Loss of Virulence of the Phytopathogen *Ralstonia solanacearum* Through Infection by ϕ RSM Filamentous Phages

Hardian S. Addy, Ahmed Askora, Takeru Kawasaki, Makoto Fujie, and Takashi Yamada

Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima 739-8530, Japan.

Accepted for publication 27 January 2012.

ABSTRACT

Addy, H. S., Askora, A., Kawasaki, T., Fujie, M., and Yamada, T. 2012. Loss of virulence of the phytopathogen *Ralstonia solanacearum* through infection by ϕ RSM filamentous phages. *Phytopathology* 102:469-477.

ϕ RSM1 and ϕ RSM3 (ϕ RSM phages) are filamentous phages (inoviruses) that infect *Ralstonia solanacearum*, the causative agent of bacterial wilt. Infection by ϕ RSM phages causes several cultural and physiological changes to host cells, especially loss of virulence. In this study, we characterized changes related to the virulence in ϕ RSM3-infected cells, including (i) reduced twitching motility and reduced amounts of type IV pili (Tfp), (ii) lower levels of β -1,4-endoglucanase (Egl) activity and extracellular polysaccharides (EPS) production, and (iii) reduced expres-

sion of certain genes (*egl*, *pehC*, *phcA*, *phcB*, *pilT*, and *hrpB*). The significantly lower levels of *phcA* and *phcB* expression in ϕ RSM3-infected cells suggested that functional PhcA was insufficient to activate many virulence genes. Tomato plants injected with ϕ RSM3-infected cells of different *R. solanacearum* strains did not show wilting symptoms. The virulence and virulence factors were restored when ϕ RSM3-encoded *orf15*, the gene for a putative repressor-like protein, was disrupted. Expression levels of *phcA* as well as other virulence-related genes in ϕ RSM3- Δ ORF15-infected cells were comparable with those in wild-type cells, suggesting that *orf15* of ϕ RSM3 may repress *phcA* and, consequently, result in loss of virulence.

Ralstonia solanacearum is a widely distributed soilborne phytopathogen belonging to the β subdivision of Proteobacteria. It causes lethal bacterial wilt of >200 plant species, including economically important crops (16,17). During infection, *R. solanacearum* cells express various virulence and pathogenicity factors resulting in typical wilting symptoms in host plants. The virulence factors produced by *R. solanacearum* consist of a consortium of plant cell-wall-degrading enzymes (CWDEs) secreted via the type II secretion system. These CWDEs include β -1,4-endoglucanase (Egl), endopolygalacturonase (PehA), exopolygalacturonases (PehB and PehC), β -1,4-cellobiohydrolase (CbhA), and a pectin methyl esterase (Pme) (8,15,20,36). Secretion of effector proteins via the type III secretion system (T3SS) is also an important process in bacterial pathogenesis. Bacteria that lose the ability to produce these secretion systems cannot infect host plants (13).

Recently, we isolated and characterized various phages that infect *R. solanacearum* strains (40). One of these phages, ϕ RSM1, is a filamentous phage (inovirus) with a circular single-stranded DNA genome of 9,004 nucleotides (nt) encoding 14 open reading frames (ORFs) (23). Sometimes, ϕ RSM1-related DNA sequences are integrated into the genome of certain strains of *R. solanacearum*. Askora et al. (3) characterized one such prophage sequence (ϕ RSM3, 8,929 nt long) and found that ϕ RSM3 is viable and produces phage particles when introduced into different strains, including MAFF 106603. The genomes of ϕ RSM1 and ϕ RSM3 are very similar to each other (93% nucleotide identity) except for two ORFs, one of which (ORF9) encodes the host recognition protein (pIII). ϕ RSM1 and ϕ RSM3 showed

different host ranges and all 15 strains of *R. solanacearum* tested were sensitive to one or the other of the phages (3). Infection by ϕ RSM1 or ϕ RSM3 (ϕ RSM phage) does not kill host cells but establishes a persistent association between the host and the phage. Upon infection by ϕ RSM phages, the host cells showed some abnormal behaviors and characteristics, such as frequent aggregation, dark coloration, and relatively small colony size. Most importantly, ϕ RSM-infected cells lost their virulence against tomato plants (3). This virulence-reducing effect of ϕ RSM phage infection contrasts with some other previously reported cases. For example, infection of *Xanthomonas campestris* pv. *oryzae* NP5850 by the filamentous phages Xf and Xf2 resulted in enhanced virulence, possibly because of overproduction of extracellular polysaccharides (EPS) by the phage-infected bacterial cells (21). Tseng et al. (37) also reported that infection of *X. campestris* pv. *campestris* by the filamentous phage ϕ Lf increased virulence via promoting EPS production. Therefore, the changes in *R. solanacearum* cells caused by ϕ RSM infection are worthy of investigating in relation to their pathogenicity.

In this study, we have further characterized the changes in phage-infected *R. solanacearum* cells that result in the reduction of virulence. We hypothesized that ϕ RSM infection might cause (i) reduction of cell motility, especially twitching motility; (ii) reduction of virulence factors such as Egl and EPS; and (iii) reduced expression of specific genes involved in virulence and pathogenicity.

MATERIALS AND METHODS

Bacterial strains and bacteriophage. *R. solanacearum* strains MAFF 106603 (race 1, biovar 3, and phylotype I) and MAFF 106611 (race 1, biovar 4, and phylotype I) were obtained from the National Institute of Agrobiological Sciences (Japan). Avirulent strain M4S (race 1, biovar 3, and phylotype I) was obtained from the Leaf Tobacco Research Center, Japan Tobacco Inc. (35). Strain MAFF 106603 was used for all experiments, and strains

Corresponding author: T. Yamada; E-mail address: tayamad@hiroshima-u.ac.jp

*The e-Xtra logo stands for "electronic extra" and indicates that the online version contains three supplemental figures.

