New bacteriophages that infect the phytopathogen
*Ralstonia solanacearum*

Takashi Yamada, Takeru Kawasaki, Shoko Nagata, Akiko Fujiwara, Shoji Usami and Makoto Fujie

Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima 739-8530, Japan

Four kinds of bacteriophage (φRSL, φRSA, φRSM and φRSS) were isolated from *Ralstonia solanacearum*, a soil-borne Gram-negative bacterium that is the causative agent of bacterial wilt in many important crops. The Myovirus-type phages φRSL1 and φRSA1 contained dsDNA genomes of 240 kbp and 39 kbp, respectively. These phages have relatively wide host ranges and gave large clear plaques with various host strains; especially φRSA1 was able to infect all 15 *R. solanacearum* strains of different races or different biovars tested in this study. Three host strains contained φRSA1-related sequences in their genomic DNAs, suggesting a lysogenic cycle of φRSA1. Two phages, φRSM1 and φRSS1, were characterized as Ff-type phages (Inovirus) based on their particle morphology, genomic ssDNA and infection cycle. However, despite their similar fibrous morphology, their genome size (9.0 kb for φRSM1 and 6.6 kb for φRSS1) and genome sequence were different. Strains of *R. solanacearum* that were sensitive to φRSM1 were resistant to φRSS1 and vice versa. Several *R. solanacearum* strains contained φRSM1-related sequences and at least one strain produced φRSM1 particles, indicating the lysogenic state of this phage. These phages may be useful as a tool not only for molecular biological studies of *R. solanacearum* pathogenicity but also for specific and efficient detection (φRSM1 and φRSS1) and control of harmful pathogens (φRSL and φRSA) in cropping ecosystems as well as growing crops.

INTRODUCTION

*Ralstonia solanacearum* is a soil-borne Gram-negative bacterium that is the causative agent of bacterial wilt in many important crops (Hayward, 2000; Yabuuchi et al., 1995). This bacterium has an unusually wide host range of over 200 species belonging to more than 50 botanical families (Hayward, 2000). It infects roots and exhibits strong tissue-specific tropism within the host, specifically invading and extensively multiplying in the xylem vessels. Recently, the complete genome sequence of *R. solanacearum* GMI1000 was reported (Salanoubat et al., 2002). The 5.8 Mbp genome is organized into two replications: a 3.7 Mbp chromosome and 2.1 Mbp megaplasmid. The genome encodes a total of 5129 predicted proteins, many of which are potentially associated with a role in pathogenicity. To identify specific genes involved in this pathogenicity and accelerate exhaustive functional analyses of them in this pathogen, unique and powerful molecular biological tools applicable to *R. solanacearum* are required.

In the field, *R. solanacearum* is easily spread via contaminated irrigation water and can survive for many years in association with alternate hosts. In cropping fields, gardens or greenhouses, once identified as being infected, plants must be destroyed and soil and water draining systems that could potentially be contaminated with the bacterium must be treated with chemical bacteriocides such as quaternary ammonia compounds, peroxygen compounds or bleaches. Due to the limited efficiency of the current integrated management strategies, bacterial wilt continues to be an economically serious problem for field-grown crops in many subtropical, tropical and warm areas of the world (Hayward, 1991, 2000).

For detection of *R. solanacearum*, a variety of methods, including typical bioassays, dilution plating on semi-selective media, fatty-acid analysis, immunofluorescence microscopy, ELISA and PCR have been developed (Elphinstone et al., 1996; Janse, 1988; Seal et al., 1993; Van der Wolf et al., 2000; Weller et al., 2000). However, none of these methods are able to reliably detect the pathogen not only in plants but also in soil or soil-related habitats.

