Mutations in the *lrpE* Gene of *Ralstonia solanacearum* Affects Hrp Pili Production and Virulence

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The *Ralstonia solanacearum* *hrpB*-regulated gene *lrpE* (*hpx5/brg24*) encodes a PopC-like leucine-rich repeat (LRR) protein that carries 11 tandem LRR in the central region. Defects in the *lrpE* gene slightly reduced the virulence of *R. solanacearum* on host plants and changed the bacterial morphology leading to the formation of large aggregates in a minimal medium. The aggregation in the Δ *lrpE* background required the presence of a functional Hrp type III secretion system. In wild-type *R. solanacearum*, Hrp pili disappeared from the bacterial surface at the end of the exponential growth phase, when the pili form into long bundles. However, even in the late growth phase, bundled Hrp pili were still observed on the cell surface of the Δ *lrpE* mutant. Such bundles were entangled and anchored the mutant cells in the aggregates. In contrast to PopC, LrpE accumulated in bacterial cells and did not translocate into plant cells as an effector protein. The expression levels of *hrp* genes increased three- to fivefold in the Δ *lrpE* background compared with those in the wild type. We propose that LrpE may negatively regulate the production of Hrp pili on the cell surface of *R. solanacearum* to disperse bacterial cells from aggregates. In turn, dispersal may contribute to the movement of the pathogen in the plant vascular system and, as a consequence, the pathogenicity of *R. solanacearum*.

Additional keywords: bacterial wilt, eggplants, *fliC*, *hpx*, *hrpY*, *pilA*.

Ralstonia solanacearum is a gram-negative soilborne bacterium that causes diseases in more than 100 plant species, including economically important crops such as tomato, potato, banana, tobacco, and eggplant (Hayward 1991). The pathogen enters plant roots through natural openings or wounds, invades xylem vessels, and spreads throughout the vascular system, resulting in the lethal wilting of plants (Kelman and Sequeira 1965; Vasse et al. 1995).

As in many other gram-negative phytopathogenic bacteria, the Hrp type III secretion system (TTSS) is essential for the multiplication of *R. solanacearum* in host plants (Boucher et al. 1987). Hrp TTSS is a specialized secretion system that allows the direct injection of virulence factors, called effector proteins, from pathogens into plant cells, in which they modulate host cellular functions and contribute to pathogenic infection

(Alfano and Collmer 1997; Galán and Collmer 1999; Hueck 1998). The components of the Hrp secretion apparatus of *R. solanacearum* are encoded in the large *hrp* (hypersensitive response and pathogenicity) gene cluster that includes at least 20 genes (Van Gijsegem et al. 1995). Ten *hrp* genes, termed *hrc* (*hrp* conserved), are highly conserved among plant and animal pathogenic bacteria and show significant homologies with the genes of the components of a flagellar basal body (Bogdanove et al. 1996; Nguyen et al. 2000). Presently, *hrp* gene products are considered to assemble into a cylinder-like secretion apparatus that penetrates bacterial outer and inner membranes as a flagellar basal body (Cornelis and Van Gijsegem 2000; He et al. 2004).

In *R. solanacearum*, *hrp* genes and most effector genes are controlled by an AraC-type transcriptional activator, HrpB, and specifically expressed in a minimal medium that probably mimics conditions in planta but not in a nutrient-rich BG medium (Arlat et al. 1994; Genin et al. 1992). On the basis of the speculation that unknown effector genes are regulated by HrpB, we have screened *hrpB*-regulated genes in *R. solanacearum* RS1000 and have identified 30 novel *hpx* (*hrpB*-dependent expression) genes (Mukaiharu et al. 2004). Recently, 48 genes belonging to the *hrpB* regulon, named *brg* (*hrpB*-regulated) genes, have also been found in the *R. solanacearum* genome-sequenced strain GMI1000 (Cunnac et al. 2004). Including *hrp* genes, HrpB therefore regulates the expression of a large virulence regulon composed of more than 70 pathogenicity genes.

An *hrp*-dependent filamentous structure, called the Hrp pilus, was identified in many plant pathogens (Jin et al. 2001; Roine et al. 1997; Van Gijsegem et al. 2000; Weber et al. 2005). All the *hrp* mutants of *R. solanacearum* that lost their ability to secrete an Hrp pilin protein (HrpY) or to polymerize it to form a pilus, both resulting in the loss of Hrp pili, cannot secrete Hrp substrate proteins such as harpins and effector proteins (Van Gijsegem et al. 2002). Therefore, the Hrp pilus is considered to extend from the Hrp secretion apparatus and to act as a molecular conduit through which bacterial effector proteins translocate into plant cells. The Hrp pili of *R. solanacearum*, with an estimated constant diameter of 6.6 ± 1 nm, are produced at one pole of the cell (Van Gijsegem et al. 2000). They have a brush-like structure at the early stage of bacterial growth but disappear from the bacterial surface and seem to be released into the culture medium at the late stage, when they have formed a long straight bundle-like structure (Van Gijsegem et al. 2000). The mechanism underlying the disappearance of Hrp pili and its significance in the interaction between *R. solanacearum* and its host plants have not yet been

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clarified. In this study, we carried out genetic and molecular analyses of an *hpx/brg* gene, *lrpE* (*hpx5/brg24*), which encodes a PopC-like leucine-rich repeat (LRR) protein that appears to be involved in hrp pili organization.

RESULTS

The *R. solanacearum* *hrpB*-regulated *lrpE* (*hpx5*) gene encodes a PopC-like LRR protein and defects in the *lrpE* gene reduce the virulence of *R. solanacearum* on host plants.

