Contribution of the \textit{murI} Gene Encoding Glutamate Racemase in the Motility and Virulence of \textit{Ralstonia solanacearum}

Kihyuck Choi\textsuperscript{1}*, Geun Ju Son\textsuperscript{1}†, Shabir Ahmad\textsuperscript{2}, Seung Yeup Lee\textsuperscript{1}, Hyoung Ju Lee\textsuperscript{1}, and Seon-Woo Lee\textsuperscript{1}*  
\textsuperscript{1}Department of Applied Bioscience, Dong-A University, Busan 49315, Korea  
\textsuperscript{2}Department of Microbiology and Biotechnology, Sarhad University of Science and Information Technology, Peshawar, Pakistan  
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Bacterial traits for virulence of \textit{Ralstonia solanacearum} causing lethal wilt in plants were extensively studied but are not yet fully understood. Other than the known virulence factors of \textit{Ralstonia solanacearum}, this study aimed to identify the novel gene(s) contributing to bacterial virulence of \textit{R. solanacearum}. Among the transposon-inserted mutants that were previously generated, we selected mutant SL341F12 strain produced exopolysaccharide equivalent to wild type strain but showed reduced virulence compared to wild type. In this mutant, a transposon was found to disrupt the \textit{murI} gene encoding glutamate racemase which converts L-glutamate to D-glutamate. SL341F12 lost its motility, and its virulence in the tomato plant was markedly diminished compared to that of the wild type. The altered phenotypes of SL341F12 were restored by introducing a full-length \textit{murI} gene. The expression of genes required for flagella assembly was significantly reduced in SL341F12 compared to that of the wild type or complemented strain, indicating that the loss of bacterial motility in the mutant was due to reduced flagella assembly. A dramatic reduction of the mutant population compared to its wild type was apparent \textit{in planta} (i.e., root) than its wild type but not in soil and rhizosphere. This may contribute to the impaired virulence in the mutant strain. Accordingly, we concluded that \textit{murI} in \textit{R. solanacearum} may be involved in controlling flagella assembly and consequently, the mutation affects bacterial motility and virulence.

\textbf{Keywords} : bacterial wilt disease, glutamate racemase, tomato plant

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\textit{Ralstonia solanacearum} is a soil-borne plant pathogen belonging to the class Betaproteobacteria and causes deadly wilt disease in over 200 plant species across 50 different plant families (Hayward, 1991). It causes diseases in various commercially important plants such as brown rot in potato; wilt in tomato, tobacco, and eggplant; and Moko disease in banana (Hayward, 1991). Due to its broad host range and wide geographical distribution, this pathogen holds the second position among the top ten plant pathogenic bacteria (Mansfield et al., 2012). \textit{R. solanacearum} invades host plants as a parasite and survives in soil or water as a saprophyte (van Elsas et al., 2001; Wallis and Truter, 1978). Its broad host range and ability to survive in soil or water for extended periods suggest that this pathogen adopts various mechanisms to combat both biotic and abiotic stress conditions.

Numerous virulence factors including extracellular polysaccharide I (Denny and Baek, 1991), the type III secretion system and effectors (Boucher et al., 1985), swimming motility (Tans-Kersten et al., 2001), cell-wall degrading enzymes and type II secretion system (Liu et al., 2005) contribute to the virulence and pathogenicity of \textit{R. solanacearum}. Among these factors, flagella-driven swimming motility in plant pathogenic bacteria play a crucial role in ecological fitness and virulence (Kang et al., 2002; Tans-Kersten et al., 2001). The significance of flagella
in an invasion of plants has also been studied in several plant pathogenic bacteria such as *Pseudomonas syringae* pv. *phaseolicola* in bean leaves and *Erwinia amylovora* in apple blossom (Bayot and Ries, 1986; Panopoulos and Schroth, 1974). Moreover, flagella perform the crucial functions of colonization of biotic and abiotic surfaces (Hikitchi et al., 2013; Taguchi et al., 2010).

