Prophage Rs551 and Its Repressor Gene orf14 Reduce Virulence and Increase Competitive Fitness of Its Ralstonia solanacearum Carrier Strain UW551

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We previously characterized a filamentous lysogenic bacteriophage, φRs551, isolated directly from the race 3 biovar 2 phylotype IIB sequevar 1 strain UW551 of Ralstonia solanacearum grown under normal culture conditions. The genome of φRs551 was identified with 100% identity in the deposited genomes of 11 race 3 biovar 2 phylotype IIB sequevar 1 strains of R. solanacearum, indicating evolutionary and biological importance, and ORF14 of φRs551 was annotated as a putative type-2 repressor. In this study, we determined the effect of the prophage and its ORF14 on the virulence and competitive fitness of its carrier strain UW551 by deleting the orf14 gene only (the UW551 orf14 mutant), and nine of the prophage’s 14 genes including orf14 and six out of seven structural genes (the UW551 prophage mutant), respectively, from the genome of UW551. The two mutants were increased in extracellular polysaccharide production, twitching motility, expression of targeted virulence and virulence regulatory genes (pilT, egl, pehC, hrPB, and phcA), and virulence, suggesting that the virulence of UW551 was negatively regulated by φRs551, at least partially through ORF14. Interestingly, we found that the wt φRs551-carrying strain UW551 of R. solanacearum significantly outcompeted the wt strain RUN302 which lacks the prophage in tomato plants co-inoculated with the two strains. When each of the two mutant strains was co-inoculated with RUN302, however, the mutants were significantly out-competed by RUN302 for the same colonization site. Our results suggest that ecologically, φRs551 may play an important role by regulating the virulence of and offering a competitive fitness advantage to its carrier bacterial strain for persistence of the bacterium in the environment, which in turn prolongs the symbiotic relationship between the phage φRs551 and the R. solanacearum strain UW551. Our study is the first toward a better understanding of the co-existence between a lysogenic phage and its carrier plant pathogenic bacterial strain by determining the effect of the prophage Rs551 and its repressor on the virulence and competitive fitness of its carrier strain UW551 of R. solanacearum.

Keywords: Ralstonia solanacearum, filamentous lysogenic phage, prophage, phage repressor, competitive fitness, race 3 biovar 2, phylotype, sequevar
INTRODUCTION

Bacterial wilt, a soil borne vascular disease caused by *Ralstonia solanacearum*, is one of the most devastating bacterial diseases in tropical, subtropical, and temperate regions of the world. The bacterium has a host range of over 450 plant species, including ornamentals such as geranium and economically important crops such as tomatoes and potatoes (Hayward, 1991; Kelman et al., 1994; Denny, 2006). *R. solanacearum* normally invades host plants from soil through wounds in roots, reproduces in the xylem vessels, and spreads rapidly through the plant's vascular system resulting in wilting and death of the plant (Vasse et al., 2000). The bacterium can be spread in soil or water or through latentely infected plant materials like potato tubers or geranium cuttings.

*Ralstonia solanacearum* is historically classified into five races and five biovars based on host range and biochemical properties, respectively. Molecular classification, however, has thus far grouped *R. solanacearum* into 4 phylotypes and 53 sequevars (Albuquerque et al., 2014; Stulberg and Huang, 2016). The race 3 biovar 2 (phylotype IIB sequevars 1 and 2) of *R. solanacearum* was grown and its inocula prepared as described (Stulberg et al., 2015). To isolate *R. solanacearum* from soil of crop fields, and their use as biocontrol agents has been explored (Yamada et al., 2007; Fujiwara et al., 2011; Bhunchoth et al., 2015). We recently isolated a filamentous lysogenic bacteriophage φRs551 directly from the race 3 biovar 2 strain UW551 of *R. solanacearum* grown under normal culture conditions (Ahmad et al., 2017). The phage has a particle size of about 1,200 nm in length and 7 nm in width, and has a genome size of 7,929 nucleotide with 14 open reading frames (Ahmad et al., 2017). In contrast with other *R. solanacearum* phages isolated from soil, φRs551 is the first isolated phage that contains a resolvase (ORF13) and a putative type-2 phage repressor (ORF14), although how this repressor maintains a prophage state and phage immunity in φRs551 had not been studied. In addition, the genome sequence of φRs551 is surprisingly found with 100% identity in the deposited genomes of 11 race 3 biovar 2 phylotype IIB sequevar 1 strains of *R. solanacearum*, indicating evolutionary importance (Ahmad et al., 2017). Infection of a susceptible *R. solanacearum* strain RUN302 by φRs551 resulted in colonies with less fluidal appearance and reduced EPS production, motility, and virulence (Ahmad et al., 2017). It is unclear, however, what effects the phage or the putative phage repressor has on its host strain *R. solanacearum* UW551.