MAFF 106611 and M4S were used as controls. The bacterial cells were cultured in casamino acid-peptone-glucose (CPG) medium containing 0.1% casamino acids, 1% peptone, and 0.5% glucose (18) at 28°C with shaking at 200 to 300 rpm. Strain MAFF 106603 carrying a green fluorescent protein (GFP)-expressing plasmid pRSS12 was described previously (24), and was cultivated in CPG containing kanamycin (50 µg/ml). In some cases, bacterial cells were cultivated in minimal medium (MM) containing 1.75 g of K₂HPO₄, 0.75 g of KH₂PO₄, 0.15 g of Na-citrate, 0.25 g of MgSO₄, and 1.25 g of (NH₄)₂SO₄ (5) per liter. For an antibiotic sensitivity assay, exponentially growing cells (10⁷ CFU/ml) in CPG medium were streaked for growth to single colonies on CPG plates containing an antibiotic (kanamycin, chloramphenicol, or ampicillin) at the concentration of 10, 20, 30, 40, 60, or 100 µg/ml. Bacteriophage ϕ RSM3 was described previously (3). ϕ RSM3 was routinely propagated using strain MAFF 106603 as the host. To collect sufficient phage particles, a total of 2 liters of bacterial culture was grown. When the cultures reached 0.1 unit at an optical density of 600 nm (OD₆₀₀), the phage was added at a dose of 0.01 to 0.05 PFU/host cell. After further growth for 16 to 18 h, cells were removed by centrifugation in an R12A2 rotor in a Hitachi Himac CR21E centrifuge (Hitachi Koki Co. Ltd., Tokyo), at 8,000 × g for 15 min at 4°C. The supernatant was passed through a 0.2-µm membrane filter and then phage particles were precipitated in the presence of 0.5 M NaCl and 5% polyethylene glycol 6000. Phage preparations were stored at 4°C until use. To isolate single colonies of MAFF 106603 infected with ϕ RSM3, single ϕ RSM3 plaques picked from assay plates covered with a MAFF 106603 lawn were streaked onto CPG plates. Single colonies were repeatedly purified. The phage genomic DNA was isolated from cells in its replicative form and confirmed by restriction enzyme digestion.

DNA and RNA isolation and manipulation. Standard molecular biological techniques for DNA isolation and digestion with restriction enzymes and other nucleases were as described by Sambrook and Russell (33). Phage DNA was isolated from purified phage particles by phenol extraction. In some cases, extra-chromosomal DNA [replicative form (RF) DNA] was isolated from phage-infected *R. solanacearum* cells by the mini-preparation method (4). Total bacterial RNA was isolated from 3 ml of a culture of ϕ RSM3-infected MAFF 106603 cells at the exponential phase (1 × 10⁸ CFU/ml) in MM using an RNAProtect Bacteria Reagent kit (Qiagen K.K., Tokyo) according to the manufacturer's protocol. Total RNA was treated with 10 U of RNase-free DNaseI (TakaraBio, Kyoto, Japan) for 30 min at 37°C to remove any genomic DNA contaminants. DNase I was inactivated by phenol/chloroform extraction. The absence of DNA

contaminants in RNA preparations was confirmed by polymerase chain reaction (PCR) with gene-specific primers (Table 1). Thirty-five rounds of PCR were performed under standard conditions in a MY Cyclor (Bio-Rad Laboratories, Hercules, CA). The genomic DNA of MAFF 106603 was used as a positive control in the PCR reaction.

Construction of ϕ RSM3- Δ ORF15. To know the role of ORF15 found in the ϕ RSM3 genome, we constructed a ϕ RSM3 mutant lacking ORF15 (designated as ϕ RSM3- Δ ORF15). The ϕ RSM3- Δ ORF15 DNA construct was generated from ϕ RSM3 DNA by PCR using forward primer 5'-GAT GAG AAC TCC TAT CAT GGC GAA ACA CTT-3' (corresponding to ϕ RSM3 DNA position 8821 to 8850) and reverse primer 5'-ACA AGG TGT GCC CGG CAC GCT GAA CG-3' (corresponding to ϕ RSM3 DNA position 8549 to 8521). With these primers and ϕ RSM3 DNA template, PCR produces ϕ RSM3 DNA fragments lacking ORF15 (positions 8527 to 8820). The PCR product (≈8.66 kbp) was extracted and purified from agarose gel after electrophoretic separation and then circularized with T4 DNA ligase (Ligation High; Toyobo, Osaka, Japan) overnight at 16°C. The resulting DNA was introduced into cells of *R. solanacearum* MAFF 106603 by electroporation. After incubation for 2 h at 28°C, bacterial cells were subjected to plaque assay. Single plaques were isolated and phage-containing cells were cultivated to obtain RF DNA. The ϕ RSM3- Δ ORF15 DNA sequence was confirmed by entire DNA sequencing.

Real-time quantitative reverse-transcription PCR. Real-time quantitative reverse-transcription (qRT)-PCR was performed as described previously (2). First-strand cDNAs were synthesized from 1 µg of total RNA with a ReverTraAce reverse-transcriptase kit (Toyobo) and gene-specific primers according to the manufacturer's instructions. Specific gene primers were designed using Primer 3 (v. 0.4.0) software (http://frodo.wi.mit.edu/primer3/#PRIMER_MAX_TEMPLATE_MISPRIMING). The negative control (to eliminate the possibility of residual DNA amplification) consisted of the same reaction except that the RT was omitted from the reaction mixture.

