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May 2012

An International Journal of The American Phytopathological Society

Phytopathology

Bacterial Wilt (*Ralstonia solanacearum*) in a Tomato Field Near Yamoussoukro, Côte d'Ivoire

APS

Phytopathology

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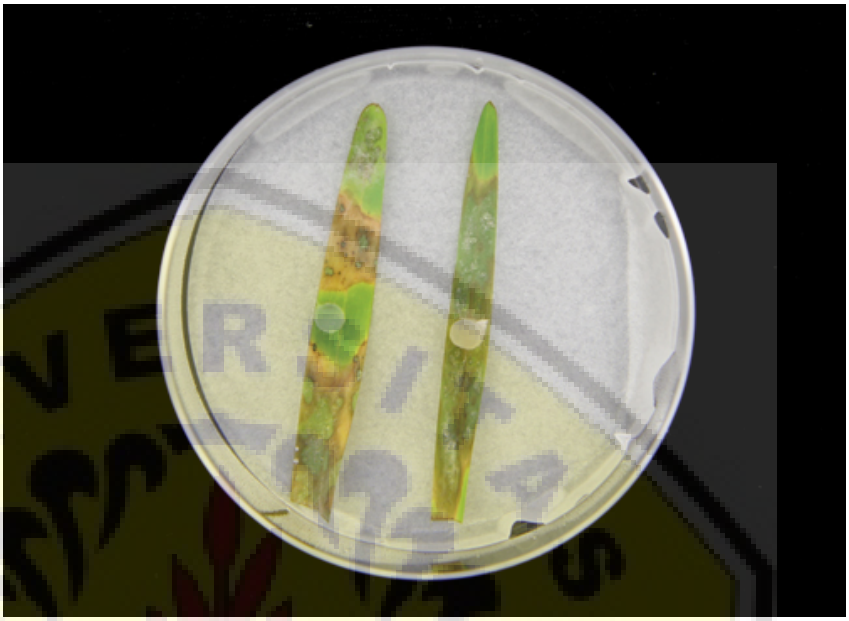
Symptoms produced on St. Augustinegrass after infection by Florida and common biotypes of *Sclerotinia homoeocarpa*. (Liberti et al., page 506)



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Phytopathology

An International Journal of The American Phytopathological Society

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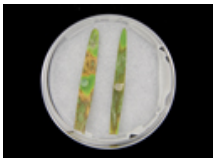

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Loss of Virulence of the Phytopathogen *Ralstonia solanacearum* Through Infection by ϕRSM Filamentous Phages

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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 ϕRSM filamentous phages. Phytopathology 102:469-477.

ϕRSM1 and ϕRSM3 (ϕRSM phages) are filamentous phages (inoviruses) that infect *Ralstonia solanacearum*, the causative agent of bacterial wilt. Infection by ϕRSM 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 ϕRSM3-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 expression of certain genes (*egl*, *pehC*, *phcA*, *phcB*, *pilT*, and *hrpB*). The significantly lower levels of *phcA* and *phcB* expression in ϕRSM3-infected cells suggested that functional PhcA was insufficient to activate many virulence genes. Tomato plants injected with ϕRSM3-infected cells of different *R. solanacearum* strains did not show wilting symptoms. The virulence and virulence factors were restored when ϕRSM3-encoded *orf15*, the gene for a putative repressor-like protein, was disrupted. Expression levels of *phcA* as well as other virulence-related genes in ϕRSM3-ΔORF15-infected cells were comparable with those in wild-type cells, suggesting that *orf15* of ϕRSM3 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), exo-polygalacturonases (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, ϕRSM1, 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, ϕRSM1-related DNA sequences are integrated into the genome of certain strains of *R. solanacearum*. Askora et al. (3) characterized one such prophage sequence (ϕRSM3, 8,929 nt long) and found that ϕRSM3 is viable and produces phage particles when introduced into different strains, including MAFF 106603. The genomes of ϕRSM1 and ϕRSM3 are very similar to each other (93% nucleotide identity) except for two ORFs, one of which (ORF9) encodes the host recognition protein (pIII). ϕRSM1 and ϕRSM3 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 ϕRSM1 or ϕRSM3 (ϕRSM phage) does not kill host cells but establishes a persistent association between the host and the phage. Upon infection by ϕRSM phages, the host cells showed some abnormal behaviors and characteristics, such as frequent aggregation, dark coloration, and relatively small colony size. Most importantly, ϕRSM-infected cells lost their virulence against tomato plants (3). This virulence-reducing effect of ϕRSM 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 ϕLf increased virulence via promoting EPS production. Therefore, the changes in *R. solanacearum* cells caused by ϕRSM 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 ϕRSM 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 1) was obtained from the Leaf Tobacco Research Center, Japan Tobacco Inc. (35). Strain MAFF 106603 was used for all experiments, and strains