Huerta et al. (2015) recently hypothesized that differences in temperature adaptation and competitive fitness account for the uneven geographic distribution of *R. solanacearum* strains, and found that lowland tropical and warm temperate strains out-compete temperate strains of *R. solanacearum*, probably due to bacteriocins produced by the tropical and warm temperate strains to specifically inhibit the growth of the temperate strains. *R. solanacearum* lytic phages encode bacteriolytic proteins (Ozawa et al., 2001) and are lytic to susceptible strains (Fujiwara et al., 2011), and lysogenic phages are known to bring novel phenotypic properties that might affect the fitness of their host bacteria (Casjens, 2003; Buisso et al., 2004; Canchaya et al., 2004; Davies et al., 2016). We therefore hypothesize that *R. solanacearum* strains prevalent under different environmental conditions may contain different phages that offer competitive fitness to their host strain, allowing the host strains to persist in the environment by preventing the establishment of susceptible strains of *R. solanacearum* that lack the lysogen.

To better understand the contribution of the putative type-2 repressor of φRs551 and the phage φRs551 itself to the virulence and competitive fitness of its carrier strain UW551 of *R. solanacearum*, we generated two *R. solanacearum* mutants by knocking out the orf14 gene, and nine of the 14 genes of φRs551 from the prophage region of UW551, respectively. The phenotype of the two mutants included increased EPS production and twitching motility, increased expression of five other genes tested, as well as increased virulence when inoculated into tomato plants alone. When co-inoculated with strain RUN302 which lacks the prophage Rs551 in its genome for infection of tomato plants, these mutants also had decreased competitive fitness in colonizing tomato stems and had little effect on the virulence of strain RUN302.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Bacterial strains and plasmids used and constructed in this study are listed in Table 1.

Growth and Isolation of Bacterial Strains

*Ralstonia solanacearum* was grown and its inocula prepared as described (Stulberg et al., 2015). To isolate *R. solanacearum* from inoculated plant samples, 0.5-cm plant stem sections were prepared and homogenized as described (Stulberg and Huang,
TABLE 1 | Bacterial strains and plasmids used in this study.

| Designation | Relevant characteristics a | Source or reference |
|-------------|---------------------------|---------------------|
| Strains     |                           |                     |
| *Ralstonia solanacearum* |                           |                     |
| UW551       | Wild-type, race 3 biovar 2, phytopathogenic | C. Allen, United States |
| UW551ΔφRs551-orf14 (UW551 orf14 mutant) | UW551 with a 342-bp prophage region containing orf14 replaced with a 616-bp Gm cassette, Gm R | This study |
| UW551ΔφRs551 (UW551 prophage mutant) | UW551 with a 3,321-bp prophage region including orf14 and orf1 to orf8 replaced with a 616-bp Gm cassette, Gm R | This study |
| RUN302      | Wild-type, biovar 1, phytopathogenic | P. Prior, France |
| *Escherichia coli* |                           |                     |
| TOP10       | F− mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ araL-lacU7697 gaiU galK ptsL (StrR) endA1 rupG | Invitrogen |
| TP997       | MG1655 lacIPΔ::bla-aadA1148 galK::aacC1067 | Addgene |
| Plasmid     |                           |                     |
| pCR Blunt II TOPO | PCR cloning vector, Kan R, Zc R | Invitrogen |
| pCR Blunt II-Gm | pCR Blunt II TOPO with a 616-bp Gm cassette, Gm R, Kan R, Zc R | This study |
| pCR Blunt II-φRs551-orf14 Up-Gm-φRs551-orf14 Down | pCR Blunt II-Gm with 994-bp upstream and 819-bp downstream fragments of the 342-bp prophage region in *R. solanacearum* inserted before and after the Gm cassette, respectively, Gm R, Kan R, Zc R | This study |
| pCR Blunt II-φRs551 Up-Gm-φRs551 Down | pCR Blunt II-Gm with 994-bp upstream and 850-bp downstream fragments of the 3,321-bp prophage region in *R. solanacearum* inserted before and after the Gm cassette, respectively, Gm R, Kan R, Zc R | This study |

aGm R, Kan R, and Zc R indicate resistance to gentamicin, kanamycin, and zeocin, respectively.

