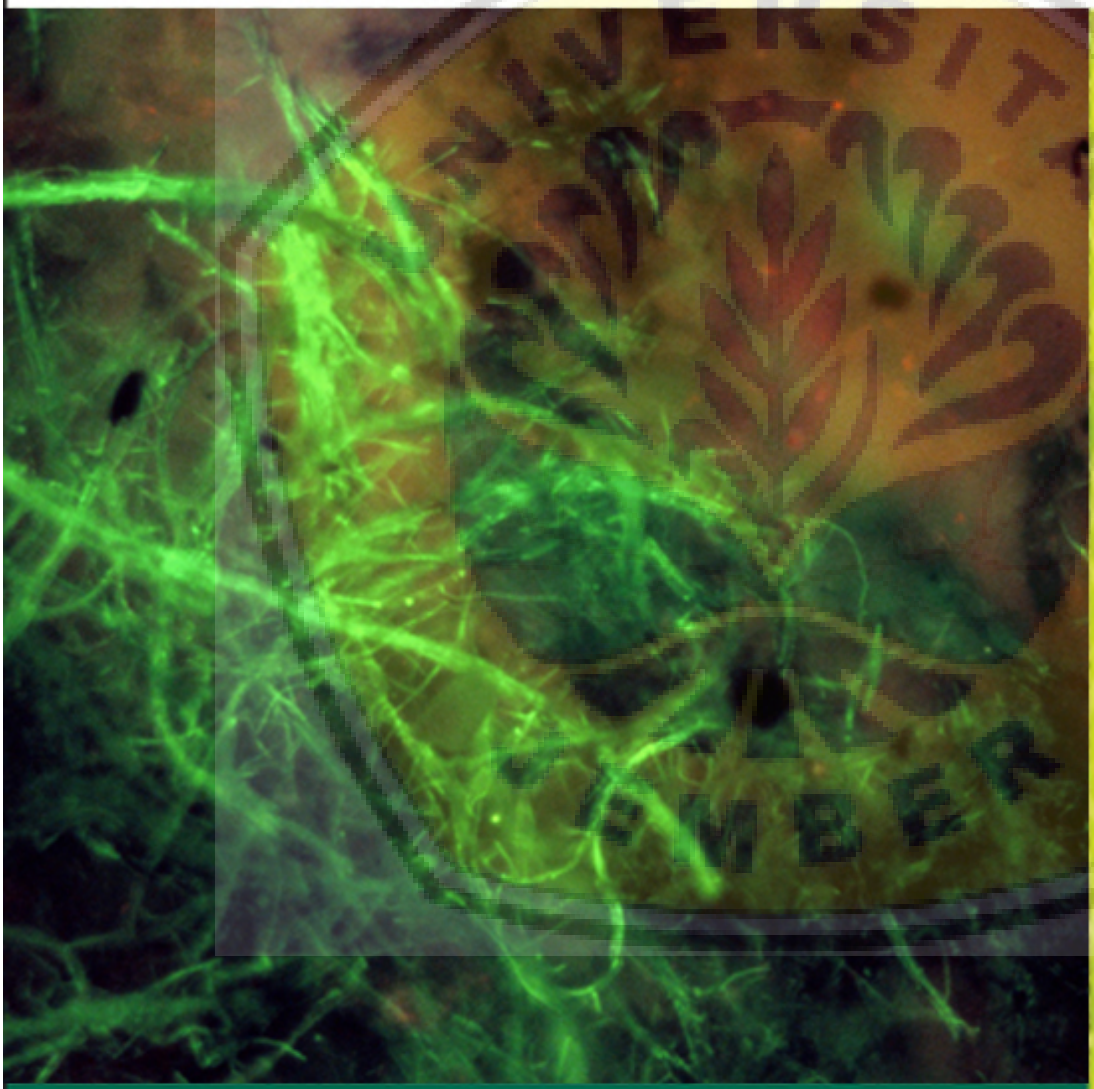
Volume 102, Number 3
March 2012

An International Journal of The American Phytopathological Society

Phytopathology



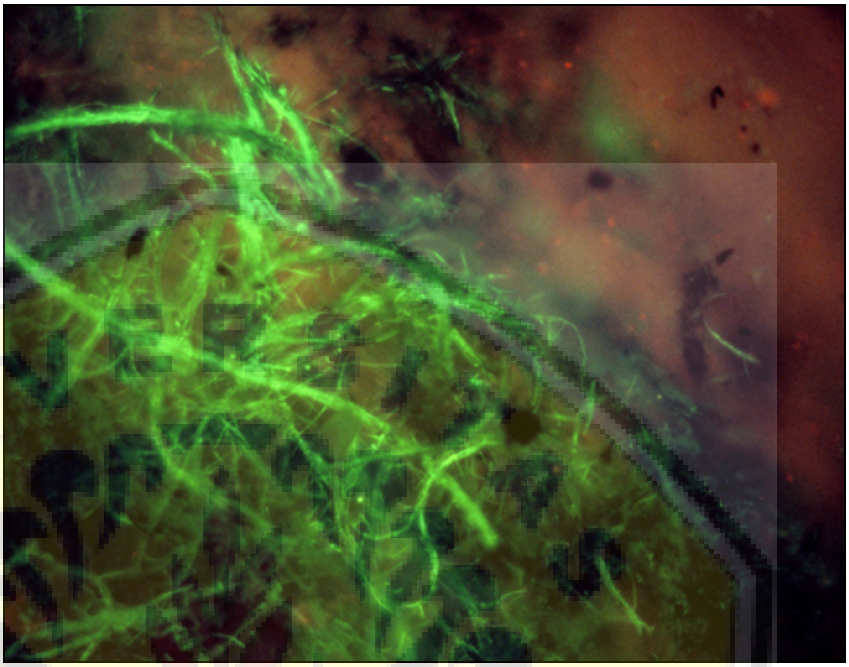
Bacterial Wilt (*Ralstonia solanacearum*) in a Tomato Field Near Yamoussoukro, Côte d'Ivoire

APS

Phytopathology

March 2012
Number 3

Proliferation of *Phaeomoniella chlamydospora* synthetic green fluorescent protein (*Pch*-sGFP)71 mycelia obtained from the basal section of inoculated 'Sangiovese' grape cuttings kept for 6 months at 4 ± 1°C and then incubated at 25 ± 1°C for 96 h. (Landi et al., page 290)



Return to March
Table of Contents

Phytopathology

An International Journal of The American Phytopathological Society

Purpose and Scope:
For nearly 100 years *Phytopathology* has been considered the premier international archival journal for publication of articles on fundamental research that advances understanding of the nature of plant diseases, the agents that cause them, their spread, the losses they cause, and measures that can be used to control them. Subject areas include bacteriology, host-parasite biochemistry and cell biology, biological control, disease control and pest management, ecology and population biology, epidemiology, disease etiology, host genetics and resistance, mycology, nematology, plant stress and abiotic disorders, postharvest pathology and mycotoxins, and virology. In addition to research articles, mini-reviews provide up-to-date summaries of recent advances in the science.

Phytopathology ranked first for impact on research and teaching efforts in the crop sciences according to *The Literature of Crop Science*, a project published by Cornell University Press.

The online version of *Phytopathology* includes *e-Xtra* articles that provide supplemental data tables and additional figures and photos as well as color versions of illustrations shown in black and white in the print journals.

Intended Readership:
Botanists, plant pathologists, horticulturists, agronomists, entomologists, physiologists, nematologists, bacteriologists, biochemists, biologists, cell biologists, ecologists, microbiologists, molecular biologists, mycologists, seed pathologists, and virologists.

The American Phytopathological Society
Leading the fight against plant diseases
for a healthier world.

Phytopathology Editorial Board

E-mail: keverts@umd.edu

Marc Fuchs
Cornell University
Plant Pathology & Plant Microbe Biology Dept.
630 W. North Street
Geneva, NY 14456
E-mail: mf13@cornell.edu

Kelly L. Ivors
North Carolina State University
Mountain Horticultural Crops Research and Extension Center
455 Research Dr.
Mills River, NC 28759
E-mail: kelly_ivors@ncsu.edu

Margaret G. Redinbaugh
USDA-ARS
OARDC/OSU–Plant Pathology Department
1680 Madison Ave.
Wooster, OH 44691
E-mail: peg.redinbaugh@ars.usda.gov

Charles M. Rush
Texas AgriLife Research
6500 Amarillo Blvd. West
Amarillo, TX 79106
E-mail: crush@ag.tamu.edu

Krishna V. Subbarao
University of California, Davis
1636 E. Alisal Street
Salinas, CA 93905-3018
E-mail: kvsubbarao@ucdavis.edu

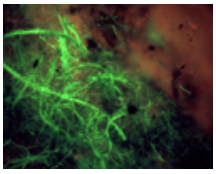
Les J. Szabo
USDA-ARS, Cereal Disease Laboratory
1551 Lindig Street
St. Paul, MN 55108-1050
E-mail: les.szabo@ars.usda.gov

Frank van den Bosch
Rothamsted Research
West Common–Harpenden
Hertsfordshire, UK
E-mail: frank.vandenbosch@bbsrc.ac.uk

Youfu (Frank) Zhao
University of Illinois
Crop Sciences Department
288A ERML, 1201 W. Gregory Dr.
Urbana, IL 61801
E-mail: zhao888@illinois.edu

Associate Editors

The American Phytopathological Society
Leading the fight against plant diseases
for a healthier world.



