designated as ϕRSM13) did not retain the host range of ϕRSM1 but showed the exactly same host range as that of ϕRSM3 (Table 2). The entire nucleotide sequence of ϕRSM13 DNA is completely the same as that of ϕRSM1, except for a 350-bp region corresponding to the pIII-D2 of ϕRSM3; therefore, the change of host range must have been determined by the D2 domain of pIII of ϕRSM3 (Askora et al., 2009). The differences in the pIII-D2 domain among ϕRSM phages correspond well to the strain-specific pili types. A minor pilin of approximately 30 kDa varies in size, depending on the strain; slightly smaller proteins correspond to ϕRSM1-susceptible strains and slightly larger proteins correspond to ϕRSM3-susceptible strains (Askora et al., 2009).

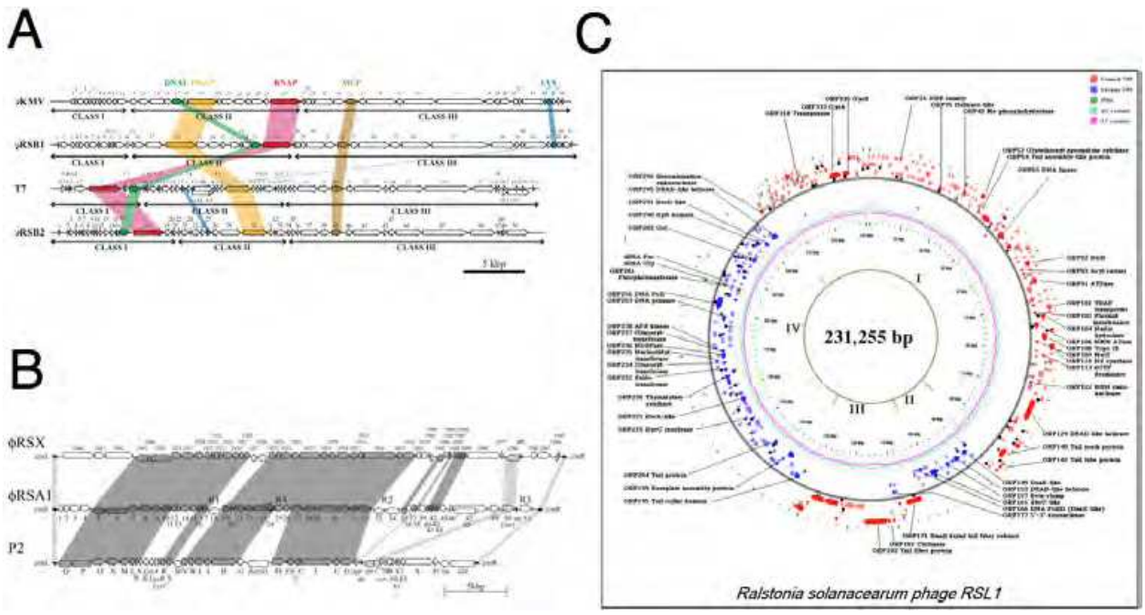
As described above, the genomes of ϕRSM phages are sometimes found to be integrated in the host genome. Askora et al. (2011) demonstrated that the integration is mediated by the phage-encoded recombinase (ORF14 of ϕRSM1), which has significant homology to resolvases/DNA invertases (small serine recombinases) at the sites, *att*P/*att*B corresponding to the 3′ end of the host serine tRNA (UCG) gene in the reverse orientation. The same unit of integration (ϕRSM Int/*att*P) was found in a *R. pickettii* 12J phage and in *B. pseudomallei* 668 prophages. Together with these phages, it is not surprising that similar *int*-containing filamentous phages occur widely in the natural world.

2.3 ϕRSB phages of the *Podoviridae*

ϕRSB1 was isolated from a soil sample from a tomato crop field and was selected for its ability to form large clear plaques on plate cultures of *R. solanacearum* strain M4S. Plaques formed on assay plates were 1.0 to 1.5 cm in diameter. This phage has a wide host range and infected 15 of 18 strains tested, including strains of races 1, 3, and 4, and of biovars 3, 4, and N2. Under laboratory conditions, host cells of *R. solanacearum* strains lyse after 2.5 to 3 h postinfection (p.i.) (with an eclipse period of 1.5 to 2 h), releasing approximately 30 to 60 pfu of new phage particles per cell (burst size). Electron microscopic observation of negatively