2015), and diluted onto modified semi-selective medium agar plates (Huang and Lakshman, 2010). *Escherichia coli* strains were cultured at 37°C in Luria-Bertani medium (Miller, 1972). When needed, antibiotics were added at 25 µg/ml for kanamycin and 15 µg/ml for gentamicin. Since *R. solanacearum* strain UW551 is a select agent pathogen in the United States, manipulation of the strain was conducted in a secured laboratory and virulence assays described below were performed in a secured greenhouse section approved for select agent research by USDA/APHIS using standard operating procedures also approved by APHIS for race 3 biovar 2 strains of *R. solanacearum*.

DNA Isolation and Manipulation

Standard molecular biology techniques were used for plasmid isolation, restriction digestion, cloning, and transformation of *E. coli* strains (Sambrook and Russell, 2001). Total bacterial DNA was extracted using Qiagen’s Blood and Tissue Kit (Qiagen, Chatsworth, CA, United States) following the manufacturer’s instructions.

Design of PCR Primers and PCR Conditions

Primers designed in this study were listed in Table 2. They were designed based on the deposited UW551 draft genome sequence in GenBank (ASM16795v1, GCA_000167955). The regions selected for primer design were entered into the free online A plasmid Editor (ApE) program. Similar design parameters (GC = 45–60%, Tm = 60–64°C, primer length 18–26) were used for primers in each pair. The specificity of each primer pair and amplicon was checked by BLASTn against the UW551 genome, and the nr and WGS databases in GenBank for specificity.

Colony PCR was performed by picking *R. solanacearum* cells using a sterile toothpick or pipette tip from a single colony grown on a plate and mixing the cells in 100 µl of sterile water. The cell suspension was boiled for 5 min and cooled on ice or stored at −20°C until use. Two to five microliters of the suspension were used for PCR.

PCR to amplify the upstream and downstream prophage regions was conducted in a 20 µl volume containing 1x KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Boston, MA, United States), 5 pmol of each primer, and approximately 20 ng of DNA template. PCR conditions were 1 cycle of 3 min at 95°C, followed by 30 cycles of 20 s at 98°C, 15 s at 58°C, and 30 s at 72°C, with a final extension of 2 min at 72°C. PCR to amplify virulence-related genes was conducted in a 20-µl volume containing 1x GoTaq Green Master Mix (Promega, Madison, WI, United States), 20 ng of template DNA, and 5 pmol of each primer. PCR conditions were 1 cycle of 4 min at 94°C, 30 cycles of 1 min at 94°C, 30 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, with a final extension of 10 min at 72°C.