The *hpx5* gene, which was identified as an *hrpB*-regulated gene in RS1000, was cloned and sequenced. The *hpx5* gene has a plant-inducible promoter (PIP) box-like motif in the upstream region and encodes a protein of 648 amino acids with a molecular mass of 70.8 kDa (Fig. 1). A homology search of databases using the BLASTP algorithm revealed that the central region (amino acids 173 to 456) of the product shows a significant homology with proteins containing the LRR motif, a typical protein motif observed in eukaryotic proteins (Kobe and Deisenhofer 1994). Hence, *hpx5* was renamed *lrpE* (LRR protein E). LrpE contained 11 tandem repeats of the LRR motif, with seven perfect LRR of 24 amino acids and four imperfect LRR of 23 amino acids in its central region, and is structurally similar to PopC, an Hrp-secreted outer protein of *R. solanacearum* (Guéneron et al. 2000) (Fig. 2A and B). The consensus LRR motif of LrpE was also similar to that of PopC (Fig. 2C). The N-terminal sequence (amino acids 1 to 172) or C-terminal sequence (amino acids 433 to 648) of LrpE did not share a high homology with PopC or any other proteins in the databases.

R. solanacearum mutants containing a transposon (Tn) insertion in the *lrpE* gene were tested for their ability to infect host plants. The strains M190 and M204 (Fig. 1) exhibited a delayed development of wilting on 8-week-old host eggplants compared

with the wild-type strain RS1002 (data not shown). To confirm this result, we constructed *R. solanacearum* RS1091, which is a Δ *lrpE* derivative of RS1002. When eggplants were inoculated with 5×10^5 bacterial cells by the stem-cutting method described below, the development of disease symptoms in RS1091-inoculated plants was delayed for two days compared with that in RS1002-inoculated plants (Fig. 3). The reduced virulence of the Δ *lrpE* mutant was restored to the wild-type level by introducing a transposon carrying the wild-type *lrpE* gene into *cis*, using pRS778 (Figs. 1 and 3). The growth rate of the Δ *lrpE* mutant in the minimal medium was the same as that of the wild type (data not shown). No differences were found in the ability of the *lrpE* mutant to elicit a hypersensitive response (HR) in nonhost tobacco plants. These results show that *lrpE* contributes to virulence of *R. solanacearum* on host plants.

Fig. 1. Genetic organization and restriction map of *lrpE* region of *R. solanacearum*. Open arrows represent penreading frames. Open and filled flags represent the transcriptional and translational fusions of the mini-Tn5*lacZYA* and mini-Tn5*lacZY* transposons, respectively. Flag heads indicate the transcriptional direction of the fusion. Numbers on the flag denote the transposon or mutant itself. A hooked arrow from a filled circle indicates the direction of transcription from the putative plant-inducible promoter box. Thick lines indicate the DNA fragments used in complementation or marker-exchange experiments. Dotted lines represent the position of the deletion in the fragment. Thin arrows indicate polymerase chain reaction (PCR) primers. Restriction sites generated by PCR are indicated by asterisks. Restriction sites derived from the transposon are shown in parentheses. B = *Bam*HI, E = *Eco*RI, ET = *Eco*T22I, P = *Pst*I, Sm = *Sma*I, and Xb = *Xba*I.

Fig. 2. Structures of LrpE and popC proteins and comparison of consensus leucine-rich repeat (LRR) motif with those of other PopC-like proteins from phytopathogenic bacteria. **A**, Structures of LrpE and PopC. Numbers indicate amino acid positions. Shaded boxes indicate leucine-rich repeats (LRR) and the number of repeats is shown above. **B**, Alignment of LRR of LrpE. Numbers on the left side indicate the number of LRR. The consensus sequence is shown below. The conserved leucine (L) and isoleucine (I) residues in LRR are shown in red. The conserved L and I are sometimes replaced by an aliphatic residue (A, V, F, Y, or M) and are shown in pink. Other conserved amino acids (more than 50% identity) are indicated in bold black letters. The consensus amino acid sequence is shown below. x = any residue and α = aliphatic residue (A, V, L, I, F, Y, or M). The region predicted to form the β -sheet or α -helix structure is underlined. **C**, Comparison of consensus sequence of the LRR motif of LrpE with those of PopC (*Ralstonia solanacearum*), HpaF (*Xanthomonas axonopodis* pv. *glycines*), XCC2565 and XCC4186 (*X. campestris* pv. *campestris*), and XAC3090 (*X. axonopodis* pv. *citri*). The consensus sequences of the LRR motifs of PopC-like proteins are shown below.

***lrpE* mutants form large bacterial aggregates in an *hrp*-dependent manner.**

Because *R. solanacearum* secretes a large amount of extracellular polysaccharides (EPS), wild-type RS1002 formed white fluid colonies on both BG and minimal medium plates (Fig. 4A). The $\Delta lrpE$ strain RS1091 formed white fluid colonies on BG plates, similar to the wild type, but formed translucent fluid colonies, including white aggregates, on minimal medium plates (Fig. 4A). When the $\Delta lrpE$ mutant was cultivated in liquid media with slow agitation, it formed into large white aggregates in the liquid minimal medium but not in the liquid BG medium (Fig. 4B). The $\Delta lrpE$ mutant complemented by the wild-type *lrpE* gene (RS1272) showed the restored colony morphology on minimal medium plates (data not shown) and did not form into aggregates in the liquid minimal medium (Fig. 4B). This indicates that a defect in the *lrpE* gene results in cell aggregation.