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

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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 K_2HPO_4, 0.75 g of KH_2PO_4, 0.15 g of Na-citrate, 0.25 g of $MgSO_4$, and 1.25 g of $(NH_4)_2SO_4$ (5) per liter. For an antibiotic sensitivity assay, exponentially growing cells (10^7 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 ϕRSM3 was described previously (3). ϕRSM3 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 (OD_{600}), 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 ϕRSM3, single ϕRSM3 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, extrachromosomal 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 ϕRSM3-infected MAFF 106603 cells at the exponential phase (1×10^8 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 Cycler (Bio-Rad Laboratories, Hercules, CA). The genomic DNA of MAFF 106603 was used as a positive control in the PCR reaction.

Construction of ϕRSM3-ΔORF15. To know the role of ORF15 found in the ϕRSM3 genome, we constructed a ϕRSM3 mutant lacking ORF15 (designated as ϕRSM3-ΔORF15). The ϕRSM3-ΔORF15 DNA construct was generated from ϕRSM3 DNA by PCR using forward primer 5′-GAT GAG AAC TCC TAT CAT GGC GAA ACA CTT-3′ (corresponding to ϕRSM3 DNA position 8821 to 8850) and reverse primer 5′-ACA AGG TGT GCC CGG CAC GCT GAA CG-3′ (corresponding to ϕRSM3 DNA position 8549 to 8521). With these primers and ϕRSM3 DNA template, PCR produces ϕRSM3 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 ϕRSM3-ΔORF15 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	*egl*	299
Egl3-R*	TCATCAGCCCGAAGATGAC		
PhcA(298)-F	GGACATGATCTTCACGGTCAACT	*phcA*	298
PhcA(298)-R*	GACTCATCCTCCTTTTCTGCATC		
PhcB(RT)-F	CTACCAGATCGTCGTCAATGAA	*phcB*	172
PhcB(RT)-R*	GTCGAGGTAGTGCTTGATCTTG		
HrpB(RT)-F	TTCTCGATGATGTAGCGATAGG	*hrpB*	238
HrpB(RT)-R*	GCTGGAATTTTCGACTTCCTCTA		
PehC(RT)-F*	GTTGTTCGGATTGCTGTACG	*pehC*	227
PehC(RT)-R	AGTCAAACGATTGCCTGAACTA		
PilT(175)-F	AAGAACAAAGCGTCTGATCTGC	*pilT*	175
PilT(175)-R*	CTTCCAGGTTTTCTTCGTAATGCT		
polA-238F	GGAATGTCGGAAAGTCAAGAAA	*polA*	238
polA-238R*	CTTGTAGGCGGGGTACAGTTC		
ace-338F	GCCTATGTGCGTGAGTTCTTCT	*aceE*	338
ace-338R*	CTTCGAACTTGACGTACGGAAC		
16SrRNA349-F	CTAGAGTGTGTCAGAGGGAGGTAGA	*16S rRNA*	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 phosphate 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 ϕRSM3 infection

Feature	Uninfected cells	ϕRSM3-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 μg/ml	40 μg/ml
Ampicillin	<40 μg/ml	<40 μg/ml
Kanamycin	<30 μg/ml	60 μg/ml