Construction of *R. solanacearum* Mutants

To study the role of orf14 of the prophage Rs551 and the prophage itself in *R. solanacearum*, two mutants of *R. solanacearum* which had deletions in the prophage were constructed in strain UW551 by homologous double recombination. Mutant UW551ΔφRs551-orf14, designated as the UW551 orf14 mutant, was generated by replacing a 342-bp prophage region, located in contig 0570 of UW551 (GenBank accession number: AAKL01000012.1), with a 616-bp gentamicin cassette. The 342-bp fragment contained the entire 291-bp prophage region...
corresponding to φRs551’s orf14, coding for a putative type-2 phage repressor, as well as 42-bp upstream and 9-bp downstream of orf14. Mutant UW551ΔφRs551, designated as the UW551 prophage mutant, was constructed by replacing a 3,321-bp prophage region with a 616-bp gentamicin cassette. The 3,321-bp fragment contained the same 342-bp in the prophage region with a 616-bp gentamicin cassette. The 3,321-bp prophage mutant, was constructed by replacing a 3,321-bp orf14 phage repressor, as well as 42-bp upstream and 9-bp downstream phcA phcA hrpB hrpB pehC pilT Egl Egl-φ φ corresponding to φ′ Rs551's orf14, between the replication and structure modules, orf2 between the replication and structure modules, and six (orf3 to orf7, and 1,005-bp of the 1,524-bp of orf8) of the seven structural genes in the structural module of the prophage (Figure 1). To make the mutants, a 616-bp gentamicin cassette was first amplified from a colony of TP997, purchased from Addgene (Cambridge, MA, United States), by PCR with primers 5′-CGAATCCATGTGGGAGTTTA-3′ and 5′-TTAGGTGGCGGTACTTGGT-3′ (Poteete et al., 2006). The cassette was then cloned into the TOPO site of the vector pCR Blunt II TOPO to generate pCR Blunt II-Gm using Invitrogen’s Zero Blunt® TOPO® PCR Cloning Kit according to the manufacturer’s instructions. Two regions of DNA, 994-bp in size located upstream and 815-bp downstream of the 342-bp prophage region, were amplified by PCR using primers in Table 2, digested with respective restriction enzymes, and cloned sequentially into the multiple cloning sites before and after the gentamicin cassette in pCR Blunt II-Gm to obtain pCR Blunt II-φRs551-orf14 Up-Gm-φRs551-orf14 Down. Similarly, the same 994-bp upstream fragment and a 850-bp downstream fragment of the 3,321-bp prophage region was cloned sequentially to obtain pCR Blunt II-φRs551-Up-Gm-φRs551 Down. The resulting plasmids were electroporated into competent cells of R. solanacearum strain UW551 as described by Ahmad et al. (2017). This was followed by selection on gentamicin-containing triphenyltetrazolium chloride (TZC) plates (Kelman, 1954) for transformants that had undergone homologous double recombination between the Up-Gm-Down region in pCR Blunt II-Up-Gm-Down and the Up and Down prophage sequences in the chromosome of UW551 of R. solanacearum. The knock-out mutants were identified by screening on TZC plates amended with gentamicin, and confirmed by PCR using primer pairs located within the sequences of the mutated regions that showed a lack of any amplified products. To determine if a phage was still produced from the UW551 mutants, an aliquot from the supernatant of the R. solanacearum mutants was subjected to the spot test and plaque-forming assay (Ahmad et al., 2017) using R. solanacearum RUN 302, a strain susceptible to φRs551 and which contained no φRs551 sequence in its genome before infection. The presence or absence of phage particles in the supernatant of the R. solanacearum mutants was also examined under transmission electron microscope (Ahmad et al., 2017).

### EPS, in Vitro Growth, and Twitching Motility Assays

Extracellular polysaccharide in the supernatant of R. solanacearum was determined quantitatively, in vitro growth of R. solanacearum strains were measured, and twitching motility examined as described by Ahmad et al. (2017), except that twitching motility was visualized using a Zeiss AxiosZoom v16 stereo zoom microscope (Carl Zeiss Microscope GmbH, Germany). Two replicates were used for each strain in the EPS assay and the experiment was repeated three times.

### RNA Isolation and Analysis of Gene Expression

Total bacterial RNA was isolated from 3 ml of R. solanacearum culture at the exponential growth phase (OD<sub>600</sub> = 0.3) using Qiagen’s RNeasy Protect Bacterial and RNeasy Mini Kits (Qiagen, Inc., CA) according to the manufacturer’s protocol. Ambion® TURBO DNA-free™ DNase Treatment and Removal Reagents (Life Technology) were used to remove contaminating DNA from the RNA preparation and to subsequently remove the DNase and divalent cations from the sample. The absence of DNA contaminants was confirmed by PCR using gene-specific primers (Table 2) on the RNA samples. The bacterial genomic DNA of

### Table 2: List of primers designed in this study.

| Primer pair       | Sequence (5′-3′, restriction enzyme sites are underlined)       | Size of PCR product (bp) |
|-------------------|----------------------------------------------------------------|
| φRs551-orf14-up-F-Xbal | TATAATGAGGTGGCGCATG                                           | 994                      |
| φRs551-orf14-up-R-Xhol  | AGTAGTCTGACATGGGAGTTTA                                         | 994                      |
| φRs551-down-F-BamHI   | TTACTGGATCCAGGACCGAAGGACGGATCCG                                  | 850                      |
| φRs551-down-R-KpnI    | AATCTGGTACGAAAGGTCACGGATCCG                                      | 850                      |
| φRs551-orf14-down-F-BamHI | ATACTGGATCCCGATGCTGACGGATCCG                                      | 819                      |
| φRs551-orf14-down-R-KpnI | ACAGGCTACACTTCCACAGGACGGACGGATCCG                                  | 819                      |