Here, the aim is to isolate bacteriophages that specifically infect races of *R. solanacearum*. These phages may be useful as a tool not only for molecular biological studies on *R. solanacearum* pathogenicity but also for specific and
efficient detection of this harmful pathogen in cropping ecosystems as well as growing crops. Moreover, they may also serve as biocontrol agents for eradication of the pathogen in contaminated soil or prevention of bacterial wilt in economically important crops. Although a few papers hitherto have documented the phages that infect R. solanacearum (Tanaka et al., 1990; Toyoda et al., 1991; Ozawa et al., 2001), unfortunately these phages were similar to each other and had very restricted host ranges. In the present work, we have demonstrated widespread occurrence of various kinds of phage that can infect and interact with R. solanacearum strains.

**METHODS**

**Bacterial strains and culture conditions.** Strains of R. solanacearum were obtained from the following culture collections: strains M45, P269, P565, P7 and P74 from the Leaf Tobacco Research Center, Japan Tobacco Inc. (JT), and strains MAFF106603, 106611, 211270, 211271, 211272, 301556, 301558, 730138 and 730139 from the National Institute of Agrobiological Sciences, Japan. Strain C319 (Furuya et al., 1997) was kindly donated by Dr N. Furuya, Kyushu University, Japan. Hosts and taxonomic features of these strains are listed in supplementary Table S1, available with the online version of this paper. The bacterial cells were cultured in CPG medium containing Casamino acids, peptone and glucose (Horita & Tsuchiya, 2002) at 28 °C with shaking at 200–300 r.p.m.

**Soil samples and plaque assay.** Soil samples for phage isolation were collected from cropping fields in Kagoshima, Nagasaki, Fukuoka, Shimane, Hiroshima, Ehime, Shizuoka and Fukushima prefectures in Japan. Sampling was performed mainly in the summer season. One gram of soil was suspended in 2 ml distilled water then, after filtration through a membrane filter (0.45 μm pore size, Steradisc, Krabo Co.), 100 μl aliquots of the suspension were subjected to plaque-forming assay with strains of R. solanacearum as the host on CPG plates containing 1.5% agar.

**Purification and characterization of phages.** Phages were propagated and purified from single-plaque isolates. Routinely, phage ϕRSS1 was propagated using strain C319 as the host, and phages ϕRSA1, ϕRSM1 and ϕRS1 with strain M45 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. After centrifugation, cells were resuspended in distilled water at a density of 10^8 cells ml⁻¹. The cell suspension was mixed with a needle into the major stem of tobacco plants (Nicotiana tabacum) SR1, 4 weeks old with 4–6 leaves at a site 1 cm above the soil level (just above the cotyledons). As a control, distilled water was injected in the same manner. Each bacterial strain was injected into five plants. Plants were cultivated in a Sanyo Growth Cabinet at 25 °C (16 h light/8 h dark) for 3–4 weeks before detailed examination. Symptoms of wilting were graded from 1 to 5 as described by Winstead & Kelman (1952).

**RESULTS**

**Detection and isolation of bacteriophages infecting strains of R. solanacearum**

Phage plaques were detected at frequencies of 1–67 plaques per plate from eight soil samples (Hiroshima, Shimane and Fukushima) and were further tested for their host specificity. Based on the infectivity to strains of R. solanacearum, they were classified into four groups, ϕRSL, ϕRSA, ϕRSM and ϕRSS. Because all phage clones belonging to the same group always gave the same patterns in the genomic analyses as described below, an arbitrarily chosen clone from each group was used for further studies, labelled as ϕRSL1, ϕRSA1, ϕRSM1, or ϕRSS1. As shown in Table 1, ϕRSL1 infected all strains of race 1 (but of biovars of 4 and N2) and MAFF301558 (race 3) but did not infect strains MAFF211271 (race 3) and MAFF211272 (race 4). In contrast, ϕRSA1 infected all strains tested in this study to Dykstra (1993). ϕ phage particles were used as an internal standard marker for size determination.

**Isolation and characterization of nucleic acids from phage particles.** Standard molecular biological techniques for DNA isolation, digestion with restriction enzymes and other nucleases, and construction of recombinant DNAs were performed according to Sambrook & Russell (2001). Genomic DNA was isolated from the purified phage particles by phenol extraction. In some cases, extrachromosomal DNA was isolated from phage-infected R. solanacearum host cells by the mini-preparation method (Ausubel et al., 1995) and analysed by PFGE with a CHEF MAPPER electrophoresis apparatus (Bio-Rad) according to Higashiyama & Yamada (1991).