stained phage particles revealed short-tailed icosahedral structures resembling those of the family *Podoviridae*. The particles consist of a head of approximately 60 nm in diameter and a stubby tail of 20 nm in length (Fig. 1). The ϕRSB1 genome is linear double-stranded DNA of 43,079 bp and includes direct terminal repeats of 325 bp (accession no. AB276040). The G + C content of the genome is 61.7%. This value is lower than the G + C values of the large and small replicons of the *R. solanacearum* GMI1000 genome (67.04% and 66.86%, respectively) (Salanoubat et al., 2002). A total of 47 potential ORFs oriented in the same direction were assigned on the genome (Fig. 3A). Patchy or local nucleotide sequence homologies were detected in the genomic sequences of various phages, including *Xanthomonas oryzae* phages Xop411 (accession no. DQ777876) and Xp10 (accession no. AY299121) (Yuzenkova et al., 2003), *Pseudomonas aeruginosa* phages ϕKMV (accession no. AJ505558) (Lavigne et al., 2003), *Erwinia amylovora* phage Era103 (accession no. EF160123), and *Burkholderia cenocepacia* phage BcepB1A (accession no. AY616033). All of these are members of the family *Podoviridae*. The genome of coliphage T7, the representative of T7-like viruses of the *Podoviridae*, generally consists of three functional gene clusters: one for early functions (class I), one for DNA metabolism (class II), and the other for structural proteins and virion assembly (class III) (Dunn & Studier, 1983). These gene clusters are essentially conserved in the ϕRSB1 genome. Figure 3A shows the putative ORFs identified on the ϕRSB1 genome compared with ORFs from other phages: *Xanthomonas* phage Xop411 (giving the highest local similarities), *Pseudomonas* phage ϕKMV (showing marginal similarity but longest regions of similarity), and coliphage T7. One of the characteristic features found in the ϕRSB1 gene organization is that the predicted gene for RNA polymerase (RNAP) of ϕRSB1 (*orf26*) is not located in the early gene region (class I), but at the end of the class II region (Fig. 3A). Another exception is the gene for DNA ligase (DNAL), *orf25*, encoding the ϕRSB1 DNAL, is in 5′ to the RNAP ORF (*orf26*), whereas the gene encoding T7 DNAL is downstream of the gene for RNAP, at the end of the class I cluster (Dunn & Studier, 1983). In *Pseudomonas* phages ϕKMV, LKD16, and LKA1, the DNAL gene is upstream of the gene for DNA polymerase in the class II gene cluster (Fig. 3A). T7-like phages are generally known as absolute lytic phages, with a few exceptions, such as integrase-coding phages, e.g., prophage 3 of *Pseudomonas putida* (Molineux, 1999) and the cyanophage P-SSP7 (Lindell et al., 2004). Occasionally, nucleotide sequences related to T7-like phages are found in conjunction with other temperate phages, such as λ-like phages that are integrated in various bacterial genomes (Brussow et al., 2004; Canchaya et al., 2003; Casjens, 2003; Hendrix et al., 2003). A sequence highly homologous to ϕRSB1 was found to be embedded in a large (85-kbp) λ like prophage sequence (ϕ1026b) integrated in the genome of *Burkholderia pseudomallei* 1710b (accession no. CP000124). The homologous region of the 1710b prophage contains eight ORFs encoding DNA primase, DNA helicase, DNAL, DNA polymerase, exonuclease, and RNAP, etc. These correspond to the class II genes of ϕRSB1, as shown in Fig. 3A. Both *Ralstonia* and *Burkholderia* belong to the Betaproteobacteria and may share common bacteriophages (Fujiwara et al., 2008).

Searching for core promoter-like sequences conserved in phages T3, T7, or SP6 in the ϕRSB1 intergenic regions did not reveal any significant homologies. Instead, a set of sequence elements (possible promoter elements) consisting of a GC-rich stretch and TTGT, TCTGG, and CGGGCAC motifs preceding an AG-rich Shine-Dalgarno sequence were found. The activity of transcriptional promoters of such elements on the ϕRSB1 genome was demonstrated using a green fluorescent protein (GFP)-expressing single-copy plasmid,



T7-like arrangements of 47 ORFs identified on the ϕRSB1 genome (43,079 bp, Kawasaki et al., 2009) and 50 ORFs on the ϕRSB2 genome are compared with those of E. coli T7 (39,937 bp, NC_00164) and *Pseudomonas aeruginosa* ϕKMV (42,519 bp, AJ50558)(A). According to the T7-gene-organaization, ORFs are grouped into three classes (Kawasaki et al., 2009). Corresponding major ORFs are connected by shading (DNAL, DNA ligase; DNAP, DNA polymerase; MCP, major capsid protein; LYS lysozyme). P2-like arrangement of 51 ORFs on the ϕRSA1 genome (38,760 bp, Fujiwara et al., 2008) and 52 ORFs on ϕRSX (40,713 bp), a prophage found on the genome of strain GMI1000 (Salanoubat et al., 2002). Shading indicates similar regions among the phages (B). In the map of ϕRSL1 genome (231,255), a total of 343 ORFs are grouped into 4 genomic regions according to their clustering with the same orientation (C).

Fig. 3. Genomic organization of ϕRSB1-related phages (A), ϕRSA1-related phages (B), and ϕRSL1 (C).

pRSS12 (Kawasaki et al., 2007b). A switch from host RNAP to ϕRSB1 RNAP occurs between 75 min p.i. and 90 min p.i., and the late stages of ϕRSB1 replication are independent of rifampin. A phage, ϕRSB3, from our phage collection also showed a similar gene arrangement to ϕRSB1, but its genome size is significantly larger (tentatively 44,242 bp) than ϕRSB1 and the nucleotide sequence similarity are entirely marginal between the two phages. The host range of ϕRSB3 is narrower (5 of 15 strains tested are susceptible). Another phage in our collection, which was obtained from a potato field, gave large clear plaques with 10 of 15 strains tested as hosts. Electron microscopy revealed a typical podoviral morphology of this phage (designated as ϕRSB2), an icosahedral head of 45 ± 5 nm in diameter, and a short tail of 12.5 ± 2 nm in length. The ϕRSB2 genome is linear double-stranded DNA of 40,411 bp and includes direct terminal repeats of 214 bp (accession no. AB597179). The G + C content of the genome is 61.7%. A total of 50 ORFs were identified along the genome. Homology searches through the databases revealed a general organization of this phage similar to T7-like phages, consisting of three gene classes; Class I (ORF1-ORF21), Class II (ORF22-ORF34), and Class III (ORF35-ORF50) (Fig. 3A). In contrast to ϕRSB1, the gene for RNAP associated with *dnal* (encoding DNAL) is located within Class I. The position of *lys* (encoding lysozyme) is within Class II in ϕRSB2, whereas it is near the right terminus in ϕRSB1.