The cell wall peptidoglycan is essential for bacterial viability. D-glutamate is a specific component of cell wall peptidoglycan, generated by two known pathways involving reactions between D-amino acid aminotransferase (D-AAT) and glutamate (Doublet et al., 1992; Liu et al., 1998). One pathway comprises of D-AAT transforming α-ketoglutarate to D-glutamate through transamination with D-alanine, which is generated by alanine racemase. The other pathway involves glutamate racemase converting L-glutamate to D-glutamate through racemization (Liu et al., 1998). Previously, transposon mutagenesis of *R. solanacearum* SL341 generated over 3,900 mutants (Wu et al., 1998). Among these mutants, the SL341F12 strain, which produces more black pigment compared to the wild type and loses motility, was selected here for further studies. The transposon disrupted a gene, *murI*, encoding glutamate racemase that transforms L-glutamate to D-glutamate.

Since the SL341F12 strain was disrupted *murI* gene encoding glutamate racemase lost its motility, we hypothesized that the strain may affect the virulence by attenuating the motility. To test this hypothesis, we investigate the motility of the mutant strain on the media and virulence phenotypes on tomato cultivars and bacterial populations associated with tomato plant, by using the wild type, the mutant strain and its complementation strain on the basis of Koch’s molecular postulate (Falkow, 2004).

**Materials and Methods**

**Bacterial strains and growth conditions.** All bacterial strains and plasmids used in this study are listed in Supplementary Table 1. *Escherichia coli* strains were routinely cultured in Luria-Bertani (LB) media at 37°C. Antibiotics were added to the medium as per need at the following concentrations: kanamycin 50 μg/ml, tetracycline 10 μg/ml, and ampicillin 100 μg/ml. *R. solanacearum* SL341 (race 1, phyotype I) (Jeong et al., 2007) was cultured in casamino acid-peptone-glucose (CPG), which contains 0.1% casamino acid, 1% peptone, 0.5% glucose, CPG supplemented with 0.005% (w/v) 2, 3, 5-triphenyltetrazolium chloride (TZC) and M9 media, which contains 20% M9 salts (5×), 2% glucose, 0.2% MgSO₄, 0.01% CaCl₂. The following antibiotic concentrations were used in CPG, TZC, and M9 media: kanamycin 25 μg/ml, and tetracycline 10 μg/ml. Yeast extract-dextrose-CaCO₃, medium, which contains 1% yeast extract, 2% glucose, 2% CaCO₃, and 1.5% agar was used for triparental mating between *R. solanacearum* mutant strains and *E. coli* donor and helper strains.

**Characterization of murI gene of *R. solanacearum* SL341.** In a previous study (Wu et al., 2015), over 3,900 transposon (Tn)-insertional mutants of SL341 were generated using the EZ-Tn5<KAN-2>Tnp Transposome kit (Epicentre, Madison, WI, USA), as per the manufacturer’s instructions. Among these mutants, the SL341F12 strain was selected for further studies. The disrupted site was identified using a previously described method (Wu et al., 2015). The genomic DNA was isolated from the mutant strain and randomly digested using Sac I. The digested fragment was ligated into pUC119 (Yanisch-Perron et al., 1985) which was digested using the same restriction enzyme. After ligation, this recombinant plasmid was transferred to a competent cell of *E. coli* DH5α for selection of transformants using LB agar supplemented with kanamycin and ampicillin. The recombinant plasmid in selected positive clones was sequenced with Tn5 specific primers (Supplementary Table 2) to identify the flanking regions of the insertion site.

For complementation of SL341F12 strain, the *murI* gene was amplified from the genomic DNA of the wild type strain, SL341, using specific primers (Supplementary Table 2). Polymerase chain reaction was performed in the following cycles: 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, and a final extension step at 72°C for 7 min. The amplicon of *murI* gene was first cloned into the pGEM-T easy vector (Supplementary Table 1) and the insert was sequenced to confirm. The *murI* gene was subcloned into pRK415, and subsequently this recombinant plasmid was introduced into the MF 12 strain through triparental mating using *E. coli* HB101 (Boyer and Roulland-Dussoix, 1969) harboring pRK2013 as a helper plasmid.