**Southern blot hybridization.** Genomic DNA of R. solanacearum cells was prepared by the minipreparation method according to Ausubel et al. (1995). After digestion with various restriction enzymes, DNA fragments were separated by agarose gel electrophoresis, blotted onto a nylon membrane (Bionyte, Pall Gelman Laboratory), hybridized with a probe (bacteriophage genomic DNA) labelled with fluorescein (Gene Images Random Prime labelling kit; Amersham Biosciences), and detected with a Gene Images CDP-Star detection module (Amersham Biosciences). Hybridization was performed in buffer containing 5 × SSC, 0.1% SDS and 5% dextran sulfate for 16 h at 65 °C. The filter was washed at 60 °C in 1 × SSC and 0.1% SDS for 15 min, and then in 0.5% SSC and 0.1% SDS for 15 min with agitation according to the manufacturer’s protocol. The hybridization signals were detected by exposing the filter onto X-ray film (RX-U, Fuji Film).

**In planta virulence assay of R. solanacearum strains.** Cells of R. solanacearum were grown in CPG medium for 1–2 days at 28 °C. After centrifugation, cells were resuspended in distilled water at a density of 10^8 cells ml⁻¹. The cell suspension was injected with a needle into the major stem of tobacco plants (Nicotiana tabacum) SR1, 4 weeks old with 4–6 leaves at a site 1 cm above the soil level (just above the cotyledons). As a control, distilled water was injected in the same manner. Each bacterial strain was injected into five plants. Plants were cultivated in a Sanyo Growth Cabinet at 25 °C (16 h light/8 h dark) for 3–4 weeks before detailed examination. Symptoms of wilting were graded from 1 to 5 as described by Winstead & Kelman (1952).
Phage sensitivity and genomic hybridization with phage DNAs as probe

Table 1. Phage sensitivity and genomic hybridization with phage DNAs as probe

<table>
<thead>
<tr>
<th>Strain</th>
<th>Race, biovar</th>
<th>Phage</th>
<th>(\phi_{\text{RSL1}}) (Myovirus)</th>
<th>(\phi_{\text{RSA1}}) (Myovirus)</th>
<th>(\phi_{\text{RSM1}}) (Inovirus)</th>
<th>(\phi_{\text{RSS1}}) (Inovirus)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(S^\ast)</td>
<td>(H)</td>
<td>(S^\ast)</td>
<td>(H^\dagger)</td>
</tr>
<tr>
<td>C319</td>
<td>1, ND</td>
<td></td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>M4S</td>
<td>1, ND</td>
<td></td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Ps29</td>
<td>1, ND</td>
<td></td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Ps65</td>
<td>1, ND</td>
<td></td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Ps72</td>
<td>1, ND</td>
<td></td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Ps74</td>
<td>1, ND</td>
<td></td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>MAFF106603</td>
<td>1, 3</td>
<td></td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>MAFF106611</td>
<td>1, 4</td>
<td></td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>MAFF211270</td>
<td>1, N2</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MAFF211271</td>
<td>3, N2</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MAFF211272</td>
<td>4, 4</td>
<td></td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>A</td>
</tr>
<tr>
<td>MAFF301556</td>
<td>1, 4</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MAFF301558</td>
<td>3, N2</td>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>MAFF730138</td>
<td>1, 3</td>
<td></td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>B</td>
</tr>
<tr>
<td>MAFF730139</td>
<td>1, 4</td>
<td></td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>B</td>
</tr>
</tbody>
</table>