Searching for core promoter-like sequences conserved in phages T3, T7, or SP6 in the ϕRSB2 intergenic region identified 14 elements with a consensus sequence 5′ ATTAACCCACACTRYAGGARRRS. The actual activity of the transcriptional promoters of some of these elements was demonstrated using GFP-expressing single-copy plasmid, pRSS12 (Kawasaki et al., unpublished data).

In early studies to control *R. solanacearum*, a few bacteriophages were isolated and their physical and physiological properties were partially characterized. The virulent phage P4282, and related phages, have a polyhedral head (69 nm in diameter) and a short tail (20 nm in length) (Tanaka et al., 1990), and contain a 39.3-kbp dsDNA genome (Ozawa et al., 2001), giving characteristic features of podoviruses. Another phage, PK-101, was isolated from soil, and characterized to have a linear 35-kbp dsDNA genome (Toyoda et al., 1991). The morphological feature of this phage is unknown. Both of these phages show very narrow host ranges and infect only a few strains of *R. solanacearum*. A recently isolated phage, ϕRSB4 showed a typical podoviral morphology by electron microscopy. Nucleotide sequence of a 40 kbp DNA fragment from the ϕRSB4 genome perfectly accorded with that of the P4282 gene encoding bacteriolytic protein (accession no. AB048798) (Ozawa et al., 2001). Genomic characterization of this phage is now underway.

2.4 ϕRSA phages of the *Myoviridae*

ϕRSA1 spontaneously appeared from strain MAFF211272 (Yamada et al., 2007). It consists of an icosahedral head of 40 ± 5 nm in diameter, a tail of 110 ± 8 nm in length and 3 ± 0.2 nm diameter, and a tail sheath (40 ± 6 nm in length and 17 ± 1.5 nm in diameter) located at the bottom of the tail (Fig. 1). This generates a racket-frame-like structure that resembles the morphology reported for *Burkholderia cepacia* phage KS5 (Seed and Dennis, 2005; Lynch et al., 2010). A tail sheath was often observed attached at the bottom of the tail, but sometimes at intermediate position along the tail, suggesting a movable nature of the sheath along the tail. Sometimes, structures resembling the tail sheath were observed connected in a chain.

ϕRSA1 has a 38,760-bp dsDNA genome (65.3% G + C) with a 19-bp 5′-extruding cohesive end (cos). The genome contains 51 open reading frames (accession no. AB276040, Fujiwara et al., 2008). Two-thirds of the ϕRSA1 genomic region on the left side encodes the phage structural modules, which are very similar to those of coliphage P2 and P2-like phages (Fig. 3B). Genes for DNA replication, host lysis, and regulatory functions have been identified on the genome, but there are no apparent pathogenesis-related genes. A late-expression promoter sequence motif (Ogr-binding sequence) was predicted for the ϕRSA1 genes as 5′ TGTTGT-$(X)_{13}$-ACAACA. It is interesting to compare this sequence with that identified for the P2 family members as TGT-$(N)_{12}$-ACA (Julien & Calendar, 1995). The entire genomic sequence of ϕRSA1 showed significant similarity to the genomes of *Burkholderia pseudomallei* phage ϕ52237 (accession no. DQ087285) and *Pseudomonas aeruginosa* phage ϕCTX (accession no. AB008550, Nakayama et al., 1999). Extended comparison of the ϕRSA1 sequence with these phage sequences by the matrix plot method revealed characteristic features of the phage gene organization: the sequence homology was broken by small AT-islands (designated R-regions). One such region (R2) on the ϕRSA1 DNA contained an ORF (ORF34) showing 100% amino acid sequence identity with transposase ISRSO15, which was found in the chromosomal DNA (positions 2,780,153 to 2,781,370; accession no. AL646070) as well as

the megaplasmid DNA (positions 111,895 to 113,185; accession no. AL646085) of *R. solanacearum* GMI1000. ORF34 is on an IS of 1,319 bp with a terminal repeat of seven A residues. These regions might have been horizontally transferred and been serving as anchor points for genome rearrangements. ϕRSA1 uses the lipopolysaccharide core as a receptor site on the cell surface and requires Ca^{2+} ions to bind to the receptor. Its lifecycle takes 60–90 min for one round and the burst size is approximately 200 pfu/cell. Like P2 phages, ϕRSA1 encodes an ORF for integrase and *att*P, suggesting a lysogenic cycle. In fact, a ϕRSA1-like phage was found to be integrated into at least three different strains of *R. solanacearum*. In addition, the chromosomal integration site (*att*B) was identified as the 3′ portion (45 bases) of the arginine tRNA (CCG) gene (Fujiwara et al., 2008). However, □RSA1 can also infect strains that contain a lysogenized ϕRSA1-like phage; all 18 strains of *R. solanacearum* tested were susceptible to this phage. Therefore, ϕRSA1 itself may be a kind of immunity-deficient or super-infective phage. This property of ϕRSA1 is very important for avoiding the problem of lysogenization by a therapeutic phage, and thus avoids passive import of pathogenicity-related genes (Merril et al., 2003; Brussow 2005). Compared to cells (strain M4S) without ϕRSA1 sequences, the lysogenic cells (newly established with ϕRSA1-original phage) showed no obvious changes in growth rate, cell morphology, colony morphology, pigmentation, or extracellular polysaccharide production in culture. No obvious enhanced pathogenicity has been observed so far with ϕRSA1-related lysogenic cells by plant virulence assays in tobacco plants. It is interesting to note that a prophage previously detected on the genome of *R. solanacearum* strain GMI1000 (Salanoubat et al., 2002) was characterized as a ϕRSA1-related prophage (ϕRSX) in the light of the ϕRSA1 genome sequence. The exact size of ϕRSX is 40,713 bp, and its ORFs share very high amino acid identity with their ϕRSA1 counterparts. The ϕRSX attachment site corresponds to a 15-base 3′ portion of the serine tRNA (GGA).