Real-time PCR was performed with a SYBR premix Ex Taq kit (TakaraBio) using a LineGene fluorescence quantitative detection system (BioFlux, Tokyo). The 10-µl reaction mixture contained 5 µl of SYBR premix Ex Taq, 1 µl of diluted cDNA, and 0.5 µM each gene primer (Table 1). PCR was performed under the following conditions: initial heating for 3 min at 95°C and 45 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 15 s. At the end of the program, the specificity of the primer set was confirmed by melting curve analysis (65 to 95°C with a heating rate of 0.5°C/min). Relative expression levels were calculated as the

TABLE 1. List of primers for *Ralstonia solanacearum* used for reverse-transcriptase polymerase chain reaction

Primer name ^a	Oligo sequences (5'→3')	Amplified gene	Product (bp)
Egl3-F	CAGCGCGACCTACTACAAGA	<i>egl</i>	299
Egl3-R*	TCATCAGCCCGAAGATGAC		
PhcA(298)-F	GGACATGATCTTCACGGTCAACT	<i>phcA</i>	298
PhcA(298)-R*	GACTCATCTCCTTTTCTGCATC		
PhcB(RT)-F	CTACCAGATCGTCGTCATGAA	<i>phcB</i>	172
PhcB(RT)-R*	GTCGAGGTAGTGCTTGATCTTG		
HrpB(RT)-F	TTCTCGATGATGTAGCGATAGG	<i>hrpB</i>	238
HrpB(RT)-R*	GCTGGAATTTTCGACTTCTCTA		
PehC(RT)-F*	GTTGTTTCGATTGCTGTACG	<i>pehC</i>	227
PehC(RT)-R	AGTCAAACGATTGCCTGAACCTA		
PilT(175)-F	AAGAACAAGCGTCTGATCTGC	<i>pilT</i>	175
PilT(175)-R*	CTTCCAGGTTTCTTCGTAATGCT		
polA-238F	GGAATGTCTGGAAAGTCAAGAAA	<i>polA</i>	238
polA-238R*	CTTGTAGGCGGGGTACAGTTC		
ace-338F	GCCTATGTGCGTGAGTTCTTCT	<i>aceE</i>	338
ace-338R*	CTTCGAACCTGACGTACGGAAC		
16SrRNA349-F	CTAGAGTGTGTGACAGGGAGGTTAGA	<i>16S rRNA</i>	349
16SrRNA349-R*	ATGTCAAGGGTAGGTAAGGTTTTTC		

^a F = forward, R = reverse, and * = gene-specific primer used for first-strand cDNA synthesis.

ratio of expression of each gene against that of the 16S rRNA gene in *R. solanacearum*.

Assays of Egl activity and EPS. Total Egl activity was determined by measuring the reducing sugars (30) released during incubation of 20% (vol/vol) culture supernatant in 120 mM phos-

phate buffer (pH 7.0) with carboxymethylcellulose at 15 mg/ml as a substrate at 50°C for 4 h according to Addy et al. (1). One unit of enzyme activity was defined as releasing glucose at 1 nmol/min. For EPS production, cells were grown in BG broth for 3 days at 28°C (8). To precipitate EPS, NaCl was added to the culture

TABLE 2. Changes in *Ralstonia solanacearum* cells caused by ϕ RSM3 infection

Feature	Uninfected cells	ϕ RSM3-infected cells
Colony appearance	Viscous	Less viscous
Colony size	Relatively large	Relatively small
Color of culture	White to yellow	Yellow to brown
Edge of the colony	Irregularly shaped	Round-shaped
Twitching motility	Highly twitching	Reduced or no twitching
Aggregate formation	Seldom	Frequently
Antibiotic sensitivity (minimum inhibitory concentration)		
Chloramphenicol	<20 μ g/ml	40 μ g/ml
Ampicillin	<40 μ g/ml	<40 μ g/ml
Kanamycin	<30 μ g/ml	60 μ g/ml

Fig. 1. Morphology of *Ralstonia solanacearum* colonies. Colonies of strain MAFF 106603 **A** and **C**, uninfected and **B** and **D**, infected with ϕ RSM3 were observed **A** and **B**, 20 h and **C** and **D**, 30 h after streaking on minimal medium (25). Bar = 100 μ m.

supernatant to a final concentration of 0.1 M, and four volumes of acetone was added. After standing overnight at 4°C, precipitated materials were recovered by centrifugation (8,000 × g, 10 min, 4°C), dissolved in 500 µl of double-distilled (dd)H₂O, heated for 10 min at 65°C, and centrifuged at 8,000 × g for 5 min to remove insoluble material. The concentration of hexosamine in the culture supernatant was estimated using a modified Elson and Morgan reaction (12). Appropriately diluted samples (0.45 ml) were mixed with 0.15 ml of concentrated HCl, hydrolyzed in sealed tubes at 110°C for 30 min, and then the colorimetric assay was conducted. The absorbance at 530 nm was determined, and the hexosamine concentration was calculated from an N-acetyl D-glucosamine standard curve. The background due to residual media components was subtracted. For a control, N-acetyl D-glucosamine standards were subjected to the entire analysis and were added before the hydrolysis step. For each assay, three independent experiments were repeated, and mean value and standard deviation value were calculated. The significance of observed differences was judged by statistical analysis (Student's *t* test).