**Quantification of extracellular polysaccharide.** Extracellular polysaccharide (EPS) was extracted from *R. solanacearum* strain SL341, SL341F12, and SL341F12C and quantified by measuring hexosamine content with the Elson Morgan Assay. All strains were grown with shaking at 200 rpm at 28°C for 48 h in CPG supplemented with appropriate antibiotics. The bacterial cell density was measured by dilution plating on TZC medium. The liquid culture was filtered using a membrane filter with a pore size of 0.2 μm to prepare cell-free culture filtrates. Precipi-
tation of EPS fraction and reaction with Ehrlich’s regent was performed as described previously (Gatt and Berman, 1966). To generate standard curve, D-(+)-galactosamine was used as a reference compound. The amount of EPS generated from *R. solanacearum* strains were displayed as the amount of galactosamine per 10^9* bacterial cells using the standard curve.

**Bacterial growth and motility assay.** To compare the growth rate of wild type strain SL341 and mutant strain SL341F12 and SL341F12C, these strains were cultured on two different types of media: CPG and M9 minimal salt at 30°C with agitation at 200 rpm for up to 72 h, respectively. Samples which had 3 replicates for each medium were harvested every 12 h from CPG media and M9 minimal media. These samples were used to plate 100 µl of serial dilution of each suspension for corresponding microbial growth (colony-forming unit [cfu]/ml) on TZC media.

To compare bacterial motility between wild type and the mutant strain, wild type strain SL341, mutant strain SL341F12, and complementation strain SL341F12C were stab-inoculated into the center of separate test tubes to about half the depth of the motility test media (TZC media containing 0.35% agar) using a sterilized needle. Cultures were incubated at 30°C for 2 weeks and then photographed. Motility was determined as positive when the growth of strains diffuses throughout the TZC medium rendering it red, and as negative when the growth of strains is confined to the top of media and in the stab-line.

**Reverse transcription quantitative PCR (RT-qPCR).** The expression levels of three genes (flgC, flgE, and flIM) involved in flagella assembly were examined in SL341, SL341F12, and SL341F12C strains along with the mutated gene in the corresponding mutant strain by RT-qPCR. Total RNA was extracted from SL341, SL341F12, and SL341F12C strains collected after 48 h of M9 culture using Trizol (Invitrogen, Carlsbad, CA, USA) and the isolated RNA was cleaned using the RNeasy-mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. The cleaned RNA was directly used as a template for cDNA synthesis using cDNA Tetro synthesis kit (Bioline, Taunton, MA, USA), following the manufacturer’s instructions. Prior to cDNA synthesis, all RNA samples were treated with DNaseI to remove any remaining traces of DNA contamination. Concentration and purity of cDNA of each sample was measured using the NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). The amplification reactions were performed in the CFX384 Real Time System (Bio-Rad, Hercules, CA, USA) using normalized RNA samples. Each reaction mixture contained SYBR Green Supermix (Bio-Rad), 20 ng of cDNA template, 5 µM of both forward and reverse primers and RNase free water. The PCR program was as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s. The quantitative PCR (qPCR) data were analyzed using the CFX Manager software ver. 3.1. Each reaction was performed in triplicate. V3 region of the 16S rRNA gene of *R. solanacearum* was used as the reference gene to normalize the expression level of each gene. The relative normalized expression of qPCR products of each gene were used to determine the target cDNA concentration based on the relative comparison with V3 gene expression.

**Tomato growth and virulence assay.** Tomato cultivars Moneymaker and Hawaii 7996 were used as susceptible and resistant cultivars to bacterial wilt, respectively. Tomato seeds were surface sterilized using 70% ethanol, 0.5% NaOCl, and 0.1% Triton X-100 followed by washing with sterilized distilled water (SDW). Completely dried seeds were germinated for 7 days in Petri dishes containing 5 ml SDW on sterilized filter paper (Advantec, Toyo Roshi Co., Ltd., Tokyo, Japan). The germinated seeds were further grown in a plug tray containing 17 g of nursery soil (Punong Co., Ltd., Gyeongju, Korea). A regimen of 14 h/10 h light/dark cycle at 28°C was set for plant growth for 3 weeks.