ND, Not determined.
\(^\ast\)Phage sensitivity (S) is shown as sensitive (+) or resistant (−).
\(^\dagger\)Hybridization (H) patterns are shown as A (similar to \(\phi_{\text{RSA1}}\)) or B (different from \(\phi_{\text{RSA1}}\)). Appearance of faint bands is shown as (+). (−) indicates no hybridization.
\(^\ddagger\)Hybridization patterns are grouped into A and B. (−) indicates no hybridization.
\(^\S\)Hybridization patterns are grouped into A1, A2, B and C.

including those of races 1, 3 and 4, although in some cases plaque appeared at a relatively low frequency (with strains Ps74, MAFF211271 and MAFF301558). \(\phi_{\text{RSM1}}\) did not infect strains of race 3 or race 4 but infected several strains of race 1 such as M4S, Ps29, Ps65, Ps74, MAFF211270 (biovar N2) and MAFF730138 (biovar 3). \(\phi_{\text{RSS1}}\) infected strains MAFF106603 (race 1, biovar 3), MAFF106611 (race 1, biovar 4) and MAFF730139 (race 1, biovar 4). These results indicate that the four bacteriophages isolated from soil samples collected in Japan had markedly different host specificities.

Morphological analysis of *R. solanacearum* phages

Bacteriophages were propagated and purified from single-plaque isolates from each group. Phage particles purified by CsCl-gradient ultracentrifugation were subjected to electron microscopic observation. The morphological features of these phages are compared in Fig. 1. \(\phi_{\text{RSL1}}\), a representative \(\phi_{\text{RSL}}\) phage, consisted of an icosahedral head of 150±14 nm in diameter and a non-contractile tail of 138±10 nm in length and 22.5±3 nm in diameter (Fig. 1A), giving a coliphage P2-like morphology (Buchen-Osmund, 2003b; ICTVdB; http://www.ictvdb.rothamsted.ac.uk). At the end of the tail a base plate was seen (Fig. 1A, inset) but tail fibres were not discernible. \(\phi_{\text{RSA1}}\), a representative \(\phi_{\text{RSA}}\) phage, consisted of an icosahedral head of 40±5 nm in diameter, a tail of 110±8 nm in length and 3±0.2 nm in diameter, and a tail sheath (40±6 nm in length and 17±1.5 nm in diameter) located at the bottom of the tail, giving a structure which resembled the morphology reported for *Burkholderia cepacia* phage KS5 (Seed & Dennis, 2005) (Fig. 1B). In contrast to these head–tail structures, particles of \(\phi_{\text{RSM1}}\), a representative \(\phi_{\text{RSM}}\) phage, showed a long fibrous shape of 1500±300 nm in length and 6±0.7 nm in width (Fig. 1C), giving a shape similar to coliphage M13 (Buchen-Osmund, 2003a; ICTVdB). \(\phi_{\text{RSS1}}\), a representative \(\phi_{\text{RSS}}\) phage, also showed a flexible filamentous shape of 1100±100 nm in length and 10±0.5 nm in width (Fig. 1D), giving a morphology like coliphage fd (Buchen-Osmund, 2003a; ICTVdB).

Genomic analysis of *Ralstonia* phages

Genomic DNA isolated from the phages was analysed by restriction enzyme digestion and agarose gel electrophoresis. When \(\phi_{\text{RSL1}}\) DNA was digested with various restriction enzymes, numerous restriction fragments were produced; for example, more than 30 fragments were visible with *PstI* or *KpnI* (Fig. 2A), whose sizes summed up to 250 kbp. All phages of the \(\phi_{\text{RSL}}\) group gave essentially the same DNA restriction fragmentation patterns (Fig. 2A). PFGE of \(\phi_{\text{RSL1}}\) DNA gave a single band of approximately 240 kbp in size (Fig. 2B), indicating that the genome of
φRSL1 is a 240 kbp linear dsDNA. Similarly, φRSA1 DNA was digested with restriction enzymes; the digestion patterns with HincII and PstI are shown in Fig. 2(C). In each case, the sum of the restriction fragment sizes was consistently 39 kbp. The size of a single band of φRSA1 DNA separated by PFGE coincided with this size, indicating that the genome of φRSA1 is a 39 kbp linear dsDNA (Fig. 2D).