Cell motility and movement monitoring. *R. solanacearum* cells were cultured in CPG broth for 1 day at 28°C. After centrifugation at 8,000 × g for 2 min at 4°C, cells were washed twice with ddH₂O and resuspended in ddH₂O (OD₆₀₀ = 1.0). For each assay, 5 µl of the suspension was dropped onto the test medium: MM for twitching motility (25), swimming medium

(SWM) for swimming motility (19), and swarming medium (SRM) for swarming motility (19). Motility was observed by measuring the diameter of the dropped culture for 6 days. To monitor the movement of bacterial cells in tomato stems, we used cells of a GFP-expressing strain (φRSM3-infected or uninfected MAFF 106603 harboring pRSS12) (11,24). For inoculation into plants, the bacterial cells grown in CPG medium for 1 to 2 days were washed and resuspended in ddH₂O at a density of 10⁸ CFU/ml. The suspension (1 µl) was injected with a needle into the major stem (between the cotyledon and the first leaf) of tomato plants (*Solanum lycopersicum* L. 'Oogata Fukuju', 4 weeks old, with four to six leaves) and incubated in a Sanyo growth cabinet (Sanyo, Osaka, Japan) at 28°C (16 h of light and 8 h of darkness). After incubation for 1 week, the plant stem was cut into slices 20 µm in thickness with a microtome and then observed under a Leica MZ16 microscope equipped with a GFP3 filter (11,24).

Bacterial surface appendages. Cells of *R. solanacearum* strains were streaked heavily onto MM plates and incubated for ≈22 to 24 h. The colonies were suspended in a small volume of 10 mM Tris-HCl buffer at pH 8, and the cell suspension (same cell density in each sample) was forced five times through a 25-gauge hypodermic needle (6). Bacterial cells were removed by centrifugation at 8,000 × g for 20 min at 4°C. The bacterial surface appendages were collected by centrifugation at 136,000 × g for 60 min. Precipitated materials were subjected to Tris-Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Schagger and von Jagow (34). For protein identification, separated protein bands were transferred to polyvinylidene difluoride nylon membranes (Immobilion; Nihon Millipore, K.K., Kyoto, Japan) using a semi-dry transfer cell (Bio-Rad Laboratories). Each protein band was subjected to N-terminal peptide sequence analysis on a protein sequencer (Model 492; Applied Biosystems, Foster City, CA) as described by Askora et al. (3).

Pathogenicity assays. Cells of *R. solanacearum* were grown in CPG medium for 1 to 2 days at 28°C. After centrifugation, cells were resuspended in ddH₂O at a density of 10⁸ CFU/ml (OD₆₀₀ = 0.3). For the virulence assay, the bacterial cell suspension (1 µl) was injected with a needle into the major stem of tomato plants (4 to 6 weeks old with four leaves) at a site between the cotyledon and the first leaf. As a control, *Escherichia coli* cells at the same density were injected in the same manner. Each bacterial strain was injected into five plants. Plants were cultivated in a Sanyo Growth Cabinet at 28°C (16 h of light and 8 h of darkness) for up to 1 week before evaluation of disease symptoms. The wilting symptoms were graded from 0 to 4 as described by Winstead and Kelman (39) and modified by Poueymiro et al. (31).

RESULTS

Cultural, physiological, and morphological changes in cells infected with φRSM3. Infection by φRSM3 does not cause host cell lysis but establishes a persistent association between the host and the phage (3,40). Although φRSM3-infected cells always showed slightly less cell density compared with wild-type cells, the growth curves were almost comparable in either rich medium (CPG) or MM between infected and uninfected cells (Supplemental Figure 1). φRSM3-infected cells of *R. solanacearum* strain MAFF 106603 produced φRSM3 particles and yielded the replicative form of φRSM3 DNA. Restriction enzyme digestion of the DNA with *Cla*I and *Hinc*II confirmed the exact genomic structure of φRSM3 recovered from the cells (data not shown). We also confirmed the changes in MAFF 106603 cells caused by φRSM3 infection, including frequent aggregation, dark coloration, and relatively small size of colonies (Table 2; Supplemental Figures 2 and 3), as previously reported by Askora et al. (3). In addition to these changes, φRSM3-infected cells showed enhanced antibiotic resistance. Wild-type MAFF 106603 cells could not grow (no

Fig. 2. Comparison of proteins from cell surface structures. Cell surface appendages were released by passing bacterial cells through a hypodermic needle and their protein components were solubilized, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and stained with Coomassie blue. Molecular size of each marker protein (from Amersham LMW gel filtration kit) is indicated on the left. FliC, PilA, and HrpY proteins were identified by their N-terminal amino acid sequence as described previously (3).

single colonies) on CPG plates containing kanamycin (Km) at 30 µg/ml or chloramphenicol (Cm) at 20 µg/ml, whereas, after ϕ RSM3 infection, cells could grow on CPG plates containing Km at 60 µg/ml and Cm at 40 µg/ml. Both ϕ RSM3-infected and uninfected cells were sensitive to ampicillin (Amp) at 40 µg/ml; no growth, including single colonies, was observed. These results were similar to those observed in strain MAFF 730138 infected with ϕ RSM1 (3).

Comparison of twitching motility and cell surface structures between ϕ RSM3-infected and uninfected cells. Colonies of MAFF 106603 cells on CPG and MM are usually viscous and glossy; however, after infection with ϕ RSM3, they became smaller and less viscous. Especially on MM, the irregular and rough colony margins of uninfected cells became smooth after ϕ RSM3 infection. *R. solanacearum* cells show twitching motility in culture (25), and our results suggested that phage infection affected this motility; therefore, we examined the micromorphology of the colonies. On MM plates, the colony margins of uninfected MAFF 106603 showed irregularly shaped spearheads, and rafts of bacteria were separated from the colonies (Fig. 1A and C), indicating active twitching motility (25). After 30 h of growth, uninfected colonies typically had thin or layered edges with multiple irregular projections (Fig. 1C), whereas colonies of ϕ RSM3-infected cells were round with smooth margins, and lacked rafts or spearheads (Fig. 1B and D). This colony morphology resembled that of a *pilQ* mutant of K60, which lacks type IV pili and does not twitch (25), suggesting a decrease or loss of twitching motility of MAFF 106603 cells infected with ϕ RSM3.