**Eps primers**

| Primer pair       | Sequence (5′-3′, restriction enzyme sites are underlined)       | Size of PCR product (bp) |
|-------------------|----------------------------------------------------------------|
| Egl-F             |TCATCAGCGCCGGAAGATGAC                                              | 140                      |
| Egl-R             |GGCTGGATCCAGGACGGATCCG                                              | 140                      |
| pilT-F            |GTAATTGCTGCGGTGACACC                                               | 147                      |
| pilT-R            |GCGCGTCTGATCGACTTGC                                                 | 147                      |
| pehC-F            |GTTGTGGGATTGCTGAGC                                                 | 227                      |
| pehC-R            |AGTCAACAGGATTGCGTTGAAC                                              | 227                      |
| hrpB-F            |TTCTGATGATTGCGAGCAGATG                                            | 123                      |
| hrpB-R            |CAGACGGAGCGTGAACCT                                                 | 123                      |
| phcA-F            |GTGATTGCGCCGACCACT                                                 | 147                      |
| phcA-R            |CGAGGGCTACAGGCGCTAAC                                              | 147                      |

**orf2 primers**

| Primer pair       | Sequence (5′-3′, restriction enzyme sites are underlined)       | Size of PCR product (bp) |
|-------------------|----------------------------------------------------------------|
| Rs551-up-F-BamHI  | ATACTGGATCCCGATGCTGACGGATCCG                                      | 819                      |
| Rs551-up-R-Xhol   | AATCTGGTACGAAAGGTCACGGATCCG                                       | 819                      |
| Rs551-down-F-BamHI | ATACTGGATCCCGATGCTGACGGATCCG                                      | 819                      |
| Rs551-down-R-KpnI | ACAGGCTACACTTCCACAGGACGGACGGATCCG                                  | 819                      |

**orf3 primers**

| Primer pair       | Sequence (5′-3′, restriction enzyme sites are underlined)       | Size of PCR product (bp) |
|-------------------|----------------------------------------------------------------|
| Rs551-up-F-BamHI  | ATACTGGATCCCGATGCTGACGGATCCG                                      | 819                      |
| Rs551-up-R-Xhol   | AATCTGGTACGAAAGGTCACGGATCCG                                       | 819                      |
| Rs551-down-F-BamHI | ATACTGGATCCCGATGCTGACGGATCCG                                      | 819                      |
| Rs551-down-R-KpnI | ACAGGCTACACTTCCACAGGACGGACGGATCCG                                  | 819                      |

**orf4 primers**

| Primer pair       | Sequence (5′-3′, restriction enzyme sites are underlined)       | Size of PCR product (bp) |
|-------------------|----------------------------------------------------------------|
| Rs551-up-F-BamHI  | ATACTGGATCCCGATGCTGACGGATCCG                                      | 819                      |
| Rs551-up-R-Xhol   | AATCTGGTACGAAAGGTCACGGATCCG                                       | 819                      |
| Rs551-down-F-BamHI | ATACTGGATCCCGATGCTGACGGATCCG                                      | 819                      |
| Rs551-down-R-KpnI | ACAGGCTACACTTCCACAGGACGGACGGATCCG                                  | 819                      |
**RESULTS**

**Confirmation of *R. solanacearum* Mutants and Determination of Phage Production by the Mutant Strains**

The 7,929-nucleotide genome sequence of φRs551 corresponds to nucleotides 73,039-80,967 in contig 0570 of the deposited genome sequence of *R. solanacearum* strain UW551 (Figure 1). *R. solanacearum* mutants were confirmed by their ability to grow on TZC plates containing gentamicin, and by PCR for the absence of the 342-bp prophage region in the UW551 orf14 mutant and the 3,321-bp region in the UW551 prophage mutant (data not shown). The UW551 orf14 mutant was found to produce phage particles spontaneously in its supernatant at a rate similar to the wt strain UW551. This was shown when the supernatant of the overnight culture of the UW551 orf14 mutant strain was subjected to the spot test and plaque-forming assay using φRs551-susceptible strain *R. solanacearum* RUN302, similar plaque formation was observed, and a similar number of plaques was obtained as with the supernatant of the wt UW551 (data not shown). On the contrary, no plaques were formed when the supernatant of the prophage mutant strain was subjected to the same plaque susceptibility assay, and no phage particles were observed under transmission electron microscope.