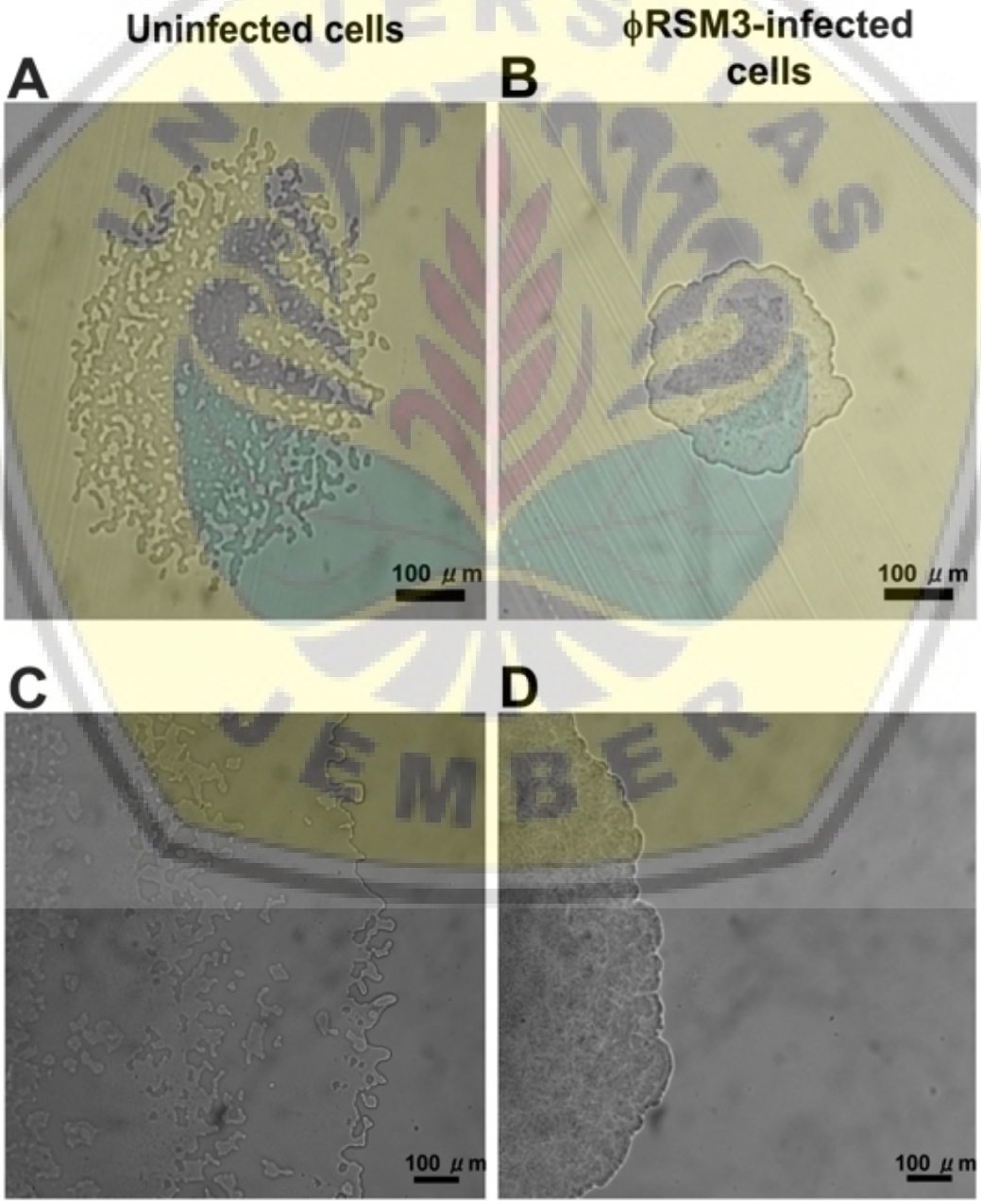

Fig. 1. Morphology of *Ralstonia solanacearum* colonies. Colonies of strain MAFF 106603 **A and C,** uninfected and **B and D,** infected with ϕRSM3 were observed **A and B,** 20 h and **C and D,** 30 h after streaking on minimal medium (25). Bar = 100 μm.

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)H_2O, 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 ddH_2O and resuspended in ddH_2O (OD_{600} = 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 ddH_2O at a density of 10^8 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).