Aggregation in the $\Delta lrpE$ background occurred under slow agitation but not in well-shaken cultures; furthermore, the resulting aggregates could be easily disrupted by vortexing (data not shown). When aggregates were incubated with proteinase

K (50 μ g/ml) at 28°C for 24 h, most of them dispersed (Fig. 4C). These observations suggest that the aggregates are connected by a proteinaceous factor.

In unshaken liquid BG medium, *R. solanacearum* forms cell aggregates via type IV pili (Kang et al. 2002). In our *R. solanacearum* RS1000, mutants of the *pilA* gene encoding the type IV pilin protein do not produce type IV pili on the cell surface or exhibit twitching motility on plates (Mukaihara et al. 2004). To investigate whether type IV pili are involved in aggregation in the $\Delta lrpE$ background, we constructed the $\Delta lrpE$ *pilA* strain RS1263. We also constructed the $\Delta lrpE$ *fliC* strain RS1266, which does not produce flagellar filaments. Cells of both strains, however, aggregated in the minimal medium (Fig. 4D), indicating that type IV pili and flagellar filaments are not involved in aggregation in the $\Delta lrpE$ background.

During the experiments, we found a paradoxical phenotype of a regulatory mutant, $\Delta(hrpB-hrcT)$ strain RS1051, which is affected in expression of all HrpB-regulated loci. Because *hrpB* specifically regulates the expression of the *lrpE* gene (Mukaihara et al. 2004), the *hrpB* mutant should have lost the function of LrpE and exhibited the same aggregation pheno-

Fig. 3. Effect of $\Delta lrpE$ mutation on pathogenicity. **A**, Disease symptoms of host eggplants inoculated with RS1002 (WT) and RS1091 ($\Delta lrpE$). Photographs were taken 8 days after inoculation. **B**, Twelve eggplants were inoculated with 5×10^5 cells of a *Ralstonia solanacearum* strain: RS1002, filled circle; RS1091, open triangle; and RS1272 ($\Delta lrpE$ Tn $lrpE$), open square. Average disease indexes from 0 (none of the leaves are wilted) to 4 (all leaves are completely wilted) scored daily after inoculation are shown. Results are from one experiment that best represents five independent assays. In this experiment, the $\Delta lrpE$ mutant showed a lower disease index than the wild type ($P < 0.05$, calculated by *U*-test) 5 days after inoculation.

type as the $\Delta lrpE$ mutant. However, cells of this strain did not aggregate in the minimal medium (Fig. 4D). HrpB regulates the expression of more than 50 genes other than *lrpE*, which include *hrp*, *pop*, and *hpx* genes (Mukaihara et al. 2004). Aggregation in the $\Delta lrpE$ background occurred only in the minimal medium in which *hrp* genes are induced but not in the BG medium in which *hrp* induction is impossible (Fig. 4A and B). Thus, we next examined the involvement of Hrp TTSS in aggregation. We constructed the $\Delta lrpE \Delta(hrpB-hrcT)$ strain RS1223 and found that the *hrpB* null mutation suppresses aggregation in the $\Delta lrpE$ background (Fig. 4D). This observation suggests that some *hrpB*-regulated genes are involved in aggregation. Therefore, we constructed the two Hrp TTSS-defective mutants $\Delta lrpE hrcT$ (RS1224) and $\Delta lrpE hrpY$ (RS1228). In both mutant strains, *hrpB* is still active but Hrp pili are not produced. Interestingly, both mutants lost their ability to form aggregates (Fig. 4D). These results suggest that a functional Hrp TTSS is required for aggregation in the $\Delta lrpE$ background.

Bacterial cells in aggregates are anchored by bundled Hrp pili.

To investigate the role of Hrp pili in aggregation, samples from the cultures of *R. solanacearum* strains grown in the minimal medium were examined by transmission electron microscopy. In this experiment, we used $\Delta epsP fliC$ (RS1265) derivatives, which are defective in both EPS production and flagellation. In this strain, Hrp pili can be more easily observed as compared with the wild type. When cells were grown up to the late exponential growth phase, the cultures of the *lrpE*⁺ strain (RS1265) contained numerous bundle-formed filaments

(Fig. 5A and B). The constant diameter of the filament was estimated to be 6 to 7 nm (Fig. 5C). Because the diameter of the filament corresponded to that of the Hrp pilus (Van Gijsegem et al. 2000) and filaments did not appear in cells in the $\Delta(hrpB-hrcT)$, *hrcT*, or *hrpY* background (Mukaihara et al. 2004), these filaments were considered Hrp pili. Under the conditions that we used, only a small percentage of bacterial cells had Hrp pili on their surfaces (Fig. 5A and B). In contrast, most of the bacterial cells in aggregates in the cultures of the $\Delta lrpE$ mutant (RS1266) had such bundles on their surfaces (Fig. 5D and E). These bundles appeared to be entangled, forming a netlike structure that anchored the bacterial cells in the aggregates (Fig. 5D and E). The $\Delta lrpE hrcT$ mutant (RS1270) did not produce such a structure on the surface (Fig. 5F), also indicating that these bundles are Hrp pili.

The $\Delta lrpE$ mutant forms aggregates in the host vascular system.

We carried out the phase-contrast microscopy of cultures of the wild type and $\Delta lrpE$ strains grown in the liquid minimal medium. Bacterial cells dispersed uniformly in the wild-type culture, but large three-dimensional cell aggregates were observed in the culture of the $\Delta lrpE$ mutant (Fig. 6A).