2.5 ϕRSL phages of the *Myoviridae*

ϕRSL1 is a large-tailed phage (jumbo phage) belonging to the family *Myoviridae*, and was isolated from crop fields (Yamada et al., 2007; Yamada et al., 2010). Phage particles consist of a 125 nm diameter icosahedral head and a long contractile tail that is 110 nm long and 22.5 nm wide (Fig. 3C). ϕRSL1 gives clear plaques with various strains of race 1 and 3 and biovar 3 and 4; 17 of 18 strains tested were susceptible to this phage. The infection cycle of ϕRSL1 with strain M4S as a host has an eclipse phase of 90 min and a latent period of 150 min, followed by a rise period of 90 min (Yamada et al., 2010). The average burst size is 80-90 pfu per infected cell. One characteristic feature of ϕRSL1 infection is a lasting host killing effect. The large□ϕRSL1 dsDNA genome of 231,255 bp (G + C = 58.2%) contains 343 ORFs and three tRNA genes (including one pseudogene) (accession no. AB366653). According to the orientation of the ORFs, four major genomic regions are apparent (Fig. 3C): The largest, region I (114.5 kbp, G + C = 58.3%), encodes 193 ORFs mostly located in a clockwise orientation. Region II (15.8 kbp, G + C = 57.4%) encodes 24 ORFs all in a counter clockwise orientation. Region III (27.9 kbp, G + C = 57.4%) encodes 23 ORFs, 19 clockwise and four counterclockwise). The last, and second largest, region is region IV (73.1 kbp, G + C = 58.4%), which encodes 102 ORFs mostly located in a counterclockwise orientation. ORFs are generally tightly organized, with little intergenic space. In many cases, the stop codon of an ORF was found to overlap the start codon of the following ORF. ϕRSL1 ORFs showed no detectable similarity at the nucleotide level to other viruses or cellular organisms. The

proportion of ORFans (i.e. genes lacking detectable homologs in the current databases) was very high; of the 343 ORFs, 251 ORFs (73%) showed no significant sequence similarity in the publicly available databases (E-value < 0.001). Of 83 homologs in UniProt or in the NCBI environmental sequence collection, 53 ORFs (15.5%) showed best sequence similarities in bacteria, 10 ORFs (2.9%) in viruses/plasmids, one ORF (0.3%) in eukaryotes, one ORF (0.3%) in archaea, and 18 ORFs (5.3%) in environmental sequences. It is notable that only a few ϕRSL1 ORFs were similar to sequences in *R. solanacearum* spp., given that the genomic sequences are available for strain GMI1000 and UW551. Viruses/plasmid best hits include five homologs in myoviruses, three in siphoviruses, one in a eukaryotic virus (Mimivirus), and one in a plasmid. These results suggest that ϕRSL1 may have access to the gene pools of largely different families of bacteria and viruses. At present, putative functions have been assigned to 47 ϕRSL1 ORFs by examination of homologous search results, including enzymes for the salvage pathway of NAD^+ and for the biosynthetic pathways of lipid, carbohydrates, and homospermidine in addition to proteins required for phage replication (Yamada et al., 2010). A chitinase-like protein was found to be a potential lysis enzyme. Expression patterns of these ϕRSL1 genes were characterized using a DNA microarray during the infection cycle. Most genes showing early expression (10-30 min p.i.) and later repression by 90 min p.i. (designated as early-intermediate genes) were confined within region I. In contrast, genes that showed increased expression during 30-90 min p.i. (designated as intermediate-late genes) are mostly concentrated around both extremities of region I, as well as in the entire region IV. The intermediate-late genes also included several genes located in region II and region III. Putative genes for phage structural proteins are intermediate-late genes. Genes involved in DNA metabolism are also intermediate-late, except for ORF065, which encodes NAD^+-dependent DNA ligase. Putative promoter elements for these differential genes' expressions have been identified (Yamada et al., 2010).

Several myoviruses are known to have large genomes over 200 kbp, and are designated as "jumbo phages" (Hendrix, 2009). These include *Pseudomonas aeruginosa* phage ϕKZ (280 kbp, Mesyanzhinov et al., 2002), EL (211 kbp, Herveldt et al., 2005), and ϕPA3 (309 kbp, Monson et al., 2011), *Vibrio parahaemolyticus* phage KVP40 (245 kbp, Miller et al., 2003), *Stenotrophomonas maltophila* phage ϕSMA5 (250 kbp, Chang et al., 2005), and *Yersinia enterocolitica* phage R1-37 (270 kbp, Kiljunen et al., 2005). Jumbo phages were also reported for *Sinorhizobium meliloti* (phage N3, 207 kbp, Martin and Long, 1984) and *Bacillus megaterium* (phage G, 670 kbp, Sun and Serwer, 1997). According to their large genomes, they contain many genes, most of which are unknown and might function in the interaction with their hosts. Jumbo phages have only recently been identified and there is too little information about them to discuss their interrelationships. Currently, only a handful of jumbo phages have been isolated. Jumbo phages might have been missed in ordinary screenings because of their large size: they diffuse too slowly in the top agar gels typically used to plaque phages. Reducing the top agar concentration from 0.7-0.8% to 0.45-0.5% allowed us to plaque ϕRSL1. A similar observation was reported by Serwer et al. (2007) for *Bacillus* phage G. It is very likely that more jumbo phages will be isolated by adapting these plaquing conditions. ϕRSL1 is highly virulent, and shows no sign of genomic integration, both of these properties making it suitable for use as a biocontrol agent. Two specific advantages of this phage are its abundant yields (10^{11} pfu/ml) and easy purification using only centrifugation (15,000 × g) from routine cultures.