About the cover for March 2012

ISSN: 0031-949X

SEARCH

Enter Keywords

Phytopathology
Plant Disease
MPMI

search

Advanced Search

Inside the Journal

BACK ISSUES
(Issues before 1997)

First Look

View Most Downloaded Articles

About Phytopathology

Editorial Board

Submit a Manuscript

Author Instructions

Online e-Xtras

= "Open" Access

Subscribe Free alerts RSS

Phytopathology

Editor-in-Chief: George W. Sundin
Published by The American Phytopathological Society

Current **All Issues** **First Look** **Pre 1997**

Favorite | Track Citations | Email a Colleague | View Abstracts
Download as Reference ☐ To select/unselect all items click here

March 2012 - Volume 102, Number 3

Bacteriology

☐ **The Filamentous Phage ΦRSS1 Enhances Virulence of Phytopathogenic *Ralstonia solanacearum* on Tomato**
Hardian S. Addy, Ahmed Askora, Takeru Kawasaki, Makoto Fujie, and Takashi Yamada
Pages 244-251
Abstract | PDF Print (455 KB) | PDF with Links (398 KB) | e-Xtra

☐ **Identification and Characterization of a Novel Gene, *hshB*, in *Xanthomonas oryzae* pv. *oryzicola* Co-Regulated by Quorum Sensing and *clp***
Yancun Zhao, Guoliang Qian, Jiaqin Fan, Fangqun Yin, Yijin Zhou, Chunhui Liu, Qin Shen, Baishi Hu, and Fengquan Liu
Pages 252-259
Abstract | PDF Print (750 KB) | PDF with Links (443 KB) | e-Xtra

Biological Control

☐ **Systemically Induced Resistance and Microbial Competitive Exclusion: Implications on Biological Control**
A. Martinuz, A. Schouten, and R. A. Sikora
Pages 260-266
Abstract | PDF Print (861 KB) | PDF with Links (366 KB)

☐ **A Rapid Microbioassay for Discovery of Antagonistic Bacteria for *Phytophthora parasitica* var. *nicotianae***
Hancheng Wang, Wenhong Li, Qingyuan Chen, Yanfei Huang, Kai Li, Haiqian Xia, Maosheng Wang, Liuti Cai, Shenghua Shang, and Junxiong Shi
Pages 267-271
Abstract | PDF Print (172 KB) | PDF with Links (183 KB) | e-Xtra

Disease Control and Pest Management

☐ **Orchard Factors Associated with Resistance and Cross Resistance to Sterol Demethylation Inhibitor Fungicides in Populations of *Venturia inaequalis* from Pennsylvania**
Emily E. Pfeufer and Henry K. Ngugi
Pages 272-282
Abstract | PDF Print (321 KB) | PDF with Links (366 KB) | e-Xtra

Ecology and Epidemiology

Larval *Bradysia impatiens* (Diptera: Sciaridae) Potential for Vectoring Pythium Root Rot Pathogens
S. E. Braun, J. P. Sanderson, and S. P. Wraight
Pages 283-289
Abstract | PDF Print (840 KB) | PDF with Links (272 KB)

Colonization of *Vitis* spp. Wood by sGFP-Transformed *Phaeomoniella chlamydospora*, a Tracheomycotic Fungus Involved in Esca Disease
Lucia Landi, Sergio Murolo, and Gianfranco Romanazzi
Pages 290-297
Abstract | PDF Print (368 KB) | PDF with Links (398 KB) | e-Xtra

Genetics and Resistance

Characterization of *CbCyp51* from Field Isolates of *Cercospora beticola*
Melvin D. Bolton, Keshav Birla, Viviana Rivera-Varas, Kurt D. Rudolph, and Gary A. Secor
Pages 298-305
Abstract | PDF Print (189 KB) | PDF with Links (199 KB)

Variation for Resistance to Kernel Infection and Toxin Accumulation in Winter Wheat Infected with *Fusarium graminearum*
Clay Sneller, Mary Guttieri, Pierce Paul, José Costa, and Ryan Jackwood
Pages 306-314
Abstract | PDF Print (171 KB) | PDF with Links (227 KB)

Population Biology

Molecular Characterization of Pyraclostrobin Resistance and Structural Diversity of the Cytochrome *b* Gene in *Botrytis cinerea* from Apple
Y. N. Yin, Y. K. Kim, and C. L. Xiao
Pages 315-322
Abstract | PDF Print (395 KB) | PDF with Links (360 KB)

Phenotypic Variation Within a Clonal Lineage of *Phytophthora infestans* Infecting both Tomato and Potato in Nicaragua
J. U. Blandón-Díaz, A.-K. Widmark, A. Hannukkala, B. Andersson, N. Högberg, and J. E. Yuen
Pages 323-330
Abstract | PDF Print (192 KB) | PDF with Links (222 KB)

Techniques

Development of an Assay for Rapid Detection and Quantification of *Verticillium dahliae* in Soil
Guillaume J. Bilodeau, Steven T. Koike, Pedro Uribe, and Frank N. Martin
Pages 331-343
Abstract | PDF Print (239 KB) | PDF with Links (288 KB) | e-Xtra

This article is from the
March 2012 issue of

Phytopathology

published by
The American Phytopathological Society

The Filamentous Phage ϕRSS1 Enhances Virulence of Phytopathogenic *Ralstonia solanacearum* on Tomato

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 1 November 2011.

ABSTRACT

Addy, H. S., Askora, A., Kawasaki, T., Fujie, M., and Yamada, T. 2012. The filamentous phage ϕRSS1 enhances virulence of phytopathogenic *Ralstonia solanacearum* on tomato. Phytopathology 102:244-251.

Ralstonia solanacearum is the causative agent of bacterial wilt in many important crops. ϕRSS1 is a filamentous phage that infects *R. solanacearum* strains. Upon infection, it alters the physiological state and the behavior of host cells. Here, we show that *R. solanacearum* infected by ϕRSS1 becomes more virulent on host plants. Some virulence and pathogenicity factors, such as extracellular polysaccharide (EPS) synthesis and twitching motility, increased in the bacterial host cells infected with ϕRSS1, resulting in early wilting. Tomato plants inoculated with ϕRSS1-infected bacteria wilted 2 to 3 days earlier than those inoculated with wild-type bacteria. Infection with ϕRSS1 induced early expression of *phcA*, the global virulence regulator. *phcA* expression was detected in ϕRSS1-infected cells at cell density as low as 10^4 CFU/ml. Filamentous phages are assembled on the host cell surface and many phage particles accumulate on the cell surface. These surface-associated phage particles (phage proteins) may change the cell surface nature (hydrophobicity) to give high local cell densities. ϕRSS1 infection also enhanced PilA and type IV pilin production, resulting in increased twitching motility.

Ralstonia solanacearum is a gram-negative β-proteobacterium that causes bacterial wilt disease in many important crop plants, such as eggplant, potato, tobacco, tomato, banana, peanut, ginger, and mulberry. Because of its wide geographic distribution and unusually broad host range (more than 50 plant families), it is responsible for significant crop losses worldwide (22). Once the bacteria enter a susceptible host, they colonize the intercellular spaces of the root cortex and vascular parenchyma. The bacteria eventually enter the xylem and spread into the upper parts of the plant, causing wilt (31,55,61). The development of bacterial wilt disease depends on the virulence and pathogenicity of its pathogen (6,10).

R. solanacearum virulence is additive, and involves the production of multiple virulence factors (17,50). For example, exopolysaccharide I (EPSI), a large nitrogen-rich acidic exopolysaccharide (42), is thought to be an important virulence factor. It enhances the speed and extent of stem colonization (46), and is presumed to cause wilting by restricting water flow through xylem vessels (15). In addition to EPSI, *R. solanacearum* secretes enzymes that degrade the plant cell wall through the type II secretion system (T2SS). Pectinolytic enzymes fragment pectin into oligomers, which act as a substrate for bacterial growth (52). The breakdown of pectin enhances virulence by facilitating bacterial movement through pectin-rich regions such as vascular bundles (19). Cellulolytic enzymes also facilitate bacterial invasion of roots and/or penetration of xylem vessels by degrading cellulosic glucans in the cell wall (38). In addition to T2SS-mediated secreted protein, the type IV pilus (Tfp) is believed to be another virulence factor of *R. solanacearum*. This protein forms a surface appendage that is responsible for twitching motility and polar attachment to host cells or plant roots, and enhances the severity of wilt (29,37).

Like many other pathogenic bacteria, *R. solanacearum* has a type III secretion system (T3SS), encoded by the *hypersensitive response and pathogenicity* (*hrp*) genes. In contrast to T2SS, which secretes degrading enzymes, the T3SS allows the injection of effector proteins such as AvrA (6,43), PopP1 (35,43), and PopP2 (13) directly into the plant cell through the *hrp*-pili. The T3SS is essential for pathogenicity, since *R. solanacearum hrp* mutants cannot induce disease in many susceptible plants (28, 54,56,63).