It is well known that type IV pili (Tfp) are involved in twitching motility as well as adhesion, aggregation, and pathogenesis of various bacteria (27,38). Therefore, we examined whether cell surface structural components were affected by ϕ RSM3 infection. Cell surface structure proteins were prepared as described in Materials and Methods, separated by SDS-PAGE, and compared between ϕ RSM3-infected and uninfected cells. Compared with uninfected cells, ϕ RSM3-infected cells had considerably decreased levels of PilA (identified by the N-terminal sequence of TLIELMI), the major component of Tfp (3), and decreased levels of HrpY (identified by a trypsin fragment sequence of TGFQAQ), the major component of type III pili (3) (Fig. 2). These results indicated that infection by ϕ RSM3 resulted in decreased formation of Tfp in host cells, resulting in decreased twitching motility.

Changes in EPS production and Egl activity. The changes in colony morphology after phage infection described above suggested that the phage affected EPS production as well as extracellular Egl activity. In uninfected cells of strain MAFF 106603, EPS production and Egl activity were 965.4 µg/ml and 0.31 U/ml, respectively. These values are comparable with those reported for pathogenic strains 82N (9) and U-7R (29). After ϕ RSM infection, the EPS production and Egl activity were decreased to 674.6 µg/ml (69.9%) and 0.13 U/ml (39.6%), respectively.

Changes in gene expression levels in ϕ RSM3-infected cells. The phenotypic changes observed for the ϕ RSM3-infected cells described above, some of which seemingly involved in virulence and pathogenicity, led us to examine expression levels of specific genes related to pathogenesis and virulence. We targeted six genes (*egl*, *hrpB*, *pehC*, *phcA*, *phcB*, and *pilT*) because these

Fig. 3. Expression analysis of *Ralstonia solanacearum* genes involved in virulence. Transcript levels of each gene were determined by quantitative reverse-transcription polymerase chain reaction from RNA extracted from MAFF 106603 cells uninfected and infected with ϕ RSM3. Expression levels were also analyzed in cells infected with ϕ RSM3- Δ ORF15. For each gene, expression level was normalized to that of 16S rRNA as an internal standard. Mean expression and standard deviation values were calculated from the results of three independent experiments. Bars within each gene are marked specifically if values differ significantly at $0.01 \leq P \leq 0.05$ (*) or at $0.001 \leq P \leq 0.01$ (**), or not significantly at $P > 0.05$ (ns) compared with uninfected cells, according to the Student's *t* test.

might be closely related to the observed changes. Two genes for housekeeping functions, *aceE* for pyruvate dehydrogenase subunit E1 and *polA* for DNA polymerase, were also included for controls. Exponentially growing cells ($OD_{600} = 1.0$) in MM (mimicking the natural environment in plant tissues) (14) were subjected to qRT-PCR analyses with specific primers for each gene (Table 1), as described in Materials and Methods. All of the genes examined except for two housekeeping genes showed decreased expression levels in ϕ RSM3-infected cells compared with uninfected cells (Fig. 3). Expression levels of housekeeping genes represented by *aceE* and *polA* in ϕ RSM3-infected cells were comparable with those of wild-type cells, suggesting that the normal basic metabolism was still functioning after phage infection. This was consistent with the comparable growth rate between wild-type and ϕ RSM3-infected cells. The *egl* and *pehC* genes encoding β -1,4-Egl and exopolysaccharuronase, respectively, showed drastically decreased expression levels ($\approx 1/100$ to $1/1,000$) compared with uninfected cells, consistent with the observation of

reduced Egl activity in ϕ RSM3-infected cells. The *pilT* gene, which has a role in twitching motility (27,38), also showed lower expression levels in ϕ RSM3-infected cells. Interestingly, ϕ RSM3-infected cells also showed decreased expression of *phcB* (to 1/136 of that in uninfected cells), which is responsible for synthesis of 3-OH palmitic acid methylester (3-OH PAME), an autoinducer of quorum sensing that controls virulence and pathogenicity (10). The two-component regulatory system PhcS/PhcR responds to threshold levels of 3-OH PAME, and elevates the level of functional PhcA, which controls expression of many virulence genes (7). Furthermore, the level of *phcA* expression itself was also significantly reduced in ϕ RSM3-infected cells (to 1/20 that in uninfected cells). The expression of *hrpB*, which regulates the T3SS, was also decreased in infected cells.

Loss of virulence in ϕ RSM3-infected *R. solanacearum*. The reduced expression of many virulence genes in ϕ RSM3-infected cells suggested a potentially decreased ability by the pathogen to cause disease. When 1 μ l of cell suspension containing 10^5 CFU

Fig. 4. Effects of ϕ RSM3 infection on virulence of *Ralstonia solanacearum*. Tomato plants (4 weeks old) were injected with cells of MAFF 106603 uninfected or infected with **A**, ϕ RSM3 or **B**, ϕ RSM3- Δ ORF15. As a control, plants were injected with cells of *Escherichia coli* JM109. Each bacterial strain was injected into 20 plants (5 are shown for each experiment in the figure). All plants injected with ϕ RSM3-uninfected or ϕ RSM3- Δ ORF15-infected *R. solanacearum* cells showed wilting symptoms 1 week after injection. All plants injected with ϕ RSM3-infected cells or with *E. coli* cells (control) failed to show any symptoms. Pictures were taken **A**, 3 weeks and **B**, 1 week after infection.