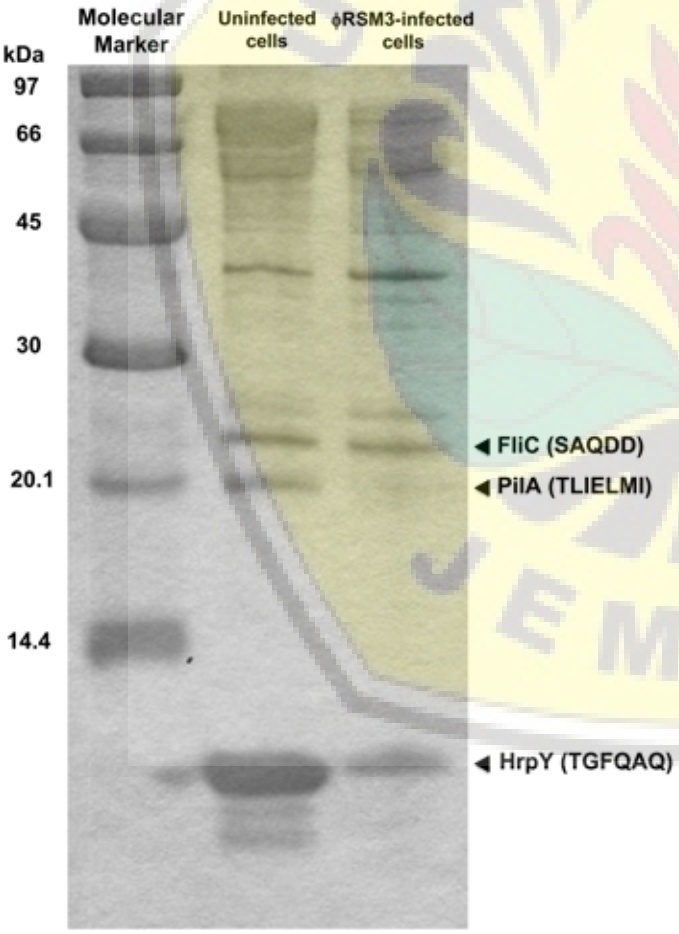

Fig. 2. Comparison of proteins from cell surface structures. Cell surface appendages were released by passing bacterial cells through a hypodermic needle and their protein components were solubilized, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and stained with Coomassie blue. 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).

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 ddH_2O at a density of 10^8 CFU/ml (OD_{600} = 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 ϕRSM phages 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 *Hin*cII 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

single colonies) on CPG plates containing kanamycin (Km) at 30 μg/ml or chloramphenicol (Cm) at 20 μg/ml, whereas, after ϕRSM3 infection, cells could grow on CPG plates containing Km at 60 μg/ml and Cm at 40 μg/ml. Both ϕRSM3-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 ϕRSM1 (3).

Comparison of twitching motility and cell surface structures between ϕRSM3-infected and uninfected cells. Colonies of MAFF 106603 cells on CPG and MM are usually viscous and glossy; however, after infection with ϕRSM3, they became smaller and less viscous. Especially on MM, the irregular and rough colony margins of uninfected cells became smooth after ϕRSM3 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 ϕRSM3-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 ϕRSM3.

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 ϕRSM3 infection. Cell surface structure proteins were prepared as described in Materials and Methods, separated by SDS-PAGE, and compared between ϕRSM3-infected and uninfected cells. Compared with uninfected cells, ϕRSM3-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 ϕRSM3 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 ϕRSM 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 ϕRSM3-infected cells. The phenotypic changes observed for the ϕRSM3-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