2.6 ϕRSC phages of the *Siphoviridae*

ϕRSC1 spontaneously appeared from a culture of strain MAFF301558. It gave turbid plaques with 15 of 18 strains tested as host (Table 1). Genomic Southern blot hybridization showed that two resistant strains, MAFF211271 and MAFF301558, are lysogenic with ϕRSC1 and that ϕRSC1 was easily induced by UV irradiation. These strains were isolated from wilted potato and classified as strains of race 3, biovar N2, and phylotype IV. Electron microscopic observation of negatively stained ϕRSC1 particles revealed an icosahedral head of 48 ± 3 nm in diameter, a non-contractile tail of 220 ± 15 nm in length, and tail fibers of 30 ± 2 nm, giving a λ-like morphology (Fig. 1). ϕRSC1 gave a single 40 kbp DNA band by pulsed-field gel electrophoresis, indicating a 40 kbp linear DNA as the genome. Partially determined nucleotide sequences of ϕRSC1 DNA fragments showed high homology with prophage sequences of *R. solanacesrum* strains; for example, RSc0863 and RSc0875 of strain GMI1000, RSMK00228 of strain Molk2, and RSIPO_02158 of strain IPO1609. Effects of lysogenic integration of ϕRSC1 on the host strain are largely unknown.

2.7 Other prophages

Recent genomic analyses of several strains of *R. solanacearum* belonging to different races, biovars, and/or phylotypes revealed many strain specific gene clusters (Remenant et al., 2010). Some of these variable regions contained phage-like sequences. There are five phage-related sequences in the chromosome of strain GMI1000 (race 1, biovar 3, and phylotype I), two of which corresponded to ϕRSX (RSc1896-RSc1948) and ϕRSC1-like sequences (around RSc0863-RSc0967) (accession no. AL646052), as described above. Another region around RSc1680-RSc1696 resembles λ-like phage HK022 and *Bacillus subtilis* temperate phage ϕ105. In the chromosome of strain UW551 (race 3, biovar 2, and phylotype II), a cluster of 38 probable prophage genes (RRSL02400-RRSL02437) is remarkable (accession no. NZ_AAKL00000000). This gene cluster was present in all race 3/biovar 2 (R3B2) strains tested, from a wide variety of geographical sources (Gabriel et al., 2006). As described above, ϕRSM4 is also a filamentous prophage of the ϕRSM group. There are at least two possible prophage regions in the chromosome of strain IPO1609 (race 3, biovar 2, and phylotype IIB); the region around RSIPO02143-RSIPO002171 (resembling ϕRSC1) and that around RSIPO04993-RSIPO05020 (accession no. CU694438). Four regions of the chromosome of strain Molk2 (race 2, biovar 1, and phylotype IIB) also contained prophage sequences (accession no. CU644397); regions around RSMK00219-RSMK00259, RSMK01452-RSMK01464 (ϕ105-like), RSMK01633-RSMK01646 (HK022-like). The recently reported genomes of three other strains, belonging to different phylotypes, contained 2-4 prophage sequences, including strains CMR15 (phylotype III, accession no. FP885895), PSI07 (phylotype IV, accession no. FP885906), and CFBP2957 (phylotype IIA, accession no. FP885897).

It is becoming increasingly clear that phages play important roles in the evolution and virulence of many pathogenic bacteria (Canchaya et al., 2003; Brussow et al., 2004). Phages are important vehicles for horizontal gene exchange between different bacterial species, as well as between different strains of the same species. When a temperate phage integrates into the host genome (lysogenization), it may affect the host cells in several ways: (i) disrupting host genes, (ii) changing the expression levels of host genes, (iii) serving as recombinational hot spots, (iv) protection from lytic infection, (v) lysis of the cells by prophage induction, and (vi) introduction of new fitness factors (lysogenic conversion

genes). Such lysogenic conversion genes may change the host phenotype drastically. Such lysogenic conversion genes (cargo genes) are sometimes called "morons". Morons are not required for the phage life cycle. Instead, many morons from prophages in pathogenic bacteria encode proven or suspected virulence factors. Therefore, prophages, especially those found commonly in a certain group of *R. solanacearum*, are vital for understanding the specific virulence of such a group. Through identification and characterization of lysogenic conversion genes, pathogenesis mechanism of *R. solanacearum* will be clarified.