In contrast to the φRSL1 and φRSA1 phages, the genomes of both φRSM1 and φRSS1 consist of ssDNA, because their genomic DNAs were not affected by digestion with various restriction enzymes and RNase A but disappeared after nuclease S1 treatment, which specifically digests ssDNA (data not shown). When extrachromosomal DNA was isolated from φRSM1-infected M4S cells, DNA molecules corresponding to covalently closed circular (ccc), linear (corresponding to 9.0 kb in size) and open circular (oc) forms were observed (Fig. 2E), indicating that the genome of φRSM1 is a circular ssDNA of 9.0 kb and may replicate in the host cells as dsDNA, presumably by a rolling-circle mechanism like many filamentous bacteriophages (Ff-type phages) (Rasched & Oberer, 1986; Model & Russel, 1988). The situation was almost the same for φRSS1: extrachromosomal DNA of φRSS1-infected C319 cells also showed ccc, linear and oc forms of genomic DNA (Fig. 2F) and the size of the linear DNA was 6.6 kbp. The genome size differences between the filamentous phages φRSM1 and φRSS1 fit well with the particle length differences.

Interaction between phage DNA and the host chromosome

In Southern blot hybridizations where R. solanacearum genomic DNA fragments produced by digestion with HincII were hybridized under high-stringency conditions with φRSL1 DNA as a probe, no hybridization signals appeared for any of the 15 bacterial strains (data not shown), indicating that φRSL1 is probably not lysogenic. In contrast, φRSA1 DNA hybridized with genomic DNA of three MAFF strains, 211272, 730138 and 730139 (Fig. 3A). In each case, more than 10 hybridizing bands appeared, the banding patterns of which were different from each other. Strain MAFF211272, from which φRSA1 was originally
detected, gave approximately 14 separated bands, which closely coincided with the *Hin*II digestion pattern of φRSA1 DNA. Two bands of 6.0 kb and 2.6 kb in size (arrowed in Fig. 3A) showing different mobility compared with φRSA1 bands were found to contain the integration junctions (A. Fujiwara and others, unpublished). These results were consistent with the finding that φRSA1 is integrated in strain MAFF211272 and suggest that the other two strains also have an integrated φRSA1-related phage on their genome, with possible DNA rearrangements or modifications. For other strains, several very faint hybridizing bands were visible; for example, strains Ps74, MAFF106603 and MAFF106611 (Fig. 3A). The nature of these bands is unclear.
In the case of φRSM1, the genomic DNAs from half the strains tested gave hybridization signals with this phage DNA probe, as seen in Fig. 3(B). The hybridizing patterns were grouped into three types: A, the five-band pattern of strains M4S, Ps74 and MAFF211270; B, the four-band pattern of strains MAFF106611 and MAFF730139; and A + B, the mixed pattern of strain MAFF211272. Sizes of the hybridizing bands were summed to 13.5 kbp (4.3, 3.5, 2.7, 1.55 and 1.45 kbp) for type A, and 9.18 kbp (4.3, 2.65, 1.3 and 0.93 kbp) for MAFF730139 (type B). The HinII restriction pattern of φRSM1 DNA consisted of four bands (3.4, 2.7, 1.55 and 1.45) summing to 9.1 kbp in size and closely related to that of type A. We detected spontaneous plaques on plate cultures of strain MAFF211270, one of the type A strains, which contained φRSM1-like phages based on the HinII restriction pattern of the genomic DNA, suggesting a lysogenic state of φRSM1 in this strain. Since the other two strains of type A contained similar patterns of φRSM1-related sequences, they may also harbour integrated φRSM1 and potentially produce φRSM1-like phages. We did not observe the production of φRSM1-like phages from type B strains, but they may also be lysogenic. From its hybridization pattern (Fig. 3B), strain MAFF211272 seemed to contain two copies of a φRSM1-like sequence.