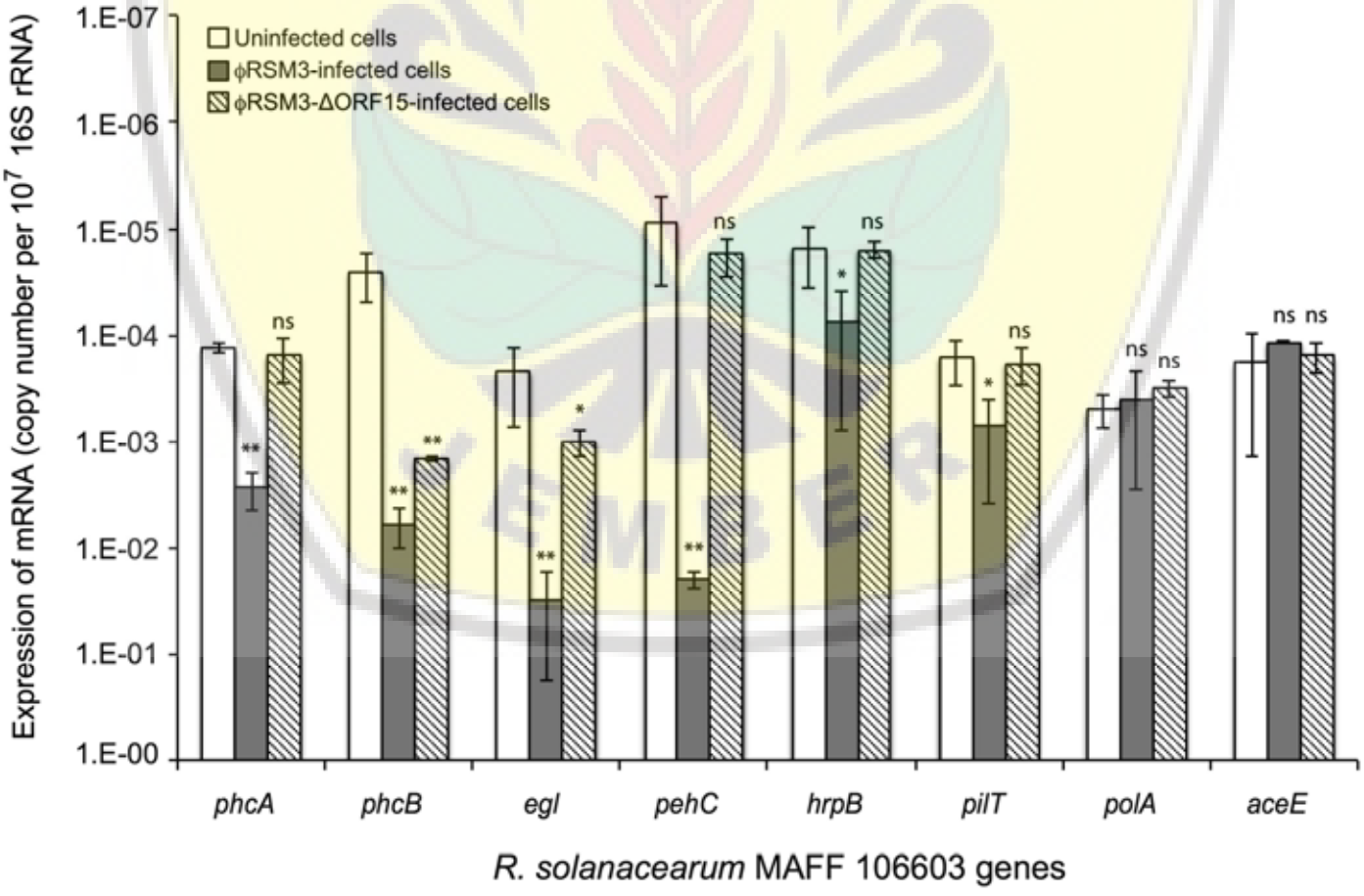

Fig. 3. Expression analysis of *Ralstonia solanacearum* genes involved in virulence. Transcript levels of each gene were determined by quantitative reverse-transcription polymerase chain reaction from RNA extracted from MAFF 106603 cells uninfected and infected with ϕRSM3. Expression levels were also analyzed in cells infected with ϕRSM3-ΔORF15. 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 ϕRSM3-infected cells compared with uninfected cells (Fig. 3). Expression levels of housekeeping genes represented by *aceE* and *polA* in ϕRSM3-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 ϕRSM3-infected cells. The *egl* and *pehC* genes encoding β-1,4-Egl and exopolygalacturonase, respectively, showed drastically decreased expression levels (≈1/100 to 1/1,000) compared with uninfected cells, consistent with the observation of reduced Egl activity in ϕRSM3-infected cells. The *pilT* gene, which has a role in twitching motility (27,38), also showed lower expression levels in ϕRSM3-infected cells. Interestingly, ϕRSM3-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 methyester (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 ϕRSM3-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 ϕRSM3-infected *R. solanacearum*. The reduced expression of many virulence genes in ϕRSM3-infected cells suggested a potentially decreased ability by the pathogen to cause disease. When 1 μl of cell suspension containing 10^5 CFU