**Physiological Changes Were Detected in *R. solanacearum* Mutants**

To characterize the UW551 orf14 and prophage mutant strains, we first compared the *in vitro* growth of the mutants with their wt strain UW551, and found all three strains grew at a similar rate (data not shown). When the three strains grew on regular TZC medium plates, however, the colonies of the mutant strains appeared more fluidal and irregular than those of the wt UW551, suggesting a high production of EPS. This observation was confirmed by an EPS quantitative assay that showed both mutant strains produced significantly higher amounts of EPS (73.6 ± 4.5 mg/10 ml for UW551 prophage mutant, and 59.6 ± 9.5 mg/10 ml for UW551 orf14 mutant) than the wt strain (44.3 ± 7.2 mg/10 ml). The difference in EPS production between the two mutant strains was not significant. The two mutant strains also displayed distinctly different twitching motility when compared with the wt strain UW551 (Figure 2). For the wt strain, we observed twitching motility under a microscope as indicated by the formation of corrugated trajectories with smooth edge around the margin of its colonies (Figure 2, left). The size of the trajectories, however, was larger with irregular edges in *R. solanacearum* mutant strains, especially in the orf14 mutant (Figure 2, middle).

**The UW551 orf14 and Prophage Mutant Strains of *R. solanacearum* Were More Virulent than the wt Strain UW551**

To study the effect of deletion of the targeted prophage regions in the virulence of *R. solanacearum*, we compared the virulence of the wt to that of the mutant strains of *R. solanacearum* (Figure 3).
The wt strain UW551 did not cause any disease symptoms until 8 days after soil drenching inoculation (DI > 0), and reached a DI of 3.1 at day 21 (Figure 3). The virulence level caused by the two mutant strains of *R. solanacearum*, however, was significantly higher (Figure 3). The mutants started to cause disease symptoms 5 days after inoculation and completely wilted all inoculated plants (DI = 4) by day 17 (Figure 3). DIs caused by the UW551 prophage mutant were statistically similar to the ones caused by the UW551 orf14 mutant, except at days 7 and 8 (Figure 3).

**Gene Expression Levels Were Increased in *R. solanacearum* Mutant Strains**

To identify the factors contributing to increased virulence of the *R. solanacearum* mutant strains, expression of five genes (*pilT*, *egl*, *pehC*, *hrpB*, and *phcA*), all known virulence factors of *R. solanacearum*, was compared between the mutant and the wt strains. The expression of all five genes was increased in the two mutant strains, with the *pilT* gene showing the greatest increase: 8-fold in the orf14 mutant and 21-fold in the prophage mutant (Figure 4). The level of expression of the *egl*, *pehC*, *hrpB*, and *phcA* genes was increased between 2.0- and 4.5-fold in the two mutants (Figure 4).

**R. solanacearum orf14 and Prophage Mutant Strains of UW551 Are Out Competed by the Phage ϕRs551-Susceptible Strain RUN302 in Plants**

The effect of UW551 and its mutant strains on plant colonization by RUN302, a strain of *R. solanacearum* lacking the prophage Rs551, was studied. RUN302 was co-inoculated with one of the UW551 strains for infection of tomato plants. The ratio of the
two mixed strains was determined in stem sections of infected tomato plants. When the wt φRs551-carrier strain UW551 was co-inoculated with the wt φRs551-lacking strain RNN302, 30 out of 50 randomly picked bacterial colonies isolated from the tomato stems belonged to UW551 (Figure 5), significantly more than the number of RUN302 colonies. On the contrary, when each of the two UW551 mutant strains was co-inoculated with RUN302, only 10 and 19 out of 50 were colonies of the prophage mutant strain and the orf14 mutant strain, respectively, significantly less than the number of RUN302 colonies in the stems (Figure 5).