of MAFF 106603 was injected into the major stem of tomato plants, all plants showed wilting symptoms as early as 3 days postinfection (p.i.) (wilting grade 1) and died 5 to 7 days p.i. (wilting grade 4) (Fig. 4A). In contrast, all 20 plants injected with ϕ RSM3-infected MAFF 106603 cells did not show any wilting symptoms until 4 weeks p.i. This was also the case with other host strains such as MAFF 106611; ϕ RSM3-infected MAFF 106611 completely lost its virulence against tomato plants (data not shown). To compare the bacterial behavior in tomato plants, ϕ RSM3-infected or uninfected MAFF 106603 cells harboring a GFP-expressing plasmid pRSS12 were injected into the stem, as described in Materials and Methods. Stem slices at intervals of 10 mm above and below the injection point were examined 1 week after the bacterial injections. As indicated by GFP fluorescence, the bacterial cells accumulated in the xylem vessels and moved both upward and downward in wilted tomato plants inoculated with phage-uninfected cells (Fig. 5A), whereas most of the ϕ RSM3-infected bacterial cells remained around the injection point, and their movement and growth were severely limited (Fig. 5B).

ORF15 encoded on the ϕ RSM3 genome is involved in the loss of virulence in infected cells. To understand the basis for the reduced virulence of ϕ RSM3-infected cells (in other words, reduce the expression levels of virulence-related genes), we considered the possibility that some gene encoded by ϕ RSM3 may directly

affect host gene expression. Fourteen ORFs were identified on the ϕ RSM3 genome, three of which (ORF2, ORF3, and ORF13) are with unknown functions (without any DNA-binding motifs) and variable among ϕ RSM phages (3). Recently, we identified ORF15, located upstream of ORF14 (Int) on the ϕ RSM3 genome (nucleotide positions 8527 to 8820, accession number AB434711). This ORF encodes a protein of 98 amino-acid residues (also corresponding to ORF15 of ϕ RSM1, accession number A0JC19) with sequence similarity to putative phage repressors (ex. *Pelobacter propionicus* DSM2379, *E* value = 0.004). When strain MAFF 106603 was infected with a ϕ RSM3 mutant whose ORF15 was removed (ϕ RSM3- Δ ORF15), the cells caused wilting on inoculated tomato plants as efficiently as wild-type cells (Fig. 4B). It was found that the expression level of *phcA* in ϕ RSM3- Δ ORF15-infected cells was comparable with that of wild-type cells (Fig. 3). The other virulence genes reduced in ϕ RSM3-infected cells, including *hrpB* and *pilT*, were also recovered to almost the same levels as the wild-type levels, except *phcB*. The potential repressor function of ORF15 suggests that it causes, directly or indirectly, the repression of *phcA* and other virulence genes and, therefore, loss of virulence in infected cells. We attempted to directly introduce ORF15 into host cells but failed to stably maintain it when ligated to pRSS12 (under the control of the *lac* promoter) (24), which was transformed into strain MAFF 106603 and other strains.

Fig. 5. Cross-sections of tomato plants injected with green fluorescent protein (GFP)-expressing *Ralstonia solanacearum* cells. Tomato seedlings (4 weeks old) were injected with uninfected cells or ϕ RSM3-infected cells. After 1 week, stem slices were cut at intervals of 10 mm **A**, above and **B**, below the injection point (0 mm). In tomato plants injected with uninfected cells, GFP fluorescence was observed in xylem vessels in sections from above and below the injection point whereas, in those injected with ϕ RSM3-infected cells, GFP fluorescence remained around the injection point. Arrowhead indicates a specific site of the tissue as position marker. The area indicated by a square is enlarged at the upper right. Numbers; distance from the injection point (+ = upward and – = downward).

DISCUSSION

Because filamentous phages such as ϕ RSM assemble on the host cell membrane and protrude from the cell surface, the nature of the host cell surface may change drastically during phage production. Among the changes observed in ϕ RSM-infected cells, the reduction of Tfp formation and decreased twitching motility are especially important. Filamentous phages infect via Tfp on the host cell surface (26,28). Askora et al. (3) suggested that minor components of Tfp of *R. solanacearum* might be involved in the host discrimination by ϕ RSM1 and ϕ RSM3. Frequent protrusion of ϕ RSM particles from the infected cell surface may somehow compete with the formation of Tfp. As reported by Kang et al. (22), Tfp is responsible for twitching motility and adherence to multiple surfaces and is required for virulence. Therefore, the loss of virulence in the ϕ RSM-infected cells seems to be at least partly due to the reduction of Tfp formation and decreased twitching motility.

However, the concomitant multiple changes in ϕ RSM-infected cells suggest that there are other complex mechanisms involved in the loss of virulence. In ϕ RSM3-infected cells, the expression of certain genes involved in virulence was reduced. Especially, the significantly lower levels of *phcA* and *phcB* expression suggested insufficient amounts of PhcA in the cells. The transcriptional regulator PhcA plays a critical role in the regulatory network of *R. solanacearum* pathogenicity (7). Abundant functional PhcA activates production of multiple virulence factors such as Egl, PehC, and EPS. ϕ RSM3 infection leads the loss of virulence in host bacterial cells while cells infected with ϕ RSM3- Δ ORF15 lacking solely ORF15 caused wilting on inoculated tomato plants as efficiently as wild-type cells. The expression levels of *phcA* and other genes in ϕ RSM3- Δ ORF15-infected cells were comparable with those of wild-type cells. The expression level of *phcB* was exceptionally retained at lower levels in ϕ RSM3- Δ ORF15-infected cells. This may be caused by changes in the cell surface nature during phage production. The gene for ORF15 is highly expressed in ϕ RSM3-infected cells (A. Askora, unpublished data). These data suggested that ORF15 of ϕ RSM3 may repress *phcA* and the other virulence genes directly or indirectly, consequently resulting in loss of virulence in infected cells. It is noteworthy that ϕ RSM3-related prophages are integrated in the genome of some *R. solanacearum* strains, including UW551 (3), IPO1609 (32), and CMR15 (32). These prophages lack an ORF15 homolog and, thus, apparently do not affect host virulence.