Fig. 4. Effects of ϕRSM3 infection on virulence of *Ralstonia solanacearum*. Tomato plants (4 weeks old) were injected with cells of MAFF 106603 uninfected or infected with **A,** ϕRSM3 or **B,** ϕRSM3-ΔORF15. 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 ϕRSM3-uninfected or ϕRSM3-ΔORF15-infected *R. solanacearum* cells showed wilting symptoms 1 week after injection. All plants injected with ϕRSM3-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 ϕRSM3-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; ϕRSM3-infected MAFF 106611 completely lost its virulence against tomato plants (data not shown). To compare the bacterial behavior in tomato plants, ϕRSM3-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 ϕRSM3-infected bacterial cells remained around the injection point, and their movement and growth were severely limited (Fig. 5B).

ORF15 encoded on the ϕRSM3 genome is involved in the loss of virulence in infected cells. To understand the basis for the reduced virulence of ϕRSM-infected cells (in other words, reduce the expression levels of virulence-related genes), we considered the possibility that some gene encoded by ϕRSM3 may directly affect host gene expression. Fourteen ORFs were identified on the ϕRSM3 genome, three of which (ORF2, ORF3, and ORF13) are with unknown functions (without any DNA-binding motifs) and variable among ϕRSM phages (3). Recently, we identified ORF15, located upstream of ORF14 (Int) on the ϕRSM3 genome (nucleotide positions 8527 to 8820, accession number AB434711). This ORF encodes a protein of 98 amino-acid residues (also corresponding to ORF15 of ϕRSM1, 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 ϕRSM3 mutant whose ORF15 was removed (ϕRSM3-ΔORF15), 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 ϕRSM3-ΔORF15-infected cells was comparable with that of wild-type cells (Fig. 3). The other virulence genes reduced in ϕRSM3-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.