To determine whether the reduced virulence of the $\Delta lrpE$ mutant results from its aggregation in host plants, we recovered bacterial cells from the vascular system of *R. solanacearum*-infected eggplants (discussed below) and observed them by phase-contrast microscopy. Most of the bacterial cells recovered from the eggplants infected with wild-type RS1002 dispersed uniformly in the collected samples (Fig. 6B). In con-

Fig. 4. Aggregation during cultivation. **A**, Colony morphology on solid media. Cells of RS1002 (WT) and RS1091 ($\Delta lrpE$) were plated on BG and minimal medium (MM) plates using toothpicks and were incubated for 30 (BG) or 60 h (MM) at 28°C. A dotted circle indicates the border of a fluid colony. **B**, Aggregation in liquid media. Cell suspensions (10^6 cells/ml) of strains RS1002, RS1091, and RS1272 ($\Delta lrpE TnlrpE^+$) were incubated in the liquid BG and liquid MM with slow agitation at 28°C. Photographs were taken after 24 (BG) or 48 h (MM) of incubation. **C**, Proteinase treatment of aggregates. Cell suspensions of RS1091 were incubated in MM with slow agitation for 24 h at 28°C. Samples were further incubated without (–K) or with (+K) proteinase K (final concentration, 50 μ g/ml) for 24 h at 28°C. **D**, Aggregation of cells of various genetic backgrounds. Cell suspensions of RS1263 ($\Delta lrpE pilA$), RS1266 ($\Delta lrpE fliC$), RS1051 ($\Delta(hrpB-hrcT)$), RS1223 ($\Delta lrpE \Delta(hrpB-hrcT)$), RS1224 ($\Delta lrpE hrcT$), and RS1228 ($\Delta lrpE hrpY$) were incubated in MM with slow agitation for 48 h at 28°C.

trast, bacterial cells from eggplants infected with the $\Delta lrpE$ strain RS1091 formed into numerous aggregates that were much larger than those of the wild type (Fig. 6B). To exclude the possibility of autoaggregation by type IV pili (Kang et al. 2002), we introduced the *pilA::Gm^r* mutation into each strain and constructed the strains RS1262 (*pilA*) and RS1263 ($\Delta lrpE$ *pilA*). Bacterial cells recovered from RS1262-infected plants did not form into aggregates, whereas those from RS1263-infected plants still formed into numerous aggregates (Fig. 6B). These observations indicate that the $\Delta lrpE$ mutant forms aggregates during infection in host plants.

LrpE is an intracellular protein but not an effector protein translocated into plant cells.

In order to examine whether LrpE is an Hrp-substrate protein, using *R. solanacearum* *hrpB^c* (*hrpB* constitutive) mutants that highly express *hrpB*-regulated genes, we analyzed proteins secreted to the supernatant and in cells of bacterial cultures grown in the *hrp*-inducing medium. The FLAG-tagged PopC protein was secreted outside the cell as a Hrp-secreted outer protein by the *hrpB^c* mutant (RS1319) but not by the Hrp-defective *hrpB^c* mutant (RS1320) (Fig. 7), as reported previously (Guneron et al. 2000). In contrast, FLAG-tagged LrpE was not secreted by the *hrpB^c* mutant (RS1323) and by the Hrp-defective *hrpB^c* mutant (RS1324) (Fig. 7), suggesting that LrpE is an intracellular protein.

Type III secretion and translocation signals are localized within the N-terminal first 50 amino acids of effector proteins from plant and animal pathogenic bacteria (Casper-Lindley et al. 2002; Lloyd et al. 2001; Schechter et al. 2004). The N-terminal first 50 amino acids of LrpE showed higher serine and proline contents, i.e., 12.0 and 10.0%, than that of randomly selected *R. solanacearum* proteins (Cunnac et al. 2004). Al-

though protein secretion analysis suggests that LrpE is an intracellular protein, the N-terminal sequence of LrpE is characteristic of a Hrp-substrate protein (Collmer et al. 2002). To further determine whether LrpE translocates into plant cells via the Hrp TTSS, using a plasmid integration system, we constructed *R. solanacearum* strains expressing an LrpE₁₋₂₃₀'-'Cya fusion protein, in which the N-terminal 230 amino acids of LrpE was fused to an adenylate cyclase domain ('Cya) (Sory and Cornelis 1994). We also constructed *R. solanacearum* strains expressing a PopC₁₋₂₃₀'-'Cya fusion protein and an Hpx19'-Cya fusion protein, in which the N-terminal 230 amino acids of PopC and full-length amino acids (180 aa) of Hpx19 was fused to an adenylate cyclase domain. The expression of *hpx19* is dependent on *hrpB* (Mukaihara et al. 2004) and its product, a glyoxalase I homolog, is not secreted outside the cell but highly expresses in cells as an intracellular protein under *hrp*-inducing conditions (Tamura et al. 2005).

Before the in planta translocation assay, using *hrpB^c* mutants, each 'Cya fusion protein was expressed in an *hrp*-inducing medium and was measured for its calmodulin-dependent adenylate cyclase activity. The adenylate cyclase activities were comparable in the *hrp⁺* and $\Delta hrpY$ strains, indicating that fusion proteins were expressed at similar levels in both strains (Table 1). As expected, the Hpx19'-Cya fusion protein highly expressed in cells compared with the LrpE₁₋₂₃₀'-'Cya and PopC₁₋₂₃₀'-'Cya fusion proteins (Table 1).