Recently, we isolated various bacteriophages that specifically infect strains of *R. solanacearum* (60). One of them, ϕRSS1, was characterized as an Ff-like phage (Inovirus) based on its particle morphology, genomic ssDNA, and infection cycle. Kawasaki et al. (30) characterized the genome organization of ϕRSS1, which has 6,662 bases of ssDNA. There are 11 open reading frames (ORFs) located on the same strand (30). In general, the genome of Ff-like phage is organized in a modular arrangement, in which functionally related genes are grouped (23,44). Three functional modules are always present. The replication module contains genes encoding rolling-circle DNA replication and single-strand DNA (ssDNA) binding proteins, *gII*, *gV*, and *gX* (40). The structural module contains genes for the major (*gVIII*) and minor coat proteins (*gIII*, *gVI*, *gVII*, and *gIX*), where gene *gIII* encodes the host recognition or absorption protein pIII (1). The assembly and secretion module contain the genes (*gI* and *gIV*) for morphogenesis and extrusion of phage particles (39). Gene *gIV* encodes protein pIV, an aqueous channel (secretin) in the outer membrane through which phage particles exit from the host cell. The ϕRSS1 genome follows the general arrangement of Ff-like phage. A database survey revealed ORFs with significant homology to Ff-like phage proteins, such as ORF2 (pII), ORF4 (pVIII), ORF7 (pIII), ORF8 (pVI) and ORF9 (pI) (30). Interestingly, 14 of 15 *R. solanacearum* strains tested contained ϕRSS1-related sequences in their genomes, and the ϕRSS1 hybridization pattern coincided well with the taxonomical grouping of these strains (60). This

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 two supplemental tables. Figures 2 and 5 appear in color online.

http://dx.doi.org/10.1094/PHYTO-10-11-0277

suggests a temperate phage of ϕRSS1 and a relation to the phylogenetic variation of host cells. Other filamentous Ff-like phage known to have a lysogenic cycle include *Xanthomonas campestris* phage Cf1c (34), Cf1t (32,33), Cf16v1 (8), and ϕLf (36), *Xylella fastidiosa* phage Xfϕf1 (51), *Yersinia pestis* phage CUSϕ-2 (20), and *Vibrio cholerae* phage VGJϕ and CTXϕ (5,26). The host bacteria of these phage are pathogenic to plants and animals, and are frequently involved in pathogenesis.

In this study, we have further characterized the effects of ϕRSS1 infection on the virulence of *R. solanacearum* cells.

MATERIALS AND METHODS

Bacterial strains, phage, media, and growth conditions. *R. solanacearum* strains MAFF 106603 (race 1, biovar 3, and phylotype I) and MAFF 106611 (race 1, biovar 4, and phylotype I) were from the National Institute of Agrobiological Sciences (Tsukuba, Japan). Bacterial cells were cultured in CPG medium containing 0.1% casamino acids, 1% peptone, and 0.5% glucose (24) at 28°C with shaking (200 to 300 rpm). In some cases, bacteria were cultivated in minimal medium (MM) containing (per liter) 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 $(NH_4)_2SO_4$ (4). Bacteriophage ϕRSS1 was described previously (30). ϕRSS1 was routinely propagated using strain MAFF 106603 as the host. To collect sufficient phage particles, a total of 2 L of bacterial culture was grown. When the cultures reached 0.1 unit at OD_{600}, phage were 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 a R12A2 rotor in a Hitachi Himac CR21E centrifuge (Hitachi Koki Co. Ltd., Tokyo, Japan), at 8,000 × *g* for 15 min at 4°C. The supernatant was passed through a 0.2-μm membrane filter, and 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 of ϕRSS1, single ϕRSS1 plaques were picked from assay plates covered with a MAFF 106603 lawn streaked onto CPG plates. Single colonies were repeatedly purified.

DNA and RNA isolation and molecular techniques. Standard molecular biological techniques for DNA isolation and digestion with restriction enzymes and other nucleases were performed as described by Sambrook and Russell (47). 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 MAFF 106603 cells by the plasmid DNA mini-preparation method (2,3). Total bacterial RNA was isolated from 3 ml culture at the exponential phase (1×10^8 cells/ml) in MM or in CPG medium using an RNA protect Bacteria Reagent kit (Qiagen K.K., Tokyo, Japan), according to the manufacturer's protocol. Total RNA was isolated when cell density reached 10^4, 10^6, and 10^8 CFU/ml. The total RNA was treated with 10 U 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 PCR with gene-specific primers (Table 1). Thirty 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. Quantitative real-time reverse transcriptase PCR (qRT-PCR) was performed using a Line Gene Fluorescence quantitative detection system (BioFlux, Tokyo, Japan) with cDNA prepared from MAFF 106603. A PCR mixture containing SYBR-green (SYBR premix ExTaq, Takara Shuzo, Kyoto) was used. The 10 μl of PCR reaction mixture contained 5 μl of PCR mixture, 1 μl of diluted cDNA, and 0.5 μM each primer (Table 1). PCR conditions were as follows: 3 min at 95°C and 45 cycles at 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°C to 95°C with a heating rate of 0.5°C/min). Relative expression levels were calculated as the ratio of expression of each gene against that of the *16S rRNA* gene in *R. solanacearum* (7).

Motility and cell surface structures. *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,

TABLE 1. List of primers used in this study

Primer name[a]	Oligo sequences (5′ → 3′)	Amplified genes	Product (bp)
phcA298-F	GGACATGATCTTCACGGTCAACT	*phcA*	298
phcA298-R*	GACTCATCCTCCTTTTCTGCATC		
phcB(RT)-F	CTACCAGATCGTCGTCAATGAA	*phcB*	172
phcB(RT)-R*	GTCGAGGTAGTGCTTGATCTTG		
epsB(RT)-F	GGGCTACCTGCACCAGTATTTC	*epsB*	194
epsB(RT)-R*	AGGTATCCATCAGCTCGACCAT		
egl3-F	CAGCGCGACCTACTACAAGA	*egl*	299
egl3-R*	TCATCAGCCCGAAGATGAC		
pglA(RT)-F	TTCATAGCCCAGGAACGTCAT	*pglA/pehA*	351
pglA(RT)-R*	CTCTTCGCACACAACCACTTCT		
pehC(RT)-F	GTTGTTCGGATTGCTGTACG	*pehC*	227
pehC(RT)-R*	AGTCAAACGATTGCCTGAACTA		
chbA(RT)-F	CGCTAGCTTCTAATGGCGAACT	*chbA*	230
chbA(RT)-R*	CGCATATTTAATGCCATCCTCA		
hrpB(RT)-F	TTCTCGATGATGTAGCGATAGG	*hrpB*	238
hrpB(RT)-R*	GCTGGAATTTTCGACTTCCTCTA		
hrpY(RT)-F	AACACGACGAACACGACGAG	*hrpY*	162
hrpY(RT)-R*	GTTCAGGGCGTTCTGCATCT		
awr2(RT)-F	TGAAATTCTTCTCATTCAGATCGTC	*awr2*	221
awr2(RT)-R*	ATCGATGGTCGTCTTGGTGAA		
dnaA(RT)-F	CTGATCGACGACATCCAGTTCT	*dnaA*	353
dnaA(RT)-R*	AAGTTCGAGTACGCCAGGATCT		
16SrRNA349-F	CTAGAGTGTGTCAGAGGGAGGTAGA	*16S rRNA*	349
16SrRNA349-R*	ATGTCAAGGGTAGGTAAGGTTTTTC		

[a] * indicates gene-specific primer used for first-strand cDNA synthesis.

5 µl of the suspension was dropped onto the test medium: MM for twitching motility (37) and SWM for swimming motility (25). Motility was observed by measuring the diameter of the dropped-culture for 6 days.

Bacterial extracellular structure proteins were isolated from 24-h-old bacterial cells grown on solid MM (37). Cells were suspended in 10 mM Tris-HCl (pH 8.0) buffer, adjusted to the same cell density (about 4.5×10^9 CFU/ml), and surface structures were shaved from the cell surfaces by passing the bacterial suspension through a 25-gauge needle. Bacterial cells were removed by centrifugation at 6,000 × *g* for 20 min at 4°C (R12A2 rotor, Hitachi Himac CR21E centrifuge). The bacterial surface structures were collected by ultracentrifugation at 136,000 × *g* for 60 min (P50S2 rotor, Hitachi Himac CP80WX centrifuge). The sedimented materials were separated by Tris-tricine SDS-polyacrylamide gel electrophoresis (PAGE) system according to Schagger and von Jagow (48).

Cell surface hydrophobicity assay. Cell surface hydrophobicity was determined according to Denny et al. (12) and compared between ϕRSS1-infected and uninfected cells of MAFF 106603. Bacterial cells were grown in CPG at 28°C for 48 h and were adjusted to OD_{600} of 0.5 and 1.0. One milliliter of bacterial suspension was added to 200 µl of xylene (Kanto Chemical Co. Inc., Japan), and was vortexed for 2 min, allowing by phases to completely separate (3 h). OD_{600} of the water phase was determined. CPG without bacterial cells was included in the same steps for a negative control. Hydrophobicity was calculated according to the equation: (OD_{600} before – OD_{600} after/OD_{600} before) × 100 = % hydrophobicity (21).