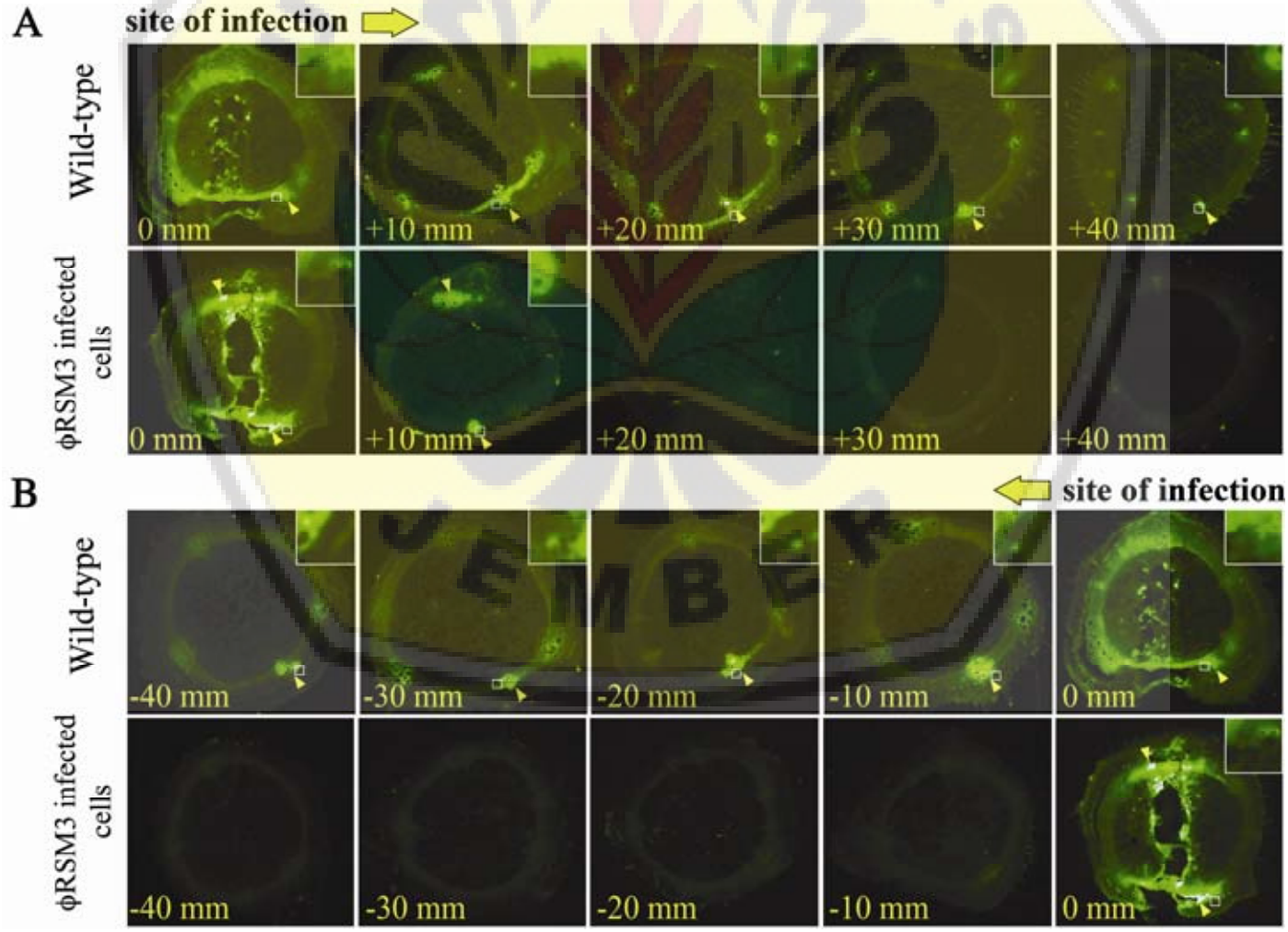

Fig. 5. Cross-sections of tomato plants injected with green fluorescent protein (GFP)-expressing *Ralstonia solanacearum* cells. Tomato seedlings (4 weeks old) were injected with uninfected cells or ϕRSM3-infected cells. After 1 week, stem slices were cut at intervals of 10 mm **A,** above and **B,** 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 ϕRSM3-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 ϕRSM 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 ϕRSM-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 ϕRSM1 and ϕRSM3. Frequent protrusion of ϕRSM 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 ϕRSM-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 ϕRSM-infected cells suggest that there are other complex mechanisms involved in the loss of virulence. In ϕRSM3-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. ϕRSM3 infection leads the loss of virulence in host bacterial cells while cells infected with ϕRSM3-ΔORF15 lacking solely ORF15 caused wilting on inoculated tomato plants as efficiently as wild-type cells. The expression levels of *phcA* and other genes in ϕRSM3-ΔORF15-infected cells were comparable with those of wild-type cells. The expression level of *phcB* was exceptionally retained at lower levels in ϕRSM3-ΔORF15-infected cells. This may be caused by changes in the cell surface nature during phage production. The gene for ORF15 is highly expressed in ϕRSM3-infected cells (A. Askora, *unpublished data*). These data suggested that ORF15 of ϕRSM3 may repress *phcA* and the other virulence genes directly or indirectly, consequently resulting in loss of virulence in infected cells. It is noteworthy that ϕRSM3-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 ϕRSM3 infection is in contrast with the previously observed effects of infection with ϕRSS1, another inovirus infecting *R. solanacearum* cells (40). ϕRSS1 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 ϕRSS1 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*. ϕRSS1, that has a small genome (6,662 nt) and lacks a regulatory gene (23), grows very abundantly up to ≈10^{11} to 10^{12} PFU/ml under usual culture conditions, whereas ϕRSM3 with a genome of 8,929 nt grows less abundantly (titer of 1/100 compared with ϕRSS1), so that the surface effects on host cells caused by phage particles may be less prominent in ϕRSM infection. Recently, it has been found that ϕRSS1 was derived from a larger phage ϕRSS0 (GenBank accession number JQ408219) by losing a 662-nt region containing ORF13 (a putative regulatory gene) (Tasaka, *unpublished data*). It is noteworthy that ϕRSS0 infection caused loss of virulence in host cells, just as observed in ϕRSM3-infected cells. In this case, ORF13 of ϕRSS0 may function like ORF15 of ϕRSM3 and ϕRSS1 may correspond to ϕRSM3-ΔORF15. Therefore, both filamentous phages ϕRSM-type and ϕRSS-type appear to exert the same general effect on the host physiology.

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

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