The loss of virulence in host cells caused by ϕ RSM3 infection is in contrast with the previously observed effects of infection with ϕ RSS1, another inovirus infecting *R. solanacearum* cells (40). ϕ RSS1 infection enhanced the virulence of *R. solanacearum* strain C319 on tobacco (40) and strains MAFF 106603 and MAFF 106611 on tomato (1). Recently, Addy et al. (1) revealed that infection with ϕ RSS1 induced early expression of *phcA*. The surface-associated phage particles may change cell surface nature (ex. hydrophobicity) and enhance cell-to-cell interactions, resulting in high local cell densities and early activation of *phcA*. ϕ RSS1, that has a small genome (6,662 nt) and lacks a regulatory gene (23), grows very abundantly up to $\approx 10^{11}$ to 10^{12} PFU/ml under usual culture conditions, whereas ϕ RSM3 with a genome of 8,929 nt grows less abundantly (titer of 1/100 compared with ϕ RSS1), so that the surface effects on host cells caused by phage particles may be less prominent in ϕ RSM infection. Recently, it has been found that ϕ RSS1 was derived from a larger phage ϕ RSS0 (GenBank accession number JQ408219) by losing a 662-nt region containing ORF13 (a putative regulatory gene) (Tasaka, unpublished data). It is noteworthy that ϕ RSS0 infection caused loss of virulence in host cells, just as observed in ϕ RSM3-infected cells. In this case, ORF13 of ϕ RSS0 may function like ORF15 of ϕ RSM3 and ϕ RSS1 may correspond to ϕ RSM3- Δ ORF15. There-

fore, both filamentous phages ϕ RSM-type and ϕ RSS-type appear to exert the same general effect on the host physiology.

Because the ϕ RSM-infected cells can grow and continue to produce infectious phage particles under appropriate conditions, ϕ RSM phages may serve as an efficient tool to control bacterial wilt in crops by decreasing the virulence of the pathogen.

ACKNOWLEDGMENTS

This study was supported, in part, by a Grant-in-Aid from the Ministry of Education, Science, Sport and Culture of Japan (21580095 to T. Yamada).

LITERATURE CITED

- Addy, H. S., Askora, A., Kawasaki, T., Fujie, M., and Yamada, T. 2012. The filamentous phage ϕ RSS1 enhances virulence of phytopathogenic *Ralstonia solanacearum* on tomato. *Phytopathology* 102:244-251.
- Alemzadeh, A., Fujie, M., Usami, S., Yoshizaki, T., Oyama, K., Kawabata, T., and Yamada, T. 2006. ZMVHA-B1, the gene for subunit B of a vacuolar H⁺-ATPase from the eelgrass *Zostera marina* L., is able to replace *vma2* in a yeast null mutant. *J. Biosci. Bioeng.* 102:390-395.
- Askora, A., Kawasaki, T., Usami, S., Fujie, M., and Yamada, T. 2009. Host recognition and integration of filamentous phage ϕ RSM in the phytopathogen, *Ralstonia solanacearum*. *Virology* 384:69-76.
- Ausubel, F., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. 1995. *Short Protocols in Molecular Biology*, 3rd ed. John Wiley & Sons, Inc., Hoboken, NJ.
- Boucher, C. A., Barberis, P. A., Trigalet, A. P., and Demery, D. A. 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: Isolation of Tn5-induced avirulent mutant. *J. Gen. Microbiol.* 131:2449-2457.
- Clough, S. J., Schell, M. A., and Denny, T. P. 1994. Evidence for involvement of a volatile extracellular factor in *Pseudomonas solanacearum* virulence gene expression. *Mol. Plant-Microbe Interact.* 7:621-630.
- Denny, T. P. 2006. Plant Pathogenic *Ralstonia* Species. Pages 573-644 in: *Plant-Associated Bacteria*. S. S. Gnanamanickam, ed. Springer, Amsterdam.
- Denny, T. P., Carney, B. F., and Schell, M. A. 1990. Inactivation of multiple virulence genes reduces the ability of *P. solanacearum* to cause wilt symptoms. *Mol. Plant-Microbe Interact.* 3:293-300.
- Denny, T. P., Makini, F. W., and Brumbley, S. M. 1988. Characterization of *Pseudomonas solanacearum* Tn5 mutants deficient in extracellular polysaccharide. *Mol. Plant-Microbe Interact.* 1:215-223.
- Flavier, A. B., Clough, S. J., Schell, M. A., and Denny, T. P. 1997. Identification of 3-hydroxypalmitic acid methyl ester as a novel auto-regulator controlling virulence in *Ralstonia solanacearum*. *Mol. Microbiol.* 26:251-259.
- Fujie, M., Takamoto, H., Kawasaki, T., Fujiwara, A., and Yamada, T. 2010. Monitoring growth and movement of *Ralstonia solanacearum* cells harboring plasmid pRSS12 derived from bacteriophage ϕ RSS1. *J. Biosci. Bioeng.* 109:153-158.
- Gatt, R., and Berman, E. R. 1966. A rapid procedure for the estimation of amino sugars on a micro scale. *Anal. Biochem.* 15:167-171.
- Genin, S., Brito, B., Denny, T. P., and Boucher, C. 2005. Control of the *Ralstonia solanacearum* type III secretion system (Hrp) genes by the global virulence regulator PhcA. *FEBS Lett.* 579:2077-2081.
- Genin, S., Gough, C. L., Zischek, C., and Boucher, C. A. 1992. Evidence that the *hrpB* gene encodes a positive regulator of pathogenicity genes from *Pseudomonas solanacearum*. *Mol. Microbiol.* 6:3065-3076.
- Gonzalez, E. T., and Allen, C. 2003. Characterization of a *Ralstonia solanacearum* operon required for polygalacturonate degradation and uptake of galacturonic acid. *Mol. Plant-Microbe Interact.* 16:536-544.
- Hayward, A. C. 1991. Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. *Annu. Rev. Phytopathol.* 29:65-87.
- Hayward, A. C. 2000. *Ralstonia solanacearum*. Pages 32-42 in: *Encyclopedia of Microbiology*. vol. 4. J. Lederberg, ed. Academic Press, San Diego, CA.
- Horita, M., and Tsuchiya, K. 2002. Pages 5-8 in: *MAFF Microorganism Genetic Resources Manual*, Number 12. National Institute of Agricultural Sciences, Tsukuba, Japan.
- Hossain, M. M., Tani, C., Suzuki, T., Taguchi, F., Ezawa, T., and Ichinose, Y. 2008. Polyphosphate kinase is essential for swarming motility, tolerance to environmental stresses, and virulence in *Pseudomonas syringae* pv. *tabaci* 6605. *Physiol. Mol. Plant Pathol.* 72:122-127.
- Huang, Q., and Allen, C. 2000. Polygalacturonases contribute to colonization ability and virulence of *Ralstonia solanacearum* on tomato plants. *Physiol. Mol. Plant Pathol.* 57:77-83.
- Kamiuntun, H., and Wakimoto, S. 1982. Effect of the infection with filamentous phage Xf-2 on the properties of *Xanthomonas campestris* var.