To study the effect of UW551 and its mutant strains on the virulence of R. solanacearum strain RUN302, tomato plants were inoculated by soil drenching with RUN302 alone, or together (1:1) with the wt UW551, the UW551 orf14 mutant or the UW551 prophage mutant, respectively (Figure 6). When RUN302 was co-inoculated with UW551, the co-inoculation caused a delayed and significantly lower DI 4 days after inoculation than inoculation with RUN302 alone (Figure 6). On the contrary, when RUN302 was co-inoculated with the UW551 prophage mutant, its overall virulence was similar to that caused by RUN302 alone (Figure 6). DIs caused by RUN302 co-inoculated with the UW551 orf14 mutant strain were lower than the ones caused by RUN302 alone or co-inoculation with RUN302 and the UW551 prophage mutant, but the difference was not significant 9 days after inoculation and all inoculated plants were completely wilted 6 days after that (Figure 6).

**DISCUSSION**

Currently, a wide range of R. solanacearum phages have been reported including filamentous phages of the family Inoviridae (Yamada et al., 2007; Murugaiyan et al., 2011; Van et al., 2014), and icosahedral phages of the families Myoviridae (Yamada et al., 2007; Bhunchoth et al., 2015), Podoviridae (Kawasaki et al., 2009, 2016; Bhunchoth et al., 2015), and Siphoviridae (Thi et al., 2015). Comparative genomics also revealed that R. solanacearum genomes contain many prophages of the families Inoviridae and Myoviridae (Yamada et al., 2007; Murugaiyan et al., 2011; Ahmad et al., 2017). The co-evolution between bacteria and bacteriophages plays a key role in driving and maintaining the ecology and evolution of microbial populations, and phages are known to change competitive dynamics among bacterial strains or species (Bohannan and Lenski, 2000a,b; Joo et al., 2006; Koskella et al., 2012; Koskella and Brockhurst, 2014). Temperate phages like the SMF phage of Streptococcus suis and the prophages of Pseudomonas aeruginosa affect their carrier bacteria in many ways (e.g., growth rate for the former and competitiveness for the latter), contributing to the fitness and virulence of the bacteria (Davies et al., 2016). Previous studies of R. solanacearum phages, however, had been focused exclusively on genomic characterization, integration mechanism, effect on their susceptible R. solanacearum strains and potential as biocontrols (Kawasaki et al., 2007a,b; Yamada et al., 2007; Askora et al., 2009, 2011; Addy et al., 2012a,b). Our study is the first toward a better understanding of the co-existence between a lysogenic phage and its carrier plant pathogenic bacterial strain by determining the effect of the prophage φRs551, through mutagenesis, on the virulence and competitive fitness of its host strain UW551 of R. solanacearum.

Recently, we found that the filamentous phage φRs551 was stably maintained in the genome of the race 3 biovar 2 strain UW551 of R. solanacearum as a prophage, and released to the supernatant of the bacterial strain under normal growth condition (Ahmad et al., 2017). In addition, infection of a φRs551-lacking R. solanacearum strain RUN302 by the phage caused integration of φRs551 into the genome of RUN302, resulting in significantly reduced EPS production, swimming, swarming, and twitching motilities, as well as virulence (Ahmad et al., 2017). In this study, we determined the effect of φRs551 on its carrier R. solanacearum strain UW551 in virulence and competitive fitness by deleting 3,321 of φRs551’s 7,929-bp prophage region from the UW551 genome. As expected, no phage particles were detected, since all but one structural genes of φRs551 were deleted in the mutant (Figure 1). The deletion also resulted in significantly increased virulence as compared to the wt strain UW551, probably due to the increased EPS production and twitching motility (as indicated by the over-expression of the pilT gene), as well as the over-expression of other virulence genes including egl and pehC, and virulence regulatory genes phcA and hrpB. Repeated attempts to delete φRs551’s entire 7,929-bp prophage region from UW551 were unsuccessful, suggesting that at least a portion of the phage may be essential to R. solanacearum UW551 for unknown reasons.