When *R. solanacearum* *hrpB⁺* strains expressing the LrpE₁₋₂₃₀'-'Cya, PopC₁₋₂₃₀'-'Cya, or Hpx19'-Cya fusion protein were inoculated into eggplant leaves, only the strain expressing the PopC'-Cya fusion protein increased the cAMP level of the inoculated leaves (Fig. 8). The cAMP level increased with time and reached its maximum 12 to 15 h after inoculation, at which it had increased to a value more than 10,000-fold that immedi-

Fig. 5. Bacterial aggregates were anchored by bundle-formed Hrp pili. **A**, Transmission electron microscopy of *Ralstonia solanacearum* $\Delta epsP$ *fliC* strain RS1265 (*lrpE⁺*) cultivated in minimal medium. Arrows in the micrograph indicate Hrp pili released from the bacterial surface and dispersed in the culture. **B**, Bundle-formed and released Hrp pili in the culture of RS1265. **C**, Enlargement of Hrp pili in the culture of RS1265. **D**, Cells of RS1266 ($\Delta lrpE$) still have Hrp pili on their surfaces. Hrp pili were entangled, forming a netlike structure, and seemed to anchor cells. **E**, Enlargement of boxed region in panel C. **F**, Cells of RS1270 ($\Delta lrpE$ *hrcT*) did not produce Hrp pili on their surfaces. Bars in A, B, D, and F represent 1 μ m. Bars in C and E represent 100 nm.

ately after inoculation (Fig. 8A). The increase in cAMP level induced by the strain expressing the PopC'-Cya fusion protein was completely abolished by the $\Delta hrpY$ mutation (Fig. 8A), indicating that the PopC'-Cya fusion protein translocates into plant cells via the Hrp TTSS. On the other hand, strains expressing the LrpE'-Cya fusion protein or the Hpx19'-Cya fusion protein did not increase the cAMP level of the inoculated leaves (Fig. 8B and C), although the in vitro adenylate cyclase activities of their cell extracts were three- to fourfold and 400-fold higher than that of PopC'-Cya cell extracts, respectively (Table 1). The result indicated that Hpx19 and LrpE are intracellular proteins and are not translocated into plant cells.

Defects in the *lrpE* gene affect the expression of *hrp* genes.

The above protein secretion and translocation analyses suggest that LrpE functions inside the cell. To investigate the role of LrpE in the regulation of the Hrp TTSS, we compared the expression levels of *hrp* operons 2, 3, 4, and 7, which are specifically regulated by the *hrp* regulator HrpB, in the *lrpE*⁺ and $\Delta lrpE$ backgrounds. In the $\Delta lrpE$ background, the expression levels of all *hrp* operons tested increased to a value four- to fivefold that in the wild type (Table 2). The enhanced expression of *hrp* genes in the $\Delta lrpE$ background was completely cancelled by a transposon carrying the wild-type *lrpE* gene

(Table 2). The expression levels of other *hrpB*-regulated genes, such as *popA* (Arlat et al. 1994) and *hpx14* (Mukaihara et al. 2004), also increased (three- to fourfold) in the $\Delta lrpE$ background (Table 2). These results suggest that defects in the *lrpE* gene have a global effect of enhancing the expression of *hrpB*-regulated genes.

DISCUSSION

In this study, we characterized the *lrpE* (*hpx5*) gene, which encodes a PopC-like LRR protein. The expression of *lrpE* was specifically regulated by an *hrp*-regulatory gene, *hrpB* (Mukaihara et al. 2004). The corresponding gene of *lrpE* in the sequenced *R. solanacearum* GMI1000, *brg24*, has been reported as an *hrpB*-regulated gene (Cunnac et al. 2004). This suggests that LrpE has an Hrp-related pathogenicity function. Indeed, the $\Delta lrpE$ mutation slightly reduced the virulence of *R. solanacearum* on host plants (Fig. 3).

Interestingly, the *lrpE*-defective mutants of *R. solanacearum* formed unusual large cell aggregates in the minimal medium (Figs. 4, 5, and 6). The aggregation of bacterial cells during cultivation was observed in several plant and animal pathogens. *R. solanacearum* cells can autoaggregate via type IV pili (Kang et al. 2002). The *bfpF*-defective mutant cells of enteropathogenic *Escherichia coli* (EPEC) aggregate with bundle-formed type IV pili (Bieber et al. 1998). The *manA*-defective mutant cells of *Xanthomonas campestris* pv. *campestris* aggregate via an extracellular matrix that strongly connects cells (Dow et al. 2003). The mechanism underlying the aggregation of *R. solanacearum* $\Delta lrpE$ mutant cells is clearly distinguished from that of the above-mentioned bacteria for the following reasons: i) type IV pili are not required for aggregation (Figs. 4D and 6B), ii) aggregated cells can be dispersed easily by vortexing or proteinase-K treatment (Fig. 4C), and iii) Hrp pili are essential for aggregation (Figs. 4D and 5).

Van Gijsegem and associates (2000) reported that *R. solanacearum* has two different states of Hrp pili production during its growth. At the beginning of the exponential growth phase, *R. solanacearum* produces Hrp pili at one pole of the cell, which

Fig. 6. $\Delta lrpE$ mutant forms large bacterial aggregates. **A**, Phase-contrast microscopy of cultures of RS1002 (WT) and RS1091 ($\Delta lrpE$) grown in liquid minimal medium. Bars represent 10 μ m. **B**, Phase-contrast microscopy of bacterial cells recovered from stems of plants inoculated with RS1002, RS1091, RS1262 (*pilA*), and RS1263($\Delta lrpE$ *pilA*). Bars represent 10 μ m.