Assays of endoglucanase activity and extracellular polysaccharides. Total endoglucanase activity was determined by measuring the amount of reducing sugars (41) released. The culture supernatant was collected by centrifugation (6,000 × *g*, 10 min, 4°C) from a 72-h-old culture in EG containing 50 mM Na-KPO_4, pH 7, 0.07% $(NH_4)_2SO_4$, 0.10% casamino acid, 0.10% yeast extract, 1.0% sucrose, 0.03% $MgSO_4 \cdot 7H_2O$, 0.0003% $ZnSO_4$, 0.0005% $Ca(NO_3)_2$, 0.0002% $MnSO_4$, and 0.0003% $FeCl_3$ (49). After filtration through a 0.45-µm-pore membrane, the supernatant was subjected to enzyme assay. The enzyme assay was performed by incubating 20% (vol/vol) culture supernatant in 120 mM phosphate buffer (pH of 7.0) with carboxymethylcellulose at 15 mg/ml as a substrate, at 50°C for 4 h. One unit of enzyme activity was defined as releasing 1 nmol glucose per min. For extracellular polysaccharide (EPS) production, cells were grown in BG broth for 3 days at 28°C (11). To precipitate EPS, NaCl was added to the culture supernatant to a final concentration of 0.1 M, and then 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 ddH_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 (16). 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, followed by colorimetric assays. The A_{530} 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 added before the hydrolysis step. EPS production in plant was quantified

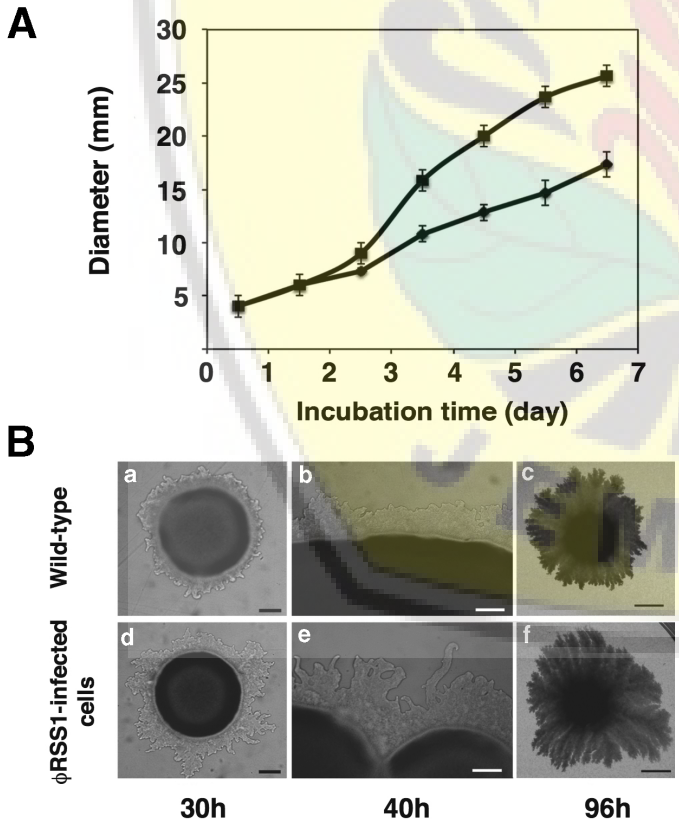


Fig. 1. Twitching motility of *Ralstonia solanacearum* MAFF 106603. **A,** Time course of colony spreading formed on MM plates. Diameter of 10 independent colonies was measured. Wild-type cells (diamonds), and ϕRSS1-infected cells (squares). **B,** Microcolonies formed on MM plates after 30 h (a and d) and 40 h (b and e) observed under a light microscope. Macroscopic views of twitching motility following 4 days of incubation (c and f). Wild-type cells (a to c) compared with ϕRSS1-infected cells (d to f). Bars, 0.1 mm (a, b, d, and e); 4 mm (c and f).

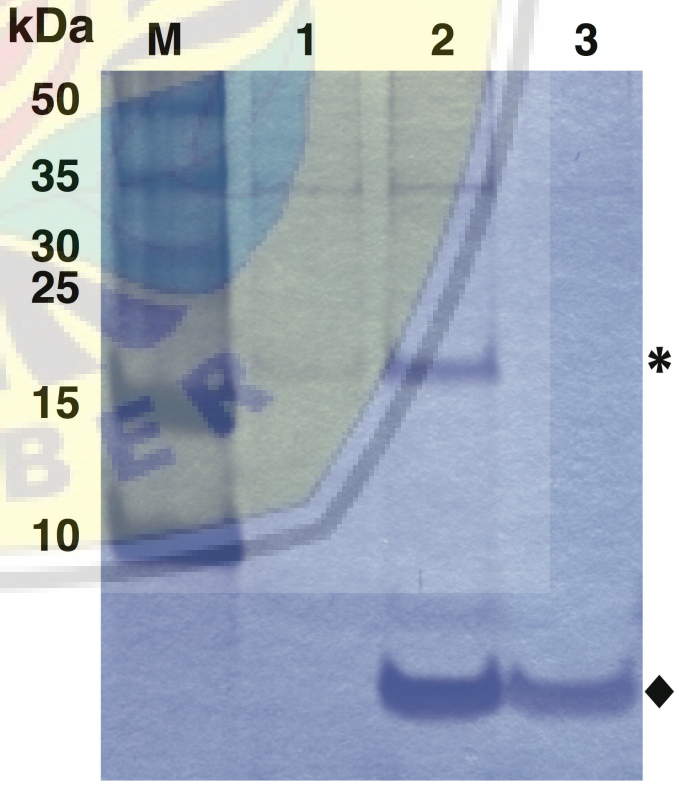


Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis separation of extracellular structural proteins of *Ralstonia solanacearum*. Lanes: M, molecular size markers; 1, wild-type cells MAFF 106603 (4.5×10^9 CFU/ml); 2, ϕRSS1-infected cells (4.5×10^9 CFU/ml); and 3, purified ϕRSS1 particles. The position of PilA (asterisk) and major capsid protein (MCP) (diamond) are indicated. Both proteins were identified by N-terminal amino acid sequencing (2).

according to Denny et al. (12) with slight modifications. Stems of tomato plant injected with 3 µl of *R. solanacearum* (10^5 CFU) were cut off about 2 cm upward and downward from the inoculation site 5 days after inoculation. The stems were sliced into small pieces with sterile razor blade, and each sample was homogenized with 5 ml of sterile water at 4°C. Plant debris was removed by centrifugation at 8,000 × *g* for 30 min at 4°C and five volumes of 99% ethanol was added immediately. The sample was incubated overnight at 4°C and the precipitate was collected by centrifugation for hexosamine assay as above.

Virulence assay of *R. solanacearum* cells. Cells of *R. solanacearum* were grown in CPG medium for 1 to 2 days at 28°C. After centrifugation, cells were washed and resuspended in sterile distilled water. An aliquot of the cell suspension (3 µl, containing 10^5 CFU) was injected with a needle into the major stem of tomato plants (*Lycopersicon esculentum* 'Oogatafukujyu', 6 to 7 weeks old with five to six leaves) at a site between the first and second leaves. As a control, distilled water was injected in the same manner. Each bacterial strain was injected into 10 plants and the virulence assay was repeated three times. Plants were cultivated in a Sanyo Growth Cabinet at 25°C (16 h light/8 h dark) for 14 days. The wilting symptoms were graded from 0 to 4 as described by Winstead and Kelman (59) and modified by Poueymiro et al. (43).

RESULTS

Effects of ϕRSS1 infection on the growth of strain *R. solanacearum* MAFF 106603. Infection by ϕRSS1 does not cause lysis of host cells, but establishes a persistent association between the host and phage, releasing phage particles from the growing host cells (60). Although ϕRSS1-type phages are frequently lysogenized in strains of *R. solanacearum*, the genome of MAFF 106603 was confirmed to be free from the ϕRSS sequence (Supplemental Figure 1). This led us to use this strain for studying the phage–host interaction in relation to plant disease. Upon infection by ϕRSS1, the ϕRSS1 genome was replicated in MAFF 106603 cells, giving a complex DNA pattern after separation by agarose gel electrophoresis. To confirm the ds replicative form (RF) ϕRSS1 genomic DNA, we used a single *Cla*I site on the genome (30). Digestion of DNA with *Cla*I gave a 6.7-kbp band corresponding to the linear dsDNA of the ϕRSS1 genome, confirming the replication of ϕRSS1 DNA. Compared with wild-type cells, MAFF 106603 cells infected by ϕRSS1 showed somewhat abnormal behavior, including less turbidity of liquid cultures, more mucoid in appearance on plates, higher motility in minimal medium, decreased growth rate (approximately 60% of the normal rate), and increased sensitivity to ampicillin (uninfected cells were resistant to 150 µg ml^{-1}, whereas infected cells were sensitive to 15 µg ml^{-1} in liquid media) (data not shown).