As for the φRSS1 sequence, all strains tested showed various hybridizing signals (Fig. 3C). Based on several characteristic signals, the hybridizing patterns could be grouped into basically three types: A, a 2.8 kbp band-bearing type; B, a 3.5 kbp band-bearing type; and C, a 4.0 kbp band-bearing type. Type A included six strains: C319 and MAFF strains 106603, 211271, 211272, 301558 and 730138. These strains could be further subdivided into two groups according to the presence (A1) or absence (A2) of a 1.5 kbp hybridizing band. Type A1 included strains MAFF211271 and MAFF301558, and all others belonged to type A2. Type B included four strains, Ps29, Ps65, Ps72 and MAFF211270, and type C included five strains, M4S, Ps74, MAFF106611, MAFF301556 and MAFF730139. Interestingly, these φRSS1-hybridizing types coincided well with the taxonomic grouping of these strains: type A1 corresponded to strains of race 3 and biovar N2; type A2 included strains of race 1 and biovar 3; and strains of race 1 and biovar 4 were included in type C with the exception of strain MAFF211272 (race 4, biovar 4), which belonged to type A2. These hybridization patterns are summarized in Table 1.

**Effects of φRSS1 infection on the pathogenic activity of R. solanacearum strain C319**

Infection with φRSS1 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. Upon infection by φRSS1, the host *R. solanacearum* cells showed several abnormal behaviours including less turbidity in the liquid cultures, less...
coloration of colonies on the plates, a decreased growth rate (approx. 60% of the normal rate) and increased sensitivity to ampicillin (uninfected cells were resistant to 150 μg ml⁻¹, while infected cells were sensitive to 15 μg ml⁻¹). More interestingly, φRSS1 infection also affected the pathogenic activities of the host cells (strain C319): when 1 μl of culture containing 10⁷ cells was injected into the major stem of tobacco plants, all five plants injected with non-infected bacterial cells showed no wilt symptoms until 10 days post-infection (p.i.), becoming wilted after 14 days p.i. (wilting grade 2–3). Meanwhile, all five plants injected with φRSS1-infected C319 cells showed wilt symptoms before 10 days p.i. (wilting grade 1–2) and had completely died at 14 days p.i. (Fig. 4). A few plants showed wilt symptoms as early as 7 days p.i. φRSS1 infection may therefore bring about changes in the expression of pathogenesis-related genes in R. solanacearum or cause drastic changes in the structure and/or nature of the cell surface, which is important in interaction with host plants. In contrast to this φRSS1 effect, no such enhanced pathogenicity was observed with φRSM1-infected cells of strain Ps29.

**DISCUSSION**

To our knowledge, only a few papers have documented the phages that infect *R. solanacearum* (Tanaka *et al.*, 1990; Toyoda *et al.*, 1991; Ozawa, *et al.*, 2001), and all of them describe similar kinds of phage, namely, a Myovirus-type particle with a dsDNA genome of 35–39 kbp such as P4282 and related phages (Tanaka *et al.*, 1990; Ozawa *et al.*, 2001), and PK-101 and P-167 (Toyoda *et al.*, 1991). These phages are lytic and have very restricted host ranges. In the present work, we demonstrated widespread occurrence of various kinds of phage that can infect and interact with *R. solanacearum* strains. Four of these phages (φRSL1, φRSA1, φRSM1 and φRSS1) were different in morphology, Fig. 4. Effects of φRSS1 infection on the pathogenic activity of *R. solanacearum*. (A) Tobacco plants (4 weeks old) were injected with cells of strain C319 (plants 2 and 3), with cells of C319 infected with φRSS1 (plants 4 and 5) and with cells of Escherichia coli (plant 1 as a control). (B) At 10 days post-infection (p.i.), only a slight change (wilting grade 1) became discernible with plants with C319 (2 and 3) but a few leaves showed wilting symptoms for plants with φRSS1-infected C319 (4 and 5). The control plant showed no changes. (C) At 14 days p.i., C319 plants showed wilting symptoms of grade 2–3 (2 and 3) and all plants injected with φRSS1-infected C319 were almost dead (4 and 5). In each experiment, five plants tested per treatment showed essentially the same results.
Bacteriophages of R. solanacearum