Fig. 7. Secretion of PopC and LrpE. *Ralstonia solanacearum* *hrpB*⁺ mutants (Hrp⁺) or their Hrp-defective derivatives (Hrp⁻) expressing the FLAG fusion were grown in the secretion medium. Equal volumes of samples of total cell extracts (Cell) and culture supernatants (Sup.) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting, using the anti-FLAG antibody. Arrows indicate the sizes of PopC-FLAG and LrpE-FLAG fusion proteins.

sometimes form into long straight bundles when cells aggregate. In contrast, most bacterial cells disperse from aggregates at the end of the exponential growth phase, which is accompanied by the disappearance of Hrp pili from the cell surface and their release into the external medium. This finding suggests that, in *R. solanacearum*, the two states of the production and disappearance of Hrp pili are controlled during its growth.

We propose that LrpE is involved in the disappearance of Hrp pili on the cell surface of *R. solanacearum* in the late exponential growth phase. The Δ *lrpE* mutant is considered to lose this ability; therefore, cells grow anchored by Hrp pili, resulting in the formation of large aggregates. We found that the Δ *lrpE* mutant also aggregates in the plant vascular system (Fig. 6B). The reduced virulence of the mutant (Fig. 3) may be explained by

the decreased extent of bacterial cell dispersal from aggregates. Aggregated bacterial cells cannot efficiently proceed to the next infection area in the vascular system, which decreases the extent of spread of the pathogen in planta, resulting in a virulence defect. As mentioned above, the *bfpF* mutant of EPEC and the *manA* mutant of *X. campestris* pv. *campestris* aggregate during their growth and show an attenuated bacterial pathogenicity (Bieber et al. 1998; Dow et al. 2003). *R. solanacearum* and *X. campestris* colonize the surface of the vascular system of plants (Bretschneider et al. 1989; Vasse et al. 1995). On the other hand, EPEC colonizes the surface of the small intestine of mammals (Nataro and Kaper 1998). Although their hosts and target organs are different, it is noteworthy that these pathogens spread throughout the host organs by repeating the infection steps of

Table 1. Adenylate cyclase activities of three 'Cya fusion proteins expressed in *Ralstonia solanacearum* *hrpB*^c mutants of *hrp*⁺ and Δ *hrpY* strains grown in minimal medium

Fusion protein	cAMP level ^a (fold increase ^b)			
	+Calmodulin		−Calmodulin	
	<i>hrp</i> ⁺	Δ <i>hrpY</i>	<i>hrp</i> ⁺	Δ <i>hrpY</i>
None	0.01 ± 0.002 (1.0)	0.01 ± 0.002 (1.0)	0.01 ± 0.002 (1.0)	0.01 ± 0.002 (1.0)
LrpE ₁₋₂₃₀ '-Cya	30.00 ± 2.52 (3,000)	28.90 ± 2.40 (2,890)	0.12 ± 0.02 (12.0)	0.07 ± 0.01 (7.0)
PopC ₁₋₂₃₀ '-Cya	8.01 ± 1.62 (801)	9.50 ± 1.19 (950)	0.02 ± 0.002 (2.0)	0.03 ± 0.002 (1.0)
Hpx19'-Cya	3,351.00 ± 317 (335,100)	3,620.00 ± 251 (362,000)	0.62 ± 0.04 (62.0)	0.71 ± 0.09 (71.0)

^a Adenylate cyclase activity (nmol cAMP per milligram of total soluble protein) is shown as an average of three independent experiments.

^b Fold increase indicates the ratio of activity of a strain expressing the 'Cya fusion protein to that of the parental strain.

Fig. 8. Time course analysis of in planta adenylate cyclase activity for *Ralstonia solanacearum* strains expressing the 'Cya fusion protein. Eggplant leaves were inoculated with 5×10^8 CFU/ml suspensions of *R. solanacearum* strains expressing the **A**, PopC₁₋₂₃₀'-Cya fusion protein, **B**, LrpE₁₋₂₃₀'-Cya fusion protein, and **C**, Hpx19'-Cya fusion protein. Leaf samples (1.6 cm²) were taken 3, 6, 9, 12, and 15 h after inoculation. Filled diamonds and open squares represent average cAMP levels of three replicates from experiments using *hrp*⁺ and Δ *hrpY* strains, respectively. Bars indicate standard deviations.

Table 2. β-Galactosidase activities of *lacZYA* translational fusion of several *hrpB*-regulated genes in *Ralstonia solanacearum* strains grown in minimal medium

Gene	Fusion	β-Galactosidase activity ^a (fold increase ^b)		
		<i>lrpE</i> ⁺	Δ <i>lrpE</i>	Δ <i>lrpE</i> / <i>TnlrpE</i> ⁺
<i>hrpF</i> (<i>hrp</i> unit 2)	M27	546.0 ± 38.7 (1.0)	2,588 ± 123 (4.7)	573 ± 36.6 (1.0)
<i>hrcV</i> (<i>hrp</i> unit 3)	M23	55.4 ± 6.5 (1.0)	280 ± 21.2 (5.1)	61.6 ± 6.4 (1.1)
<i>hrcR</i> (<i>hrp</i> unit 4)	M35	109.0 ± 13.9 (1.0)	555 ± 26.5 (5.1)	108 ± 5.3 (1.0)
<i>hpaZ</i> (<i>hrp</i> unit 7)	M46	250.0 ± 11.4 (1.0)	1,214 ± 37.4 (4.9)	231 ± 11.6 (0.9)
<i>popA</i>	<i>popA-lacZYA</i>	563.0 ± 53.9 (1.0)	1,767 ± 72.0 (3.1)	758 ± 45.9 (1.3)
<i>hpx14</i>	M21	127.0 ± 16.9 (1.0)	463 ± 23.2 (3.6)	140 ± 10.6 (1.1)

^a β-Galactosidase activity (Miller's unit ± SD) is shown as an average of four independent experiments.