The discovery that the genome of φRs551 was present with 100% identity in the deposited genomes of 11 race 3 biovar 2 phytype IIB sequevar 1 strains of R. solanacearum isolated from different countries at different times and sequenced independently by different research groups (Ahmad et al., 2017) raised the question about the evolutionary and biological significance of the prophage in the sequevar 1 strains of
The UW551 prophage mutant, however, had little effect caused by infection of RUN302 by φRs551. In virulence, the wt UW551 significantly reduced the virulence of RUN302 when the ORF14 of the prophage mutant was only 8.4-fold higher than the wt strain. Under a microscope, the size of the corrugated trajectories around the plant was determined by serial dilution plating of ground 0.5-cm stem, followed by a multiplex PCR (Sturberg et al., 2015) to differentiate the strains. Values are means of three experiments, each containing 50 randomly picked colonies from five wilted plants per treatment. Bars indicate standard errors. *Indicates significant difference (p < 0.05) between RUN302 and the wt or mutant UW551 strains by the Student’s t-test.

Since φRs551 contains a putative type-2 repressor gene orf14, it is possible that the observed physiological changes in the UW551 prophage mutant of \( R. \ solanacearum \) are due to the lack of transcriptional repression of bacterial virulence-related genes by the phage’s repressor. Such a hypothesis has also been proposed by Addy et al. (2012a) for the type-1 phage repressor. Since the UW551 prophage mutant contains a mutation of orf14, we generated the UW551 orf14 mutant by deleting a 342-bp prophage region from the UW551 genome (which includes the 291-bp orf14 of φRs551) to determine if orf14 solely played a role in the observed physiological changes in the prophage mutant. In contrast with the UW551 prophage mutant, the UW551 orf14 mutant produced phage particles like the wt strain UW551. Similar to the prophage mutant strain, however, the orf14 mutant was significantly increased in production of EPS and expression of the virulence and virulence regulatory genes assayed. This may lead to increased virulence of the orf14 mutant as compared to the wt strain UW551, although the level of increase was not as high as the prophage mutant strain, suggesting that the observed physiological changes in the prophage mutant are at least partially caused by deletion of the orf14 gene. The partial effect of the type 2 repressor encoded by orf14 of φRs551 is different from the type 1 repressor encoded by orf15 of another filamentous \( R. \ solanacearum \) phage RSM3, since the loss of virulence caused by infection with φRSM3 can be fully restored when the ORF15 of φRSM3 was deleted (Addy et al., 2012a). This suggests that the type 2 phage repressor in φRs551 may not regulate virulence as tightly as the type 1 repressor in φRSM3. Results from our competition assays revealed that like the UW551 prophage mutant, the UW551 orf14 mutant was significantly out competed by strain RUN302 in colonizing tomato stems (Figure 5), and had little effect on the virulence of RUN302 9 days after mixed inoculation of tomato plants (Figure 6). These results suggest that orf14 may only be partially responsible for offering competitive fitness to the wt strain UW551 in tomato stems and in reducing virulence of the φRs551-lacking strain RUN302 in mixed infection. This partial effect of the UW551 orf14 mutant may be explained by continued production of phage particles in the mutant, thereby exhausting the energy of the mutant or triggering other physiological changes in the mutant and/or host plant.

It is unclear why the expression of the pilT gene in the UW551 orf14 mutant was 21.1-fold higher, but the UW551 prophage mutant was only 8.4-fold higher than the wt strain. Under a microscope, the size of the corrugated trajectories around the colonies of the mutant also looked bigger in the orf14 mutant than in the prophage mutant (Figure 2). Future studies are necessary to determine the importance of pilT gene expression in relation to virulence.
needed to determine how ORF14 regulates the expression of the pilT gene and the significance of PilT in other biological functions other than twitching motility in strain UW551 of R. solanacearum.

CONCLUSION

We demonstrated that the prophage Rs551 affects multiple important physiological functions of and offers competitive fitness to its carrier R. solanacearum strain UW551, at least partially through the type 2 phage repressor encoded by orf14. Future research, however, is needed to determine exactly how the phage repressor regulates these functions in the bacterial strain, and what other phage factors contribute to the virulence and competitiveness fitness of the carrier bacterial strain against other Rs551-lacking R. solanacearum strain occupying the same environment and competing for the same ecological niche for plant infection and survival. A better understanding of the relationship between the phage and the bacterium will facilitate effective control of R. solanacearum.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: AA, MS, and QH. Performed the experiments: AA and MS. Analyzed the data: AA and QH. Contributed reagents/materials/analysis tools: QH. Wrote the paper: AA and QH.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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