Virulence of *R. solanacearum* strains were tested by using both soil-soaking and petiole injection methods. For preparing bacterial inoculum, *R. solanacearum* SL341, SL341F12, and SL341F12C were cultured on TZC plates with appropriate antibiotics as mentioned in the above paragraph. The plates containing bacterial cells were suspended using SDW and OD600 was adjusted to 0.3 (ca. 2 × 10^8* cfu/ml) for soil-soaking method. Tomato plants in the plug tray were inoculated using this bacterial cell suspension to reach the bacterial density of 1 × 10^8* cfu/g of soil. For petiole injection, the tomato cultivars were directly inoculated to 4-week-old plants with the wild type and mutant strain (1 × 10^8* cfu/ml) through the petiole of the second true leaf. Three replications of each bacterial suspension included 10 tomato plants and bacterial wilt disease severity was scored through 14 days using the following method: number of wilted leaves/total number of leaves × 100%.

**Quantification of the population of SL341 and SL341F12 strains.** Susceptible and resistant cultivars of tomato were grown as described above. Bacterial suspensions of SL341 and SL341F12 at a final concentration of 2 × 10^8* cfu/g were poured into the nursery soil of 4-week-old tomato
plants. To quantify the population of both strains, rhizosphere soil was collected at 5 and 10 days post inoculation (dpi). The tomato plants were removed from their plug pots and chunks of soil attached to the roots were removed by tapping, leaving only soil particles adhered to the roots. The roots were submerged in 50 ml falcon tubes containing 5 ml of 2.5 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.7) and these tubes were sonicated at 135 W for 5 min (Branson 5500DTH, Danbury, CT, USA). After sonication, the suspended soil in the buffer was centrifuged at 13,000 rpm for 15 min and subsequently weighed for serial dilution. For the measurement of population in the endosphere, 3 cm segments were cut from the roots 1 cm below the hypocotyl and washed three times with SDW. These washed roots were transferred to fresh falcon tubes containing 10 ml of 80% EtOH. The tubes were shaken vigorously for 30 s and subsequently the roots were placed into new falcon tubes containing 10 ml of 3% bleach followed by shaking the tubes for 30 s again. The roots were washed with SDW to thoroughly remove the ethanol and bleach. These roots were weighed and grounded in a MES buffer to conduct serial dilution plate. The serial dilutions from rhizosphere samples and root samples were spread onto semi-selective medium (SMSA) (Elphinstone et al., 1996) media as a selective media for *R. solanacearum* to enumerate the population of both strains.

**Statistical analysis.** Statistical analysis was performed using the R software (version 3.2.2, http://www.r-project.org/) employing the car package. The repeated-measures analysis of variance (ANOVA) and Student’s t-test were performed for virulence test, bacterial growth and quantification data.

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**Fig. 1.** (A) Genomic position and surrounding regions of the insertion site of transposon insertion mutant, SL341F12. (B) Phylogenetic tree of glutamate racemase homologues from *Ralstonia solanacearum* and others bacteria inferred from MurI comparisons. The tree was constructed by using the maximum likelihood method. Bootstrap values are shown for each node that had >50% support in a bootstrap analysis of 1,000 replicates. The scale bar indicates the genetic distance of 0.20 substitution per site.
Results

Identification of murI gene of R. solanacearum SL341. Random transposon mutagenesis of R. solanacearum SL341 using EZ-Tn5<KAN-2>Tnp Transposome generated over 3,900 mutants previously (Wu et al., 2015). From these mutants, we selected one strain, SL341F12, which produced more black pigment than the wild type (Supplementary Fig. 1). Moreover, the amount of produce exopolysaccharide was not significantly different between the mutant and wild type strain (P = 0.12) (Supplementary Fig. 2). To identify the disrupted gene corresponding to the murI gene encoding glutamate racemase, selected subclones of SL341F12 were sequenced with Tn-specific primers. The full-length murI gene was amplified from SL341 using murI_F and murI_R primers (Supplementary Table 2) to determine complete sequence of murI of the SL341 strain and to construct complementation vector. Further, BLAST search revealed that the murI gene of the SL341 strain was highly homologous to murI genes in Burkholderia pseudomallei, Pseudomonas syringae pv. syringae, and Pseudomonas aeruginosa (Fig. 1).