^b Fold increase indicates the ratio of activity to the wild type.

attachment, multiplication (including aggregation and dispersal), and movement on the surface of host tissues. Repeating the step with a decreased dispersal extent may amplify the defect and result in a reduced virulence.

LrpE contained tandem repeats of the PopC-like LRR motif in the central region but showed no significant homology with known proteins in the N-terminal and C-terminal regions. We examined whether the *popC* mutants of *R. solanacearum* show the same aggregation phenotype as the *lrpE* mutant; however, we found that they did not aggregate in the minimal medium (data not shown). This clearly demonstrates that the function of LrpE is different from that of PopC. Recently, PopC-like LRR proteins, namely, HpaF, XCC2565, and XCC4186/XAC3090, have been identified in *Xanthomonas* species (da Silva et al. 2002; Kim et al. 2003). They contain PopC-like LRR in their central region, as in LrpE (Fig. 2C). The upstream region of genes encoding these proteins contains a PIP box-like motif (da Silva et al. 2002; Kim et al. 2003); indeed, the expression of *hpaF* is regulated by the *Xanthomonas* hrp regulatory proteins HrpG and HrpX (Kim et al. 2003). The *hpaF* mutant of *X. axonopodis* pv. *glycines* exhibits a delayed pathogenicity on host plants (Kim et al. 2003). It would be interesting to determine whether the mutants of *hpaF* and other *popC*-like genes show an Hrp pili-dependent aggregation phenotype similar to the *lrpE* mutant.

The precise function of LrpE in the disappearance of Hrp pili remains unclarified. Protein secretion analysis revealed that LrpE is not secreted into the extracellular milieu under in vitro conditions in which PopC is effectively secreted outside the cell via the Hrp TTSS (Fig. 7). Furthermore, protein translocation analysis using a *Cya* reporter revealed that PopC translocates into plant cells but LrpE accumulates in the bacterial cell (Fig. 8). These results indicate that PopC is a type III effector protein that is translocated into the plant cell and probably manipulates its metabolism but that LrpE is an intracellular protein. Although LrpE contains a typical eukaryotic LRR motif, it may interact with bacterial proteins. Defects in the *lrpE* gene enhanced the expression of *hrpB*-regulated genes in both the Hrp functional (*hpaZ*, *popA*, and *hpx14*) and Hrp-defective (*hrpF*, *hrcV*, and *hrcR*) backgrounds (Table 2), suggesting that LrpE negatively regulates the expression of *hrp* genes. In such a case, LrpE may interact with Hrp regulatory proteins and inhibit their functions to repress the expression of *hrp* genes, resulting in the disappearance of Hrp pili.

We hypothesized that the virulence defect in the *lrpE* mutants results from Hrp pili-dependent aggregation, probably caused by the enhanced expression of *hrp* genes. This is also supported by the observation that *hrpB*^c mutants that constitutively express *hrp* genes at high levels also form aggregates in fluid colonies or in liquid cultures and have reduced virulence (N. Tamura and T. Mukaiharu, unpublished results). In *Erwinia carotovora*, the high basal-level expression of the Hrp TTSS has been shown to reduce bacterial infection, presumably resulting from the improper activation of plant defense responses even in host plants (Lehtimäki et al. 2003). However, the levels of translocation of the PopC₁₋₂₃₀'-Cya fusion protein into plant cells are similar for the wild type and *ΔlrpE* mutants (data not shown), suggesting that LrpE does not affect the Hrp TTSS in the early stage of infection. Because *lrpE* is also an *hrpB*-regulated gene (Mukaiharu et al. 2004), LrpE may act as a negative feedback regulator in the late exponential-growth phase, during which it highly accumulates in cells.

Recently, Genin and associates (2005) have reported that PhcA, a LysR-type transcriptional regulator whose activity is regulated by cell density through a quorum-sensing mechanism mediated by the autoinducer 3-hydroxy palmitic acid ester (Flavier et al. 1997), represses the expression of *hrp* genes of *R.*

solanacearum under a nutrient-rich condition. LrpE is not expressed at high levels in a nutrient-rich BG medium (Mukaiharu et al. 2004), and the *ΔlrpE* mutant did not show the Hrp pili-dependent aggregation phenotype on BG plates (Fig. 4A). These suggest that the function of LrpE is independent of PhcA.

In this study, under careful consideration, we could not exclude other possibilities regarding the function of LrpE. LrpE may release Hrp pili from the cell surface by inhibiting or disrupting the assembly of Hrp secretion apparatuses through interaction with some of its components or LrpE may negatively regulate an unidentified proteinaceous aggregation factor other than Hrp pili, whose activity is controlled by the Hrp TTSS. In either case, defects in the LrpE function result in aggregation. The identification of the working site and the target protein of LrpE in future studies will help us clarify the molecular function of LrpE.

MATERIALS AND METHODS

Bacterial strains, media, and culture conditions.