Twitching motility and cell-surface structural proteins of ϕRSS1-infected cells. Since the colonies of ϕRSS1-infected cells showed more ragged edges on MM plates, we suspected that infection of ϕRSS1 may have affected host motility. We investigated the motility, such as swimming and twitching, and found that both wild-type and ϕRSS1-infected MAFF 106603 cells exhibited similar swimming motility (not shown). However, as described above, they were significantly different in twitching motility (Fig. 1A). The wild-type MAFF 106603 cells growing on the surface of MM agar plates formed colonies with irregular rafts or tendrils (Fig. 1Ba and Bd), a reticulate appearance, and ragged edges that were thin or layered with multiple irregular projection ('spearheads') (Fig. 1Bb and Be). However, colonies of ϕRSS1-infected cells showed more ragged edges, and were larger than those of wild-type MAFF 106603 (Fig. 1Bc and Bf). These results indicate that host bacteria exhibit more active twitching motility after infection with ϕRSS1. We also isolated extracellular structural proteins from cells used in the twitching motility assay, and compared them by SDS-PAGE analyses. The results showed that a band of approximately 18 kDa, corresponding to major type 4 pilin (PilA) was more abundant in ϕRSS1-infected cells than in wild-type cells (Fig. 2; asterisk).

Host extracellular polysaccharides production and endoglucanase activity. On agar plates, colonies of ϕRSS1-infected cells contained more mucoid in appearance than those of wild-type cells after 48 to 72 h, suggesting enhanced EPS production (Supplemental Figure 2). EPS quantification based on hexosamine amounts showed that ϕRSS1-infected cells produced 3.53 times higher EPS compared with uninfected cells (Table 2). Moreover, in bacterial inoculated plant stems, ϕRSS1-infected cells produced 2.47 times higher EPS compared with uninfected cells 5 days after inoculation (Table 2). However, no significant difference was observed in Egl activity between ϕRSS1-infected and uninfected cells. EPS is positively regulated by the transcriptional regulator PhcA, which is known to activate many virulence genes in *R. solanacearum* (10,50,62). Therefore, we compared expression levels of *phcA* between wild-type and ϕRSS1-infected cells

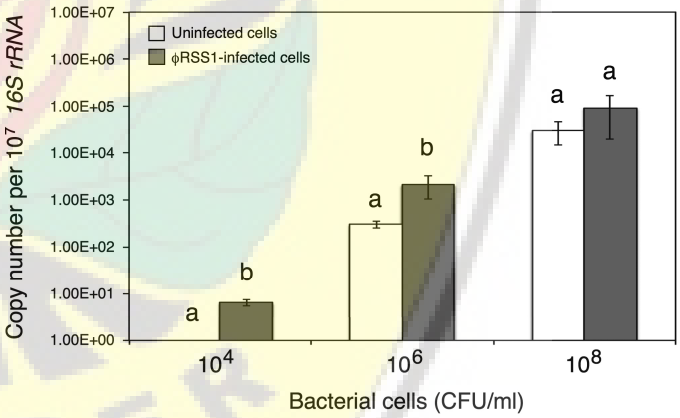


Fig. 3. Expression of *phcA* depending on the cell density in *Ralstonia solanacearum*. Transcript level of *phcA* was determined by quantitative real-time polymerase chain reaction from total RNA isolated from wild-type cells of MAFF 106603 (white) and ϕRSS1-infected cells (gray). Expression level was normalized to that of *16S rRNA* as an internal standard. Mean expression and SD values were calculated from three independent experiments. Bars within each cell density are marked with different letters if values differ significantly at $P \leq 0.05$ according to the Student's *t* test.

TABLE 2. Endoglucanase activity and extracellular polysaccharides (EPS) of *Ralstonia solanacearum* MAFF 106603

Strains	EPS rating[a]	Hexosamine concentration in culture supernatant (µg/ml)[b]	Hexosamine concentration in plant stem (µg/g)[c]	Endoglucanase activity (U/ml)[d]
Uninfected	++	926.84 ± 139.44	24.43 ± 10.01	0.31 ± 0.02
ϕRSS1-infected cells	++++	3,270.00 ± 825.65	60.26 ± 11.28	0.39 ± 0.02

[a] Rating of EPS produced by a colony growing on CPG plate for 72 h by comparing mucosity of the colony surface.
[b] Hexosamine concentration was determined with 3-day-old culture fluid in BG medium (10) broth by measuring N-acetyl-D-glucosamine content. Average of five independent experiments.
[c] Hexosamine concentration was determined in plant stem 5 days postinoculation. Average of three replications.
[d] One unit (1U) released 1 µmole/min of reduction sugar per minute. Average of four independent experiments.

at different growth phases (different cell density) (14). Interestingly, cell density-gene expression relationship showed that *phcA* was expressed at earlier growth stages (at lower cell densities) in ϕRSS1-infected cells compared with wild-type cells (Fig. 3). Apparent *phcA* expression was detected in 10-h-old cultures (10^4 cells/ml) of ϕRSS1-infected cells, but expression in wild-type cells was not detectable. At higher cells densities (>10^6 cells/ml) both cell types expressed *phcA*. Expression was always higher in ϕRSS1-infected cells than wild-type cells. These data suggest that ϕRSS1 infection enhances expression of *phcA*, and thereby activates production of virulence factors, such EPS.

Expression of virulence and pathogenicity genes. To clarify the mechanism by which ϕRSS1 enhances *R. solanacearum* virulence and pathogenicity, we performed qRT-PCR and compared the transcription profiles of ϕRSS1-infected cells to wild-type cells. We used cells grown in minimal medium (approximately 10^6 CFU/ml), because pathogenicity and virulence factors are known to be expressed under these conditions mimicking the natural environment in plant tissues (18). We targeted ten major genes that are related to virulence and pathogenicity and one house keeping gene (*dnaA*) as a control. Our data showed that expression of several genes including *phcB*, *phcA*, *egl*, *eps*, *pglA*, *pehC*, and *awr2* were significantly increased (Fig. 4), many of which are known to be under *phcA* regulation (10,50). Therefore, enhanced expression of *phcA* caused by ϕRSS1 infection may resulted in activation of these virulence and pathogenicity genes. On the other hand, four genes were not significantly affected by infection with ϕRSS1. Interestingly, *egl* and *hrpB* were retained at comparatively low levels, indicating less significance in ϕRSS1-induced virulence enhancement.

Plant virulence assays of ϕRSS1-infected MAFF 106603 cells. Previously, we demonstrated that when strain C319 was infected with ϕRSS1, it became more virulent in tobacco (60). To see whether ϕRSS1 infection also affects the virulence of MAFF 106603, we injected 3-µl cultures containing 10^5 cells into the major stem of tomato plants, and observed for wilting symptoms for 14 days. All three plants inoculated with wild-type bacterial cells showed wilt symptoms starting 3 days postinfection and then completely wilted after 8 days (wilting grade 4). Meanwhile, the three plants inoculated with ϕRSS1-infected MAFF 106603 cells showed wilting symptom as early as 2 days postinfection and died at 5 days postinfection (wilting grade 4) (Fig. 5A and B).

Increase cell surface hydrophobicity in ϕRSS1-infected cells. Filamentous phages like ϕRSS1 assemble on the host cell membrane and accumulate on the cell surface (39,40). Sometimes, host cells are surrounded by high densities of filamentous phages (often tightly interwoven into complex-lattice-works surrounding the cells) (58). We considered the possibility that ϕRSS1-infection might change the cell surface nature, which results in local cell aggregation giving a high cell density. This might be the cause to enhance *phcA* expression at a low cell density (10^4 CFU/ml compared with ~10^6 CFU/ml for wild type cells). To see such changes in surface nature, we examined the cell surface hydrophobicity. As shown in Figure 6, ϕRSS1-infected MAFF 106603 cells showed significant increase in the cell surface hydrophobicity.

DISCUSSION

Like other filamentous Ff-type phages that infect plant pathogenic bacteria, including *X. campestris* phage Cf1c (34), Cf1t (32,33), Cf16v1 (8), and ϕLf (36,53), and *X. fastidiosa* phage Xfϕf1 (51), infection with ϕRSS1 does not cause host cell lysis. Instead, it establishes a persistent association between the host and phage, releasing phage particles from the growing host cells. Therefore, infection by phage can mediate conversion of the host bacterial phenotype. Upon infection by ϕRSS1, host cells demonstrate enhanced EPS production and enhanced twitching motility (Table 2 and Fig. 1). More interestingly, virulence in tomato plants was also enhanced compared with wild-type cells (Fig. 5).