Attenuation in the bacterial growth, motility, and flagella assembly of murI disrupted R. solanacearum. To test whether the mutation of murI affect bacterial growth in vitro, the wild type (SL341), mutant (SL341F12), and complemented strain (SL341F12C) were cultured on two different types of media, rich (CPG) and minimal (M9) one. The growth rates of all strains in a rich (Fig. 2A) and minimal (Fig. 2B) media were similar.

The motility of the SL341F12 strain was investigated with respect to its ability to spread on soft agar. SL341 retained its motility on soft agar, with visible growth in every direction of the soft agar while SL341F12 stayed only on top of the soft agar since it had lost its motility (Fig. 3). Meanwhile, complementation strain, SL341F12C regained its motility on soft agar (Fig. 3). This result invokes the possibility of defects in the flagella assembly in the mutant SL341F12. To test this possibility, we investigated the...
expression of genes such as \textit{flgC} encoding flagellar basal-body rod protein FlgC, \textit{flgE} encoding flagellar hook protein FlgE and \textit{fliM} encoding flagellar motor switch protein FliM involved in the flagella assembly by RT-qPCR. RT-qPCR revealed that the expression level of \textit{flgC}, \textit{flgE}, and \textit{fliM} in SL341F12 declined by 39%, 40%, and 70%, respectively compared to those of SL341 and by 65%, 65%, and 71%, respectively compared to those of SL341F12C (Fig. 4).

**Reduced virulence of SL341F12 on tomato.** Since SL341F12 lost its motility on media and showed down-regulation of genes required for flagella assembly, we speculated that SL341F12 would also be affected in its virulence. We tested this hypothesis by inoculating SL341, SL341F12, and SL341F12C into a susceptible tomato cultivar (Moneymaker) and a resistant tomato cultivar (Hawaii 7996) using soil-soaking and petiole injection methods.

In soil-soaking method, disease severity of bacterial wilt disease in the resistant cultivar was 48%, 33%, and 9% respectively, for inoculation by SL341, SL341F12C, and SL341F12 strains (Fig. 5A). Moreover, in the susceptible cultivar, SL341F12 showed significantly lower virulence (40%) compared to that of SL341 (80%) and SL341F12C (100%) (Fig. 5B). In petiole injection method, the tomato plants inoculated by SL341F12 showed less disease severity compared to the plants inoculated by SL341, but not as much as soil-soaking method (Supplementary Fig. 3). This confirms that \textit{murI} mutation affected bacterial virulence in \textit{R. solanacearum}, probably due to the defects in the motility.

**Quantification of SL341 and SL341F12 in bulk soil, tomato rhizosphere, and roots.** To further understand the reduced virulence in the SL341F12 strain, we investigated
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the population of SL341 and SL341F12 in bulk soil, tomato (cv. Moneymaker) rhizosphere and tomato roots at 5 dpi. The populations of both strains were higher in the rhizosphere than in bulk soil. Both in bulk soil and tomato rhizosphere, bacterial population was not significantly different between SL341 and SL341F12 (Fig. 6A and B). Meanwhile, the population of SL341F12 was undetectable in the tomato root endosphere while SL341 was detected at the rate of 1.8 × 10^6 cfu of *R. solanacearum*/g of root (Fig. 6C). This suggested that SL341F12 has significant defects in host invasion.