- oryzae*. Ann. Phytopathol. Soc. Jpn. 47:627-636.
22. Kang, Y., Liu, H., Genin, S., Schell, M. A., and Denny, T. P. 2002. *Ralstonia solanacearum* requires type 4 pili to adhere to multiple surfaces and for natural transformation and virulence. Mol. Microbiol. 46:427-437.
 23. Kawasaki, T., Nagata, S., Fujiwara, A., Satsuma, H., Fujie, M., Usami, S., and Yamada, T. 2007. Genomic characterization of the filamentous integrative bacteriophage ϕ RSS1 and ϕ RSM1, which infect *Ralstonia solanacearum*. J. Bacteriol. 189:5792-5802.
 24. Kawasaki, T., Satsuma, H., Fujie, M., Usami, S., and Yamada, T. 2007. Monitoring of phytopathogenic *Ralstonia solanacearum* cells using green fluorescent protein-expressing plasmid derived from bacteriophage ϕ RSS1. J. Biosci. Bioeng. 104:451-456.
 25. Liu H., Kang, Y., Genin, S., Schell, M. A., and Denny, T. P. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. Microbiology 147:3215-3229.
 26. Marvin, D. A. 1998. Filamentous phage structure, infection and assembly, Curr. Opin. Struct. Biol. 8:150-158.
 27. Merz, A. J., So, M., and Sheetz, M. P. 2000. Pilus retraction powers bacterial twitching motility. Nature 407:98-102.
 28. Model, P., and Russel, M. 1988. Filamentous bacteriophages. Pages 375-456 in: The Bacteriophages, Vol. 2. R. Calendar, ed. Plenum Press, New York.
 29. Negishi, H., Yamada, T., Shiraishi, T., Oku, H., and Tanaka, H. 1993. *Pseudomonas solanacearum* plasmid pJTPS1 mediates a shift from the pathogenic to nonpathogenic phenotype. Mol. Plant-Microbe Interact. 6:203-209.
 30. Nelson, N. 1944. A photometric adaptation of the Somogyi method for determination of glucose. J. Biol. Chem. 153:375-380.
 31. Poueymiro, M., and Genin, S. 2009. Secreted proteins from *Ralstonia solanacearum*: A hundred tricks to kill a plant. Curr. Opin. Microbiol. 12:44-52.
 32. Remenant, B., Coupat-Goutaland, B., Guidot, A., Cellier, G., Wicker, E., Allen, C., and Fegan, M. 2010. Genomes of three tomato pathogens within the *Ralstonia solanacearum* species complex reveal significant evolutionary divergence. BMC Genomics 11:379.
 33. Sambrook, J., and Russell, D. W. 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
 34. Schagger, H., and von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166:368-379.
 35. Tanaka, H., Negishi, H., and Maeda, H. 1990. Control of tobacco bacterial wilt by an avirulent strain of *Pseudomonas solanacearum* M4S and its bacteriophage. Ann. Phytopathol. Soc. Jpn. 56:243-246.
 36. Tans-Kersten, J., Guan, Y., and Allen, C. 1998. *Ralstonia solanacearum* pectin methylesterase is required for growth on methylated pectin, but not for bacterial wilt virulence. Appl. Environ. Microbiol. 64:4918-4923.
 37. Tseng, Y. H., Lo, M. C., Lin, K. C., Pan, C. C., and Chang, R. Y. 1990. Characterization of filamentous bacteriophage ϕ Lf from *Xanthomonas campestris* pv. *campestris*. J. Gen. Virol. 71:1881-1884.
 38. Wall, D., and Kaiser, D. 1999. Type IV pili and cell motility. Mol. Microbiol. 32:1-10.
 39. Winstead, N. N., and Kelman, A. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathology 42:628-634.
 40. Yamada, T., Kawasaki, T., Nagata, S., Fujiwara, A., Usami, S., and Fujie, M. 2007. New bacteriophages that infect the phytopathogen *Ralstonia solanacearum*. Microbiology 153:2630-2639.