3. Phage prophylaxis and treatment of bacterial wilt

For phage biocontrol or therapy, only lytic phages are usually used, thereby avoiding the problem of lysogeny. A phage cocktail has been recommended to prevent the problem of resistance, which contains several phages with different host specificities, different replication mechanisms, and/or different infection cycles (Gill and Abedon, 2003; Jones et al., 2007). Fujiwara et al. (2011) used three lytic phages, ϕRSA1, ϕRSB1, and ϕRSL, for biocontrol of tomato bacterial wilt caused by *R. solanacearum*. Although ϕRSA1 and ϕRSB1 infection resulted in quick lysis of host cells, multi-resistant cells arose approximately 30 h post infection. By contrast, cells infected solely with ϕRSL1 kept a steady low level of cell density for a long period. Under laboratory culture conditions, when host *R. solanacearum* cells were quickly lysed by treatment with ϕRSA1 or ϕRSB1, resistant cells (presumably pre-existing in the population at a very low frequency) were raised after 30 h post infection (pi). Killing susceptible cells, the majority of the cell population, by phages may allow minor cells to predominate in subsequent generations. The recovering cells were somehow resistant to both ϕRSA1 and ϕRSB1: mixed treatment with these phages resulted in the same killing and recovering pattern of bacterial cells as did sole treatment. The resistance mechanisms used by these cells are unknown. A cocktail containing three phages, ϕRSA1, ϕRSB1, and ϕRSL1, also failed to stably prevent bacterial growth. By contrast, cells infected solely with ϕRSL1 kept a steady low level of cell density for a long period. Pretreatment of tomato seedlings with ϕRSL1 drastically limited the penetration, growth, and movement of inoculated bacterial cells. Treated plants survived for as long as four months. Either ϕRSA1 or ϕRSB1, which kills cells quickly, could not bring about similar plant-protecting effects. Using these observations, Fujiwara et al. (2011) proposed 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 completely, but a sustainable state of phage-bacteria coexistence (with a low level of bacterial population) is maintained.

Phages are utilized for controlling plant pathogens either in the rhizosphere or phylosphere. Application of phages to the phylosphere, namely directly to aerial tissues of the plant, must involve a serious phage stability problem (Jones et al., 2007). Field and laboratory studies have demonstrated that phages are inactivated rapidly by exposure to sunlight (UV-A and UV-B, 280-400 nm), high temperature, high and low pH, oxidative conditions, and washing-down by water (Ignoffo and Garcia, 1992, Iriarte et al., 2007). However, in the case of bacterial wilt, phages for biocontrol can be applied to the rhizosphere. Sunlight UV, the most destructive environmental factor, and oxidative inactivation, can be relieved in this case. ϕRSL1 was shown to be relatively stable in soil conditions, especially at higher temperatures (bacterial wilt occurs at higher temperatures). Prolonged disease control may be possible if ϕRSL1 is applied to plants at the seedling stage.

There is another method of phage biocontrol of bacterial wilt. As described above, filamentous ϕRSM phages cause loss of virulence in the infected host cells. Infection with ϕRSM phages does not cause host cell lysis, but establishes a persistent association between the host and phage, releasing phage particles from the growing and dividing host cells. Therefore, these phages are also good candidates for bacterial wilt biocontrol agents. In addition, ϕRSM-infected bacterial cells protect their pre-treated tomato plants from a second infection of virulent cells (Addy et al., unpublished data). Once plants are treated with ϕRSM-infected cells, the prevention effect lasts for up to two months. Two months after treatment with ϕRSM-infected cells, the second infected virulent cells could not cause wilting symptoms. Using a mixture of ϕRSM1 and ϕRSM3 seems to be especially effective, because the two phages have different host ranges (complementary to each other) and most strains of different races and/or biovars are expected to be susceptible to either of the two phages (Askora et al., 2009). Furthermore, plants inoculated with ϕRSM-infected *R. solanacearum* cells showed stable resistance to virulent bacterial cells inoculated thereafter. This resistance was induced as early as one day post inoculation and lasted for up to two months. Therefore, ϕRSM phages also give an additional possibility to prevent bacterial wilt, namely utilization as "vaccine against bacterial wilt of many crops".

An additional potential application is the use of phage genes or gene products as therapeutic agents (Loesnner, 2005; Hermoso et al., 2007; Fischetti, 2005). Ozawa et al. (2001) isolated the bacteriolytic gene from a *R. solanacearum* phage P4282. The 71 kDa phage protein consists of 687 amino acids and showed strong bacteriolytic activities against several field-isolated strains of *R. solanacearum*. Although the biochemical and enzymatic nature of this protein is not fully characterized, homologous sequences are integrated in several bacterial genomes (database accession no. A1H7Z4 and A4JD43). This phage gene was suggested to be useful for generating transgenic plants that are resistant to bacterial wilt (Ozawa et al., 2001). Phage-encoded endolysin, which disrupts the peptidoglycan matrix of the bacterial cell wall, and phage-encoded holins, which permeabilize the bacterial membrane, could also be effective against bacterial pathogens. Practically, these phage proteins are probably not effective for field use, but could have applications in local plant therapy or disease prevention.

4. Detection of *R. solanacearum* cells in plants and soil and diagnosis of bacterial wilt

Effective bacterial diagnosis is always required for successful biocontrol. A variety of methods have been developed to detect *R. solanacearum*, including typical bioassays, dilution plating on semi-selective media, fatty-acid analysis, immunofluorescence microscopy, enzyme-linked immunosorbent assay (ELISA), and polymerase chain reaction (PCR) (Janse,1988; Seal et al. 1993; Elphinstone et al., 1996; Van der Wolf et al., 2000; Weller et al., 2000; Priou et al., 2006; Kumar et al., 2002). However, none of these methods can reliably detect the pathogen both in plants and soils, and in soil-related habitats. Recently, interest in the use of phages for direct detection and identification of bacterial pathogens has rapidly increased. A number of phage-based bacterial diagnoses exist: (i) Lysis of bacterial cells by specific phages results in release of intracellular molecules that may be assayed using various methods. For example, ATP release can be easily detected by the use of firefly luciferase/luciferin system (Entis et al., 2001). The presence of bacterial pathogens can also

be monitored by measuring specific enzyme activities released by phage-mediated cell lysis. (ii) The phage amplification assay detects the increase in phage particles in target bacterial cells after infection (Mole & Maskell, 2001). (iii) The phage-tagging method is another phage-based approach for bacterial detection. In this method, which is well established with coliphage M13 (Smith 1985; Smith 1991), a tag protein, for example green fluorescent protein (GFP), can be displayed on the phage particle and may then be detected by several methods, including epifluorescence microscopy, flow cytometry, or by a fluorescent plate reader after adsorption into host cells (Goodridge et al., 1999). (iv) Reporter phages, defined here as recombinant phages expressing reporter genes in host cells after infection, are also used to monitor phage infection. A variety of reporter genes are available, such as those encoding GFP and luciferase.