gene structure, genome size and host specificity not only from each other but also from those described previously. The Myovirus-type phages φRSL1 and φRSA1 contained dsDNA genomes of 240 kbp and 39 kbp, respectively. The size of 240 kbp for a phage genome is remarkable, corresponding to approximately 1.5 times that of T4 DNA. A few bacteriophages have been reported to have such a large DNA genome; for example, Pseudomonas aeruginosa phages EL (with a genome of 211 kbp; Hertvedt et al., 2005) and φKX (280 kbp; Krylov, 2001), and Vibrio phage KVP40 (245 kbp; Miller et al., 2003). Possible relationships between φRSL1 and these large phages are interesting. φRSL1 and φRSA1 have relatively wide host ranges and gave large clear plaques with various host strains; φRSA1 was especially remarkable because it was able to infect all 15 R. solanacearum strains of different races and different biovars tested in this work. Although three host strains, MAFF211272, MAFF730138 and MAFF730139, contained φRSA1-related sequences in their genomes (Fig. 3A), and φRSA1 was actually integrated in strain MAFF211272, these strains remained sensitive to secondary infection by φRSA1. It is therefore possible that secondary infection induces the lytic cycle of φRSA1. A possible explanation is that φRSA1 is an immunity-deficient mutant of this kind of virus as reported for Xanthomonas campesiris cf-tv2, which can superinfect the Ff phage cf immune (lysogen) host (Cheng et al., 1999). In the biocontrol of plant diseases caused by pathogenic bacteria using virulent phages, a narrow host range or ineffective contact between the phage and host bacteria largely restricts practical application (Okabe & Goto, 1963; Vidaver, 1976). Therefore, φRSA1 as well as φRSL1 may offer an effective means for enhancing biological control of bacterial wilt as well as for detecting pathogenic bacteria in natural environments. However, considering φRSA1 for phage therapy, it is important to mention that obligate virulent derivatives should be selected to avoid the problem of lysogenization by a therapeutic phage and thus passive import of pathogenicity genes.

Two Ff-type phages (Inoviruses) that infected the R. solanacearum strains were also found in this work (φRSM1 and φRSS1). Despite their similar filamentous morphology, their genome sizes (9.0 kb for φRSM1 and 6.6 kb for φRSS1) and genome sequences were different. Interestingly, these phages also had incompatible host specificity; strains sensitive to φRSM1 were resistant to φRSS1 and vice versa (Table 1). It is well established that Ff-like filamentous phages adsorb to pili as a phage receptor on the host cells (Model & Russel, 1988). φRSS1 and φRSM1 may recognize different pili that are specific to R. solanacearum strains. The R. solanacearum strain GMI1000 contains a large number of genes coding for components of pili (Salanoubat et al., 2002). At least 35 genes, distributed in five gene clusters, are involved in the biogenesis of a type IV pilus, giving a considerable variation of pili. Southern blot hybridization revealed frequent occurrence of φRSS1-related sequences in the genome of R. solanacearum strains irrespective of φRSS1 phage-sensitivity. The hybridizing band patterns coincided well with phylogenetic groups of the bacterial strains, and thus the nature of φRSS1-related sequences on the bacterial genome will likely be interesting. Our preliminary results showed that the nucleotide sequence of the hybridizing bands obtained with C319 DNA (type A; Fig. 3C) coincided with that of φRSS1. The bands that appeared with M4S DNA (type B; Fig. 3C) also showed the φRSS1 sequence but its region corresponding to pIII, the host-adsorbing protein, was considerably changed (T. Kawasaki and others, unpublished). From these results, we speculate that φRSS-type phages with different host specificity frequently infect and are integrated in a variety of strains of R. solanacearum, which may explain the different hybridization patterns seen in Fig. 3(C).