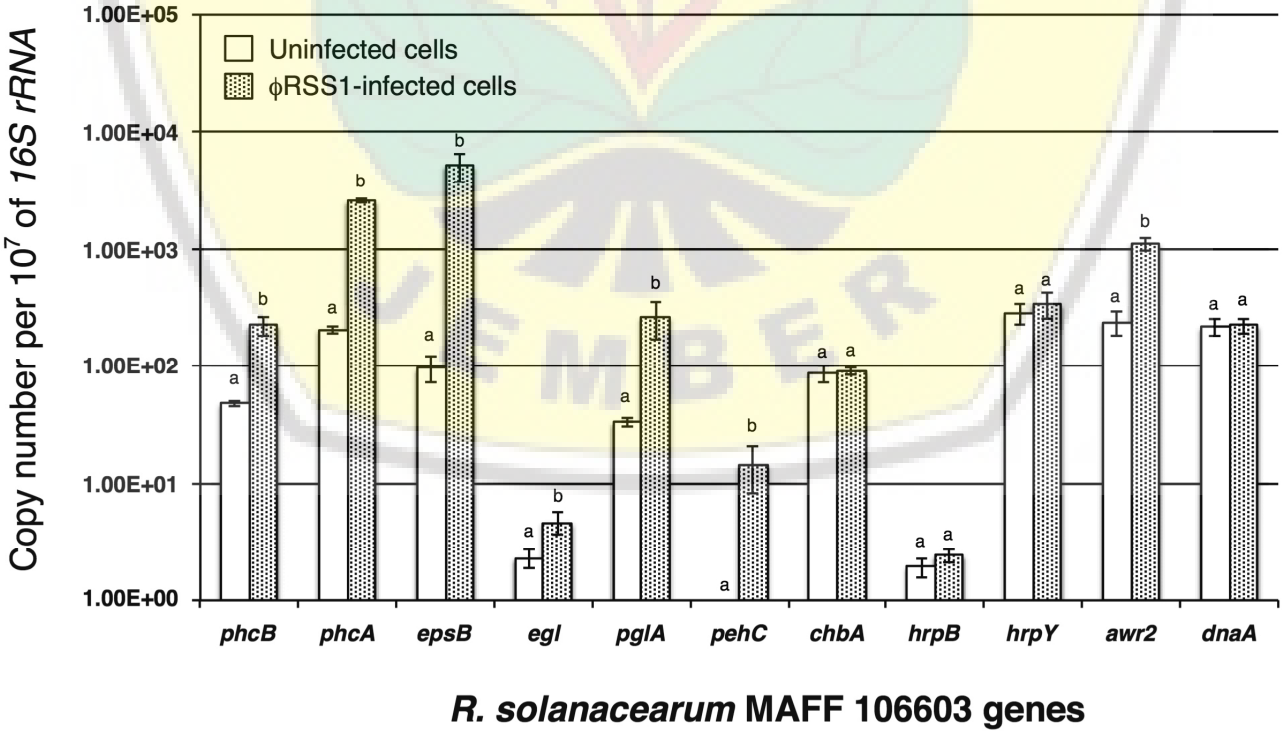


Fig. 4. Expression analysis of *Ralstonia solanacearum* genes involved in virulence. Bacterial cells were grown in MM with initial cell density of approximately 10^2 CFU/ml and incubated at 28°C with shaking (150 rpm) until the cells reached 10^6 CFU/ml. Transcript levels of each gene were determined by quantitative real-time polymerase chain reaction from wild-type MAFF 106603 (white) and ϕRSS1-infected cells (dot-filled). For each gene, expression level was normalized to that of *16S rRNA* as an internal standard. Mean expression and SD values were calculated from three independent experiments. Bars within each gene are marked with different letters if values differ significantly at $P \leq 0.05$ according to the Student's *t* test.

Several filamentous phage are involved in plant pathogenesis (27,53). However, the molecular mechanisms have not been well characterized. One possible mechanism by which phage may contribute to virulence is through the provision of virulence determinants. For example, with animal pathogens, virulence can be transmitted between strains of *Vibrio cholerae* by the filamentous phage CTXϕ, which carries the genes coding for cholera and RTX toxin (9,57).

The ϕRSS1 genomic DNA encodes 11 ORFs in three functional modules as in many Ff-phage (30). However, ORF10 and ORF11, without known functions, appear to replace *gIV* (corresponding to phage secretin) in the assembly-secretion module. Introduction of these genes connected to an expression vector (31) into the host cells did not bring any obvious changes in the cells (Y. Nakahama, T. Kawasaki, M. Fujie, and T. Yamada, *unpublished data*). Therefore, it is unlikely that these genes are involved in virulence. Instead, filamentous phage assembled on the host cell surface and phage particles accumulated on the cell surface. These surface-associated phage particles may change cell surface nature (ex. hydrophobicity) and enhance cell to cell interactions, resulting in high local cell densities. A similar situation was reported for small colony variants (SCVs) formation in *Pseudomonas aeruginosa* that was dependent on phage Pf4 activity (45,58). We found that *phcA*, the gene for the transcriptional regulator PhcA playing a critical role in pathogenicity (10,50,62), was expressed at a very early stage of growth in ϕRSS1-infected cells (at a cell density of ≈10^4 cells/ml) (Fig. 3). Usually, higher cell densities are required for *phcA* expression (Fig. 3, wild type cells). Early expression of *phcA* and accumulation of functional PhcA may have resulted in activation of many virulence factors, especially the production of EPS. Tomato plants inoculated with ϕRSS1-infected cells started to wilt as early as 2 days postinoculation (Fig. 5A). This possible effects of surface-accumulated filamentous phage particles to induce host virulence genes may also explain other cases of phage-enhanced virulence in phytopathogenic bacteria. For example, infection of *X. campestris* pv. *oryzae* NP5850 by the filamentous phages Xf and Xf2, resulted in enhanced virulence, possibly because of overproduc-

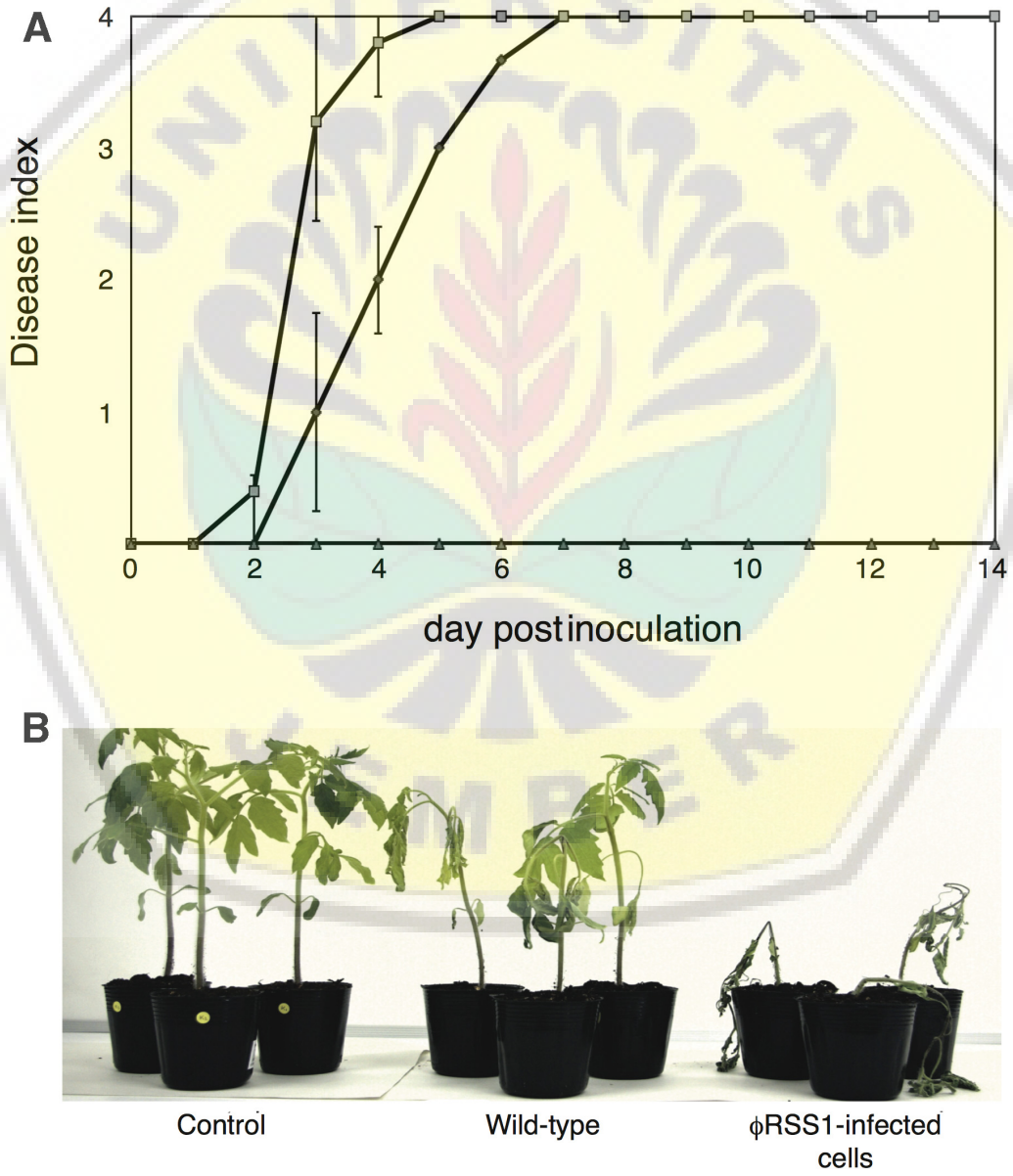


Fig. 5. In planta virulence assay of *Ralstonia solanacearum* cells. Tomato plants (6 to 7 weeks old) were inoculated with bacterial cells (10^5 CFU) into the stem at a site between the first and second leaves. Distilled water injected in the same manner served as a control. The wilting symptoms were graded from 0 to 4 according to Winsted and Kelman (59). **A,** Time course of wilting. Each experiment included 10 plants and was repeated three times (three plants of each treatment as representative picture). Wild-type cells (diamonds), ϕRSS1-infected cells (squares), and H_2O-control (triangles). **B,** Tomato plants injected with bacterial cells 5 days postinoculation.