The bacterial strains used in this study are listed in Table 3. All the *R. solanacearum* strains are derivatives of RS1000 (Mukaiharu et al. 2004). *E. coli* S17-1 (Simon et al. 1983) was used as the host for *oriT/mob* plasmids. *R. solanacearum* strains were cultivated at 28°C in the BG medium (Boucher et al. 1985) or one-fourth strength M63 glucose minimal medium (Miller 1992). In secretion assay, a secretion medium [50 mM potassium phosphate buffer, 7.6 mM (NH₄)₂SO₄, 1.7 mM MgCl₂, and 1.7 mM NaCl supplemented with 10 mM glutamate and 10 mM sucrose] (Tamura et al. 2005) containing 100 μg of Congo red per milliliter was used. *E. coli* strains were cultivated at 37°C in the LB medium (Miller 1992). Antibiotics were added to the media as described previously (Mukaiharu et al. 2004).

For aggregation, *R. solanacearum* strains were cultivated in the liquid media (approximately 10⁶ CFU/ml) at 28°C with slow agitation, using 24-well tissue culture plates (Falcon, Franklin Lakes, NJ, U.S.A.) and a plate mixer (Iuchi, Osaka, Japan). Samples were analyzed after 24 (for the BG medium) or 48 h (for the minimal medium) of incubation. Aggregates in the *ΔlrpE* background became visible after 24 h of incubation in the minimal medium. For the proteinase treatment of aggregates, proteinase K (Takara, Tokyo) was added to a 24-h incubated culture at a final concentration of 50 μg/ml and the culture was further incubated for 24 h at 28°C with slow agitation.

Molecular cloning of the *lrpE* (*hpx5*) gene.

The chromosomal DNA of M29 (Mukaiharu et al. 2004) was digested with *Bam*HI (for upstream rescue) or *Sma*I (for downstream rescue) and was self-ligated and transformed into JM109. The resulting pRS227 contained the 3.7-kb *Bam*HI fragment upstream of the M29 transposon (Tn-M29) (Fig. 1). The 3.7-kb *Bam*HI fragment of pRS227 was subcloned into pUC118 to yield pRS340. pRS268 contained the 0.7-kb *Sma*I fragment downstream of Tn-M29. The sequence analysis of pRS268 and pRS340 revealed three open reading frames, *lrpE* (*hpx5*), *hpx6*, and RS05353, in this region (Fig. 1).

Construction of *R. solanacearum* mutants.

For the construction of *ΔlrpE* mutants, a 0.9-kb fragment upstream of *lrpE* was polymerase chain reaction (PCR)-amplified from pRS340, using the primers P353 (5'-GGAATTCTC ACCAGCACCGTGTTGCCGT-3') and P354 (5'-CGGGATCC TGCGCCAGGATGCGCGCGGC-3') with *Eco*RI (underlined) and *Bam*HI (double underlined) sites, respectively. Similarly, a 0.8-kb fragment downstream of *lrpE* was amplified using the primers P355 (5'-GCTCTAGATGAAGCGGCTCACCCATCT C-3') and P356 (5'-AACTGCAGTCGCGCACCACCGCCCT

GGC-3') with *Xba*I (underlined) and *Pst*I (double underlined) sites, respectively. PCR amplification was performed using the GC-RICH PCR system (Roche, Mannheim, Germany). Each fragment, double-digested with *Eco*RI and *Bam*HI or *Xba*I and *Pst*I, was tandemly inserted into pK18*mobsacB* to yield pRS349 (Fig. 1). Using pRS349, Δ *lrpE* strains were constructed by the marker-exchange method (Schäfer et al. 1994). PCR and Southern blot analyses were carried out to select Δ *lrpE* mutants.

For the construction of Δ (*hrpB-hrcT*) mutants, a 1.8-kb *Eco*T22I-*Sph*I fragment including the *hrcC* gene was subcloned from pRS103 (Mukaihara et al. 2004) into pK18*mobsacB* to yield pRS174. A 2.7-kb *Eco*RI-*Bgl*II fragment including the *hrpD* and *hrcN* genes was subcloned from pRS103 into pRS174. The resulting pRS175 contains a 2.2-kb deletion, including the entire *hrpB* gene and the 3' half of the *hrcT* gene. A 2.9-kb *Sal*II gentamycin-resistance (*Gm*^r) gene cassette was

Table 3. Bacterial strains used in this study

Strain	Description ^a	Reference
<i>Ralstonia solanacearum</i>		
RS1002	RS1000 Nal ^r	Mukaihara et al. 2004
RS1085	RS1002 <i>DepsP</i>	Mukaihara et al. 2004
RS1087	RS1085 <i>popA-lacZYA</i>	Mukaihara et al. 2004
RS1051	RS1002 Δ (<i>hrpB-hrcT</i>):Gm ^r	This study
RS1091	RS1002 Δ <i>lrpE</i>	This study
RS1204	RS1085 Δ (<i>hrpB-hrcT</i>)	This study
RS1223	RS1002 Δ <i>lrpE</i> Δ (<i>hrpB-hrcT</i>):Gm ^r	This study
RS1224	RS1002 Δ <i>lrpE hrcT</i> :Gm ^r	This study
RS1225	RS1085 Δ <i>lrpE</i>	This study
RS1228	RS1002 Δ <i>lrpE hrpY</i> :miniTn5' <i>lacZY</i> -M175	This study
RS1239	RS1085 RSc2836::mini-Tn5 <i>hrpB</i> [constitutively expressing <i>hrpB</i> (<i>hrpB</i> ^c)]	Tamura et al. 2005
RS1254	RS1085 Δ <i>hrpY</i>	Mukaihara et al. 2004
RS1262	RS1002 <i>