In the case of *R. solanacearum*, the filamentous phages ϕRSS1 and ϕRSM1 may be useful tools for phage-based diagnoses of bacterial wilt. In general, the genomes of inoviruses (Ff-like phages) are organized in a three-module structure in which functionally related genes are grouped (Rasched & Oberer, 1986; Model & Russel, 1988). The replication module contains the genes encoding rolling-circle DNA replication and the ssDNA binding proteins, gpII, gpV, and gpX. The structural module contains genes for the major (gpVIII) and minor (gpIII, gpVI, gpVII, and gpIX) coat proteins. Among these, gpIII is the host recognition or adsorption protein (Armstrong et al., 1981). The assembly and secretion module contains genes gI and gIV for morphogenesis and extrusion of the phage particles. The genome of ϕRSS1 is 6,662 nt and encodes 11 ORFs arranged in a generally conserved module structure (Kawasaki et al., 2007a). The genomic DNA of ϕRSM1 is a little longer (9,004 nt), and encodes 14 ORFs, 12 of which are located on the same strand in a similar manner to ϕRSS1, and two of which are in the opposite orientation in the replication module (Kawasaki et al., 2007a). All strains tested were susceptible to either ϕRSM1 or ϕRSM3. Similar compensating host ranges are also detected between ϕRSS1 and ϕRSS2. Selective recognition of the host with different types of pili is mediated by the minor coat protein pIII of these phages. When the gene for GFP was inserted in the intergenic region (IG) of both ϕRSS1 and ϕRSM1 genomic DNAs, the resulting phages exhibited strong green fluorescence in phage-infected host cells, and each phage caused large plaques to appear on the host bacterial lawn (Fig. 4). The efficiency of infection and host specificity of the phages were unchanged (unpublished results). These phages can be used as reporter phages to quantify bacterial cell number in the natural environment, because they propagate in the host cells, but do not cause death. A

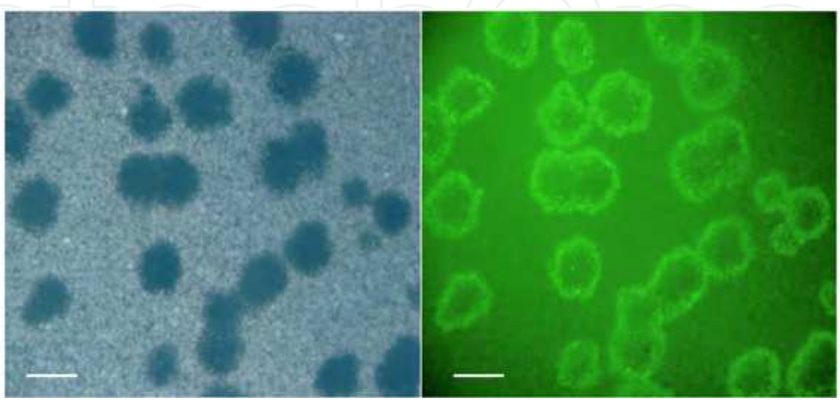


Fig. 4. Green fluorescence emission from plaques of ϕRSS1-GFP-infected *R. solanacearum* cells (right). ND, Not determined.

tag protein on ϕRSS1 and ϕRSM1 particles, such as GFP or luciferase, would lead to rapid and direct detection of the pathogen. For practical use of this method, the following three requirements should be met: (i) a set of phages with different host specificities covering all phylotypes of *R. solanacearum* strains should be prepared; (ii) the intensity of tag-signals should be increased; and (iii) an appropriate, simple device for sensitive detection of the tag-signals should be developed.