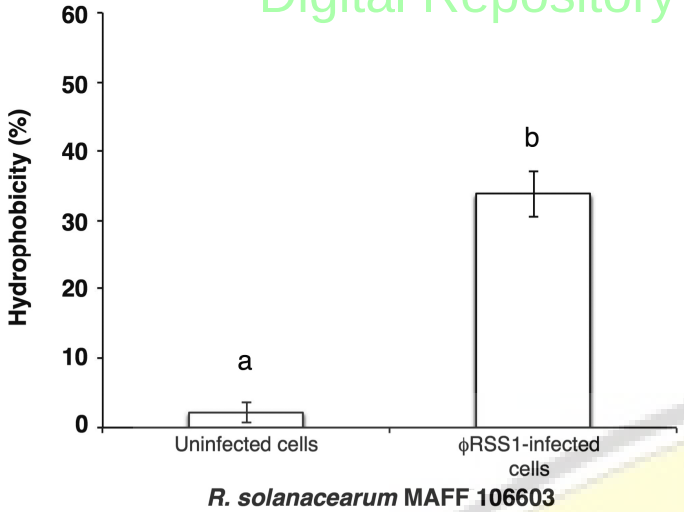


Fig. 6. Cell surface hydrophobicity. Mean hydrophobicity and standard deviation values were calculated from three independent experiments. For experimental details, see Materials and Methods. Bars are marked with different letters if values differ significantly at $P \leq 0.05$ according to the Student's *t* test.

tion of extracellular polysaccharides (EPS) by the phage-infected bacterial cells (27). Tseng et al. (53) also reported that infection of *X. campestris* pv. *campestris* by the filamentous phage Lf increased virulence via enhanced EPS production.

Another effect on the cells caused by ϕRSS1-infection was enhanced-twitching motility (Fig. 1). Twitching motility driven via Tfp in *R. solanacearum* was reported by Liu et al. (37). Its importance to infection processes, especially invasion and colonization of the bacterial cells in plants has been suggested previously. Kang et al. (29) showed that a *pilA* mutant lacking the major Tfp pilin of strain AW1 did not exhibit polar attachment to cultured tobacco cells, or to tomato roots, and became less virulent. We detected elevated levels of PilA protein in ϕRSS1-infected cells compared with wild-type cells (Fig. 2), consistent with enhanced twitching motility in ϕRSS1-infected cells. This result indicates the importance of Tfp-mediated functions, and twitching motility in bacterial virulence.

As shown in the previously, ϕRSS1-related sequences were detected at a high frequency (to date, 17 of 18 strains tested) in the chromosomal DNA of *R. solanacearum* strains isolated from wilted plants in Japan (60). Under favorable infection conditions, ϕRSS1-infected bacterial cells might have become dominant in the population because of growth advantages conferred by the phage, as described above. However, database searches for the ϕRSS1 sequence revealed only three other examples of *R. solanacearum* strains containing a significant ϕRSS1 homolog (strains CFBP2959, IPO1609, and Z04), although there may be local sequence variation.

ACKNOWLEDGMENTS

This study was supported by the Industrial Technology Research Grant Program for the New Energy and Industrial Technology Development Organization (NEDO) of Japan (81030 to T. Yamada).

LITERATURE CITED

1. Armstrong, J., Perham, R. N., and Walker, J. E. 1981. Domain structure of the bacteriophage fd adsorption protein. FEBS Lett. 135:167-172.
2. Askora, A., Kawasaki, T., Usami, S., Fujie, M., and Yamada, T. 2009. Host recognition and integration of filamentous phage ϕRSM in the phytopathogen, *Ralstonia solanacearum*. Virology 384:69-76.
3. Ausubel, F., Brent, R., Kjngston, 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.
4. Boucher, C. A., Barberis, P. A., Trigalet, A. P., and Demery, D. A. 1985. Transposon mutagenesis of *Pseudomonas solanacearum*: Isolation of Tn5 induced avirulent mutants. J. Gen. Microbiol. 131:2449-2457.
5. Campos, J., Martinez, E., Suzarte, E., Rodriguez, B. L., Marrero, K., Silva, Y., Ledon, T., del Sol, R., and Fando, R. 2003. VGJϕ, a novel filamentous phage of *Vibrio cholerae*, integrates into the same chromosomal site as CTXϕ. J. Bacteriol. 185:5685-5696.
6. Carney, B. F., and Denny, T. P. 1990. A cloned avirulence gene from *Pseudomonas solanacearum* determines incompatibility on *Nicotiana tabacum* at the host species level. J. Bacteriol. 172:4836-4843.
7. Carrillo-Casas, E. M., Hernandez-Castro, R., Suarez-Cuemes, F., and de la Pena-Mactezuma, A. 2008. Selection of the internal control gene for real-time quantitative RT-PCR assays in temperature treated *Leptospira*. Curr. Microbiol. 56:539-546.
8. Dai, H., Chow, T. Y., Liao, H. J., Chen, Z. Y., and Chiang, K. S. 1988. Nucleotide sequence involved in the neolysogenic insertion of filamentous phage Cf16-v1 into the *Xanthomonas campestris* pv. *citri* chromosome. Virology 167:613-620.
9. Davis, B. M., Lawson, E. H., Sandkvist, M., Sozhamannan, S., Ali, A., and Waldor, M. K. 2000. Convergence of the secretory pathways for cholera toxin and the filamentous phage, CTXϕ. Science 288:333-335.
10. Denny, T. P. 2006. Plant pathogenic *Ralstonia* species. Pages 573-644 in: Plant-Associated Bacteria. S. S. Gnanamanickam, ed. Springer, Amsterdam, Netherlands.
11. Denny, T. P., Carney, B. F., and Schell, M. A. 1990. Inactivation of multiple virulence genes reduces the ability of *Pseudomonas solanacearum* to cause wilt symptoms. Mol. Plant-Microbe Interact. 3:293-300.
12. Denny, T. P., Makini, F. W., and Brumbley, S. M. 1988. Characterization of *Pseudomonas solanacearum* Tn5 mutants deficient in extracellular polysaccharides. Mol. Plant-Microbe Interact. 1:215-223.
13. Deslandes, L., Olivier, J., Peeters, N., Feng, D. X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S., and Marco, Y. 2003. Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. Proc. Natl. Acad. Sci. USA 100:8024-8029.
14. Flavier, A. B., Clough, S. J., Schell, M. A., and Denny, T. P. 1997. Identification of 3-hydroxypalmitic acid methyl ester as a novel autoregulator controlling virulence in *Ralstonia solanacearum*. Mol. Microbiol. 26:251-259.
15. Garg, R. P., Huang, J., Yindeeyoungyeon, W., Denny, T. P., and Schell, M. A. 2000. Multicomponent transcriptional regulation at the complex promoter of the exopolysaccharide I biosynthetic operon of *Ralstonia solanacearum*. J. Bacteriol. 182:6659-6666.
16. 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.
17. Genin, S., and Boucher, C. 2002. *Ralstonia solanacearum*: Secrets of a major pathogen unveiled by analysis of its genome. Mol. Plant Pathol. 3:111-118.
18. 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.
19. 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.
20. Gonzalez, M. D., Lichtensteiger, C. A., Caughlan, R., and Vimr, E. R. 2002. Conserved filamentous prophage in *Escherichia coli* O18:K1:H7 and *Yersinia pestis* biovar orientalis. J. Bacteriol. 184:6050-6055.
21. Haußler, S., Ziegler, I., Lottel, A., van Gotz, F., Rohde, M., Wehmhohner, D., Saravanamuthu, S., Tummler, B., and Steinmetz, I. 2003. Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. J. Med. Microbiol. 52:295-301.
22. Hayward, A. C. 2000. *Ralstonia solanacearum*. Pages 32-42 in: Encyclopedia of Microbiology, Vol. 4. J. Lederberg, ed. Academic Press, San Diego, CA.
23. Hill, D. F., Short, J., Perharm, N. R., and Petersen, G. B. 1991. DNA sequence of the filamentous bacteriophage Pf1. J. Mol. Biol. 218:349-364.
24. Horita, M., and Tsuchiya, K. 2002. MAFF Microorganism Genetic Resources Manual, No. 12. Pages 5-8. National Institute of Agricultural Sciences, Tsukuba, Japan.
25. 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.
26. Huber, K. E., and Waldor, M. K. 2002. Filamentous phage integration requires the host recombinases XerC and XerD. Nature 417:656-659.
27. Kamiunten, 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. Japan 47:627-636.
28. Kanda, A., Yasukohchi, M., Ohnishi, K., Kiba, A., Okuno, T., and Hikichi,