Discussion

The gene disrupted by insertion of transposon in the SL341F12 genome was identified as *murI* gene, encoding glutamate racemase. To investigate the role of *murI* gene in *R. solanacearum*, complementation strain was generated using pRKM vector (Supplementary Table 1) containing *murI* gene from SL341 using triparental mating. Since identification of the gene encoding glutamate racemase named *murI* in *E. coli*, *murI*-like genes have been characterized in the several bacterial strains such as *Staphylococcus haemolyticus* and *Bacillus pumilus* (Liu et al., 1997; Pucci et al., 1995). Phylogenetic analysis of *murI*-like genes revealed that this gene has been highly conserved in prokaryotes (Fig. 1).

Glutamate racemase catalyzes the conversion of L-glutamate to D-glutamate. D-glutamate is a crucial, biosynthetic building block for all Gram-positive and negative bacteria in the production of bacterial cell wall component peptidoglycan (murin) (Van Heijenoort et al., 2001). Even though *murI* gene is essential for the viability of bacterial growth according to previously published reports, disruption of this gene in *R. solanacearum* did not affect bacterial viability (Doublet et al., 1993; Kada et al., 2004; Kimura et al., 2004; Liu et al., 1998). The growth of SL341F12 strain in rich and minimal media was similar to the growth of wild type and complementation strain (Fig. 2).

The SL341F12 strain showed relatively low levels of expression of three flagellar biosynthetic genes *flgC*, *flgE*, and *fliM* (Fig. 4). The roles of these genes in motility, bacterial attachment, and virulence have been investigated in several animal and plant pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium, *Xanthomonas axonopodis* pv. *glycines*, and *Mesorhizobium tianshanense* (Chuaboon et al., 2014; Ding et al., 2009; Zheng et al., 2015). The motility was lost in two strains of *X. cannabis*: NCPPB3753 strain which lost two large regions of the flagellar biosynthesis gene (Jacobs et al., 2015) as well as in *flgC* mutant strain of *X. axonopodis* pv. *glycines* (Chuaboon et al., 2014). Therefore, it is likely that down-regulation of flagella genes in *murI* mutant affected the motility. Likewise, test of bacterial motility in *R. solanacearum* strains by means of stab inoculation revealed that *murI* mutant strain SL341F12 was non-motile whereas the wild type and complementation strain was motile (Fig. 3). Defects in *flgC* and *fliM* resulted in decreased virulence of *X. axonopodis* pv. *glycines* and *S. typhimurium*, respectively (Chuaboon et al., 2014; Ding et al., 2009). Flagella are involved not only in motility but also in virulence (Moens and Vanderleyden, 1996).

In *Streptococcus mutans*, *murI* deficiency caused reduction in its virulence (Zhang et al., 2016). The mutated *murI* gene encoding glutamate racemase in SL341F12 led to significantly lower disease severity compared to that of
The evident phenotypes of pleiotropic mutations arising out of defects in a single gene such as rgg gene in Erwinia carotovora subsp. atroseptica, mopB in Xanthomonas campestris pv. campestris, and tatC in R. solanacearum have been studied in several bacteria (Chen et al., 2010; González et al., 2007; Toth et al., 1999). The SL341F12 strain with a single mutated gene, murI, displayed pleiotropic phenotypes exhibiting increased production of black pigment on the media containing tyrosine, impaired motility, and attenuated virulence. SL341F12 produces more black pigment than SL341 and the complementation strain SL341F12C, visible as precipitation in the tyrosine-supplemented minimal medium (Supplementary Fig. 1). This black pigment has not been characterized; however, high performance liquid chromatography analysis revealed that this pigment is not similar to melanin (data not shown).

Although we have not examined the concentration of L-glutamate and D-glutamate both in wild type and the murI mutant of R. solanacearum, mutation in the glutamate racemase activity may cause imbalance in the glutamate isomers in the bacterial cell. If this is the case, further investigation is necessary how the isomer balance is linked with the regulation of bacterial flagella assembly in R. solanacearum. In summary, glutamate racemase encoded by the murI gene contributes to the regulation of diverse physiological functions in R. solanacearum and D-glutamate might be a key signaling molecule to control bacterial phenotypic traits in this plant pathogenic bacteria.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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