These phages would also be very useful in both basic and applied research for monitoring infection of bacterial cells in plant tissues, and for observing bacterial behavior in ecological systems. Kawasaki et al. (2007b) obtained mini-replicons from ϕRSS1 and ϕRSM1. pRSS11 is a 2.2 kbp-region of ϕRSS1 containing the entire replication module (ORF1-ORF3) and IG connected to the Km-cassette (1.5 kbp). pRSM12 is a 3.4 kbp-ϕRSM1 fragment containing ORF1, ORF12, and IG connected to the Km cassette (1.5 kbp). Both plasmids are very stably maintained in *R. solanacearum* cells of different races and biovars, even without selective pressure. Almost 100% of transformed cells retained the plasmids after cultivation for 100 generations (12 d) in CPG medium without Km. This stability makes pRSS11 and pRSM12 valuable vectors for studies on *R. solanacearum* in natural ecosystems, where selective pressure cannot be applied. To demonstrate the usefulness of these plasmids, a GFP-expressing plasmid (pRSS12, 4.7 kbp) was derived from pRSS11 by connecting the GFP gene, and was introduced into various strains of *R. solanacearum* (Kawasaki et al., 2007b). As expected, pRSS12 was stably maintained in all the transformants and expressed strong green fluorescence. To monitor cell behavior, pRSS12-transformed cells were infected into tomato plants and tobacco BY-2 cells, and were also introduced into soil samples. The strong green fluorescence emitted from pRSS12-transformed cells was easily observed in tomato stems, petioles, and roots (Kawasaki et al., 2007b; Fujie et al., 2010). Bacterial cells adhered to BY-2 cell surfaces preferentially by one pole, possibly via pili on the cell surface. These phage-derived plasmids can serve as an easy-to-use GFP-tagging tool for any given strain of *R. solanacearum* in cytological or field studies. Although there have been several reports on the expression of GFP-fused proteins in *R. solanacearum* cells, all the vectors used were selective-pressure-dependent (Huynh, 1989; Aldon et al., 2000). Transposons have also been used for the constitutive expression of GFP in *R. solanacearum* cells. Random chromosomal insertion of the pAG408 mini-transposon (Suarez et al., 1997) was used to label the wild-type strain GMI1000 (Aldon et al., 2000). To monitor the movement of individual cells, and their chemotactic behaviors, Tn5-GFP-tagged *R. solanacearum* strains were examined (Liu et al., 2001; Yao & Allen, 2006). However, there are intrinsic problems in using transposition techniques. Transposon insertion may affect the genetic background of the host cells. The GFP expression itself may be affected by the genetic environment around the insertion site (position effects). Moreover, under natural environmental conditions with various physical and biological stresses, some transposons are unstable, and are easily moved or lost. pRSS12 is easily introduced by electroporation and is stably maintained in *R. solanacearum* cells of different races and biovars; therefore, it serves as an easy-to-use GFP-tagging tool for any given *R. solanacearum* strain in the wild-type background. By monitoring pRSS12-transformed cells, the following may be studied in detail: (i) differences in the virulence traits among strains; (ii) differences in the resistance level (responses) of plant hosts against a given bacterial strain; (iii) effects of environmental factors during establishment of infection; and (iv) evaluation of therapeutic effects in the development of new agricultural chemicals for bacterial wilt disease.

5. Phage biocontrol in other phytopathogen systems

To date, phage-mediated biocontrol of plant pathogens has been successfully attempted in several other pathogen–plant systems. Historical applications in this area are described in the reviews by Gill and Abedon (2003) and Jones et al., (2007). Effective phage applications have been observed in systems using *Streptomyces* phage to disinfest *Streptomyces scabies*-infected potato seed-tubers (McKenna et al., 2001), in *Xanthomonas pruni*-associated bacterial spot of peaches (Sacchardi et al., 1993; Civerolo & Kiel, 1969; Randhawa & Civerolo, 1986), in *Xanthomonas* leaf bright of onion (Lang et al., 2007), to control soft rot caused by *Erwinia* spp. (Eayre et al., 1990), in fire blight of pear and apple associated with *Erwinia amylovora* (Gill et al., 2003; Schnabel et al., 1998; Schnabel, 2001), and using phage Xav to manage bacterial leaf spot of mungbean (Borah et al., 2000). Phage biocontrol has also been successfully extended using host-range phage mutants (h-mutants) of *Xanthomonas* to bacterial blight of geraniums caused by *Xanthomonas hortorum* pv. *pelargonii* (Flaherty et al., 2000) and bacterial spot of tomatoes caused by *X. perforans* (Balogh et al., 2003). Moreover, phages have been used against bacterial blotch of mushrooms caused by *Pseudomonas tolassii* (Munisch & Olivier, 1995).

6. Future prospects

To meet increasing food demands, there is a need to reconstruct agricultural systems that are much more efficient, economical, sustainable, and free from the problems arising from agrichemical use. Phage biocontrol has many advantages: the application is relatively easy; it is relatively low cost; it does not disturb larger ecological systems; and it is safe for humans, animals, and plants. However, it should be acknowledged that phage biocontrol is not a panacea against plant pathogens. Extrapolation of practices from one pathogen-plant system, even if fully successful, to other systems may not always be applicable. Several factors can influence the success of phage biocontrol: (i) the niche where the target pathogen population resides; (ii) stability, decay, and diffusion of phage particles in the applied ecosystems; (iii) timing of phage application during the crop-growing cycle; (iv) phage host-range and emergence of phage-resistant host derivatives; and (v) the density of target bacteria and applied phages. The optimal conditions for the most effective phage biocontrol should be established for each pathogen–host system. Furthermore, detailed understanding of the properties and behavior of each specific phage-bacterium system will help to optimize phage biocontrol. Like an arms race, both phages and their host bacteria have evolved a variety of mechanisms to resist each other during their long history of interaction. Such mechanisms can be deduced from the genomes of both phages and host bacteria. Genomic information of major pathogenic bacterial species is accumulating rapidly, and newly isolated phages are also subjects for immediate genomic analysis. Molecular mechanisms deduced from genomic information on phage–host interactions, no matter how general or specific, could be useful for establishing long-lasting phage biocontrol systems. These include, for example, general interactions (Comeau & Krisch, 2007), restriction/antirestriction systems (Tock & Dryden, 2005; Hoskisson & Smith, 2007), and phage receptor/host adsorption interactions (Goldberg et al., 1994; Tetart et al., 1998; Thomassen et al., 2003).

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Bacteriophages have received attention as biological control agents since their discovery and recently their value as tools has been further emphasized in many different fields of microbiology. Particularly, in drug design and development programs, phage and prophage genomics provide the field with new insights. Bacteriophages reveals information on the organisms ranging from their biology to their applications in agriculture and medicine. Contributors address a variety of topics capturing information on advancing technologies in the field. The book starts with the biology and classification of bacteriophages with subsequent chapters addressing phage infections in industrial processes and their use as therapeutic or biocontrol agents. Microbiologists, biotechnologists, agricultural, biomedical and sanitary engineers will find Bacteriophages invaluable as a solid resource and reference book.

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