Y. 2003. Ectopic expression of *Ralstonia solanacearum* effector protein PopA early in invasion results in loss of virulence. Mol. Plant-Microbe Interact. 16:447-455.
29. 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.
30. Kawasaki, T., Nagata, S., Fujiwara, A., Satsuma, H., Fujie, M., Usami, S., and Yamada, T. 2007. Genomic characterization of the filamentous integrative bacteriophage ϕRSS1 and ϕRSM1, which infect *Ralstonia solanacearum*. J. Bacteriol. 189:5792-5802.
31. 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 ϕRSS1. J. Biosci. Bioeng. 104:451-456.
32. Kuo, T. T., Chao, Y. S., Lin, Y. H., Lin, B. Y., Liu, L. F., and Feng, T. Y. 1987. Integration of the DNA of filamentous bacteriophage Cf1t into the chromosomal DNA of its host. J. Virol. 61:60-65.
33. Kuo, T. T., Lin, Y. H., Huang, C. M., Chang, S. F., Dai, H., and Feng, T. Y. 1987. The lysogenic cycle of the filamentous phage Cf1t from *Xanthomonas campestris* pv. *citri*. Virology 156:305-312.
34. Kuo, T. T., Tan, M. S., Su, M. T., and Yang, M. K. 1991. Complete nucleotide sequence of filamentous phage Cf1c from *Xanthomonas campestris* pv. *citri*. Nucleic Acids Res. 19:2498.
35. Lavie, M., Shillington, E., Eguiluz, C., Grimsley, N., and Boucher, C. 2002. PopP1, a new member of the YopJ/AvrRxv family of type III effector proteins, acts as a host-specificity factor and modulates aggressiveness of *Ralstonia solanacearum*. Mol. Plant-Microbe Interact. 15:1058-1068.
36. Lin, N. T., Chang, R. Y., Lee, S. J., and Tseng, Y. H. 2001. Plasmids carrying cloned fragments of RF DNA from the filamentous phage ϕLF can be integrated into the host chromosome via site-specific integration and homologous recombination. Mol. Genet. Genom. 266:425-435.
37. 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.
38. Liu, H., Zhang, S., Schell, M. A., and Denny, T. P. 2005. Pyramiding unmarked deletions in *Ralstonia solanacearum* shows that secreted proteins in addition to plant cell-wall-degrading enzymes contribute to virulence. Mol. Plant-Microbe Interact. 18:1296-1305.
39. Marvin, D. A. 1998. Filamentous phage structure, infection and assembly. Curr. Opin. Struct. Biol. 8:150-158.
40. Model, P., and Russel, M. 1988. Filamentous bacteriophages. Pages 375-456 in: The Bacteriophages, Vol. 2. R. Calendar, ed. Plenum Press, New York.
41. Nelson, N. 1944. A photometric adaptation of the Somogyi method for determination of glucose. J. Biol. Chem. 153:375-380.
42. Orgambide, G., Montrozier, H., Servin, P., Roussel, J., Trigalet-Demery, D., and Trigalet, A. 1991. High heterogeneity of the exopolysaccharides of *Pseudomonas solanacearum* strain GMI1000 and the complete structure of the major polysaccharide. J. Biol. Chem. 266:8312-8321.
43. Poueymiro, M., Cunnac, S., Barberis, P., Deslandes, L., Peeters, N., Cazale-Noel, A. C., Boucher, C., and Genin, S. 2009. Two type III secretion system effectors from *Ralstonia solanacearum* GMI1000 determine host-range specificity on tobacco. Mol. Plant-Microbe Interact. 22:538-550.
44. Rasched, I., and Oberer, E. 1986. Ff coliphages: Structural and functional relationships. Microbiol. Rev. 50:401-427.
45. Rice, S. A., Tan, C. H., Mikkelsen, P. J., Kung, V., Woo, J., Tay, M., Hauser, A., McDougald, D., Webb, J. S., and Kjelleberg, S. 2009. The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. ISME J. 3:271-282.
46. Saile, E., McGarvey, J. A., Schell, M. A., and Denny, T. P. 1997. Role of extracellular polysaccharide and endoglucanase in root invasion and colonization of tomato plants by *Ralstonia solanacearum*. Phytopathology 87:1264-1271.
47. Sambrook, J., and Russell, D. W. 2001. Molecular Cloning: A Laboratory Manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
48. 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.
49. Schell, M. A. 1987. Purification and characterization of an endoglucanase from *Pseudomonas solanacearum*. Appl. Environ. Microbiol. 53:2237-2241.
50. Schell, M. A. 2000. Control of virulence and pathogenicity genes of *Ralstonia solanacearum* by an elaborate sensory array. Annu. Rev. Phytopathol. 38:263-292.
51. Simpson, A. J., Reinach, F. C., Arruda, P., Abreu, F. A., Acencio, M., Alvarenga, R., Alves, L. M., Araya, J. E., Baia, G. S., Baptista, C. S., Barros, M. H., Bonaccorsi, E. D., Bordin, S., Bové, J. M., Briones, M. R., Bueno, M. R., Camargo, A. A., Camargo, L. E., Carraro, D. M., Carrer, H., Colauto, N. B., Colombo, C., Costa, F. F., Costa, M. C., Costa-Neto, C. M., Coutinho, L. L., Cristofani, M., Dias-Neto, E., Docena, C., El-Dorry, H., Facincani, A. P., Ferreira, A. J., Ferreira, V. C., Ferro, J. A., Fraga, J. S., França, S. C., Franco, M. C., Frohme, M., Furlan, L. R., Garnier, M., Goldman, G. H., Goldman, M. H., Gomes, S. L., Gruber, A., Ho, P. L., Hoheisel, J. D., Junqueira, M. L., Kemper, E. L., Kitajima, J. P., Krieger, J. E., Kuramae, E. E., Laigret, F., Lambais, M. R., Leite, L. C., Lemos, E. G., Lemos, M. V., Lopes, S. A., Lopes, C. R., Machado, J. A., Machado, M. A., Madeira, A. M., Madeira, H. M., Marino, C. L., Marques, M. V., Martins, E. A., Martins, E. M., Matsukuma, A. Y., Menck, C. F., Miracca, E. C., Miyaki, C. Y., Monteriro-Vitorello, C. B., Moon, D. H., Nagai, M. A., Nascimento, A. L., Netto, L. E., Nhani, A. Jr., Nobrega, F. G., Nunes, L. R., Oliveira, M. A., de Oliveira, M. C., de Oliveira, R. C., Palmieri, D. A., Paris, A., Peixoto, B. R., Pereira, G. A., Pereira, H. A. Jr., Pesquero, J. B., Quaggio, R. B., Roberto, P. G., Rodrigues, V., de Rosa, A. J., de Rosa, V. E. Jr., de Sá, R. G., Santelli, R. V., Sawasaki, H. E., da Silva, A. C., da Silva, A. M., da Silva, F. R., da Silva, W. A. Jr., da Silveira, J. F., Silvestri, M. L., Siqueira, W. J., de Souza, A. A., de Souza, A. P., Terenzi, M. F., Truffi, D., Tsai, S. M., Tsuhako, M. H., Vallada, H., Van Sluys, M. A., Verjovski-Almeida, S., Vettore, A. L., Zago, M. A., Zatz, M., Meidanis, J., and Setubal, J. C. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406:151-157.
52. Tans-Kersten, J., Huang, H. Y., and Allen, C. 2001. *Ralstonia solanacearum* needs motility for invasive virulence on tomato. J. Bacteriol. 183:3597-3605.
53. Tseng, Y. H., Lo, M. C., Lin, K. C., Pan, C. C., and Chang, R. Y. 1990. Characterization of filamentous bacteriophage ϕLf from *Xanthomonas campestris* pv. *campestris*. J. Gen. Virol. 71:1881-1884.
54. Vailleau, F., Sartorel, E., Jardinaud, M. F., Chardon, F., Genin, S., Huguet, T., Gentzbittel, L., and Petitprez, M. 2007. Characterization of the interaction between the bacterial wilt pathogen *Ralstonia solanacearum* and the model legume plant *Medicago truncatula*. Mol. Plant-Microbe Interact. 20:159-167.
55. Vasse, J., Frey, P., and Trigalet, A. 1995. Microscopic studies of intercellular infection and protoxylem invasion of tomato roots by *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 8:241-251.
56. Vasse, J., Genin, S., Frey, P., Boucher, C., and Brito, B. 2000. The *hrpB* and *hrpG* regulatory genes of *Ralstonia solanacearum* are required for different stages of the tomato root infection process. Mol. Plant-Microbe Interact. 13:259-267.
57. Waldor, M. K., and Mekalanos, J. J. 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science 272:1910-1914.
58. Webb, J. S., Lau, M., and Kjelleberg, S. 2004. Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. J. Bacteriol. 186:8066-8073.
59. Winstead, N. N., and Kelman, A. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathology 42:628-634.
60. 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.
61. Yao, J., and Allen, C. 2007. The plant pathogen *Ralstonia solanacearum* needs aerotaxis for normal biofilm formation and interactions with its tomato host. J. Bacteriol. 189:6415-6424.
62. Yoshimochi, T., Hikichi, Y., Kiba, A., and Ohnishi, K. 2009. The global virulence regulator PhcA negatively controls the *Ralstonia solanacearum hrp* regulatory cascade by repressing expression of the PrhIR signaling proteins. J. Bacteriol. 191:3424-3428.
63. Zolobowska, L., and Van Gijsegem, F. 2006. Induction of lateral root structure formation on petunia roots: A novel effect of GMI1000 *Ralstonia solanacearum* infection impaired in *hrp* mutants. Mol. Plant-Microbe Interact. 19:597-606.