Similarly, six of the 15 strains of R. solanacearum tested in this work showed strong hybridization signals for φRSM1-related sequences in their genome. Of these, strain MAFF211270 was shown to be lysogenized with φRSM1 phage and occasionally produced phage particles, while two other strains showed the same hybridization signals as those of strain MAFF211270. These results clearly demonstrate the lysogenic cycle of φRSM1. This was confirmed by determining and comparing the nucleotide sequences of the φRSM1 genomic DNA and the hybridizing fragments of the genomic DNA of strain MAFF211270 (T. Kawasaki and others, unpublished). In those experiments, both attP and attB sequences were determined. Other filamentous Ff-type phages known to have a lysogenic cycle include X. campesiris phages Cf1c (Kuo et al., 1991), Cf1t (Kuo et al., 1987a, b), Cf16v1 (Dai et al., 1988) and φLf (Lin et al., 2001), Xylella fastidiosa phage Kiφ (Simpson et al., 2000), Yersinia pestis phage CUSφ-2 (Gonzalez et al., 2002), and Vibrio cholerae phages VGφ (Campos et al., 2003) and CTXφ (Huber & Waldor, 2002). These host phages are pathogenic for plants and animals, and the host bacteria are frequently involved in this pathogenesis. Therefore, infection and/or lysogenization with φRSS1 and φRSM1 may be related to the pathogenicity of R. solanacearum. In fact, infection by φRSS1 enhanced the virulence of strain C319 to tobacco plants (Fig. 4). Recently, the 6662 base sequence of φRSS1 DNA has been determined and a total of 11 ORFs were identified on it (T. Kawasaki and others, unpublished). As in the genomes of Ff phages (Hill et al., 1991), φRSS1 ORFs are essentially organized in three functional modules, the replication, structure and assembly–secretion modules, but a few additional unknown ORFs are also present in an extra region, which might be involved in the host pathogenesis. Various phage-encoded virulence genes in animal/plant bacterial pathogens have been extensively discussed (Canchaya et al., 2003; Brussow et al., 2004), and prophage effects on bacterial pathogenicity are thus a more general phenomenon than previously anticipated. The virulence-enhancing effect found for φRSS1 indicates that such filamentous phages are not suitable for biocontrol of R. solanacearum.
On the other hand, as previously demonstrated, filamentous phages are very useful as a vector to display proteins on the virion surface in the bacteriophage-display method (Smith, 1985, 1991). In the same way, ϕRSS1 and ϕRSM1 may be utilized to display specific proteins on the virion surface. For example, a minor capsid protein corresponding to gp3 of M13 can be fused to green fluorescent protein (GFP) and the resulting phage particles can be easily detected by its fluorescence. Therefore, GFP-ϕRSS1 or GFP-ϕRSM1 may serve as a tool for detecting harmful pathogens not only in plants but also in soil or soil-related habitats.

In handling R. solanacearum strains for isolation from natural environments, routine culture maintenance and culture storage, one may sometimes encounter problems in culturing the cells. In our experience, some strains studied in this work, for example, MAFF strains 211270, 211272, 730138 and 730139 were somewhat difficult to revive from lyophilized stock cultures (Horita & Tsuchiya, 2001). Moreover, other strains such as M45, P574 and MAFF106611 were sometimes unstable and grew very slowly in routine cultivation. This culture instability might be partly explained in light of the new information given in this work about the frequent occurrence of R. solanacearum phages, some of which are possibly lysogenized in certain strains. Such temperate phages may be spontaneously induced to grow in the host cells, affecting the growth rate. In the future, we recommend determination of the occurrence or association with phages similar to those described in this work for strains stocked in the culture collections of R. solanacearum.

ACKNOWLEDGEMENTS

We thank Naruto Furuya and the Leaf Tobacco Research Center, Japan Tobacco Inc., for providing us with R. solanacearum strain C319 and strains M45, P574, P665, P572 and P574, respectively. This study was supported by the Industrial Technology Research Grant Program in 04A09505 from the New Energy and Industrial Technology Development Organization (NEDO) of Japan.

REFERENCES


Edited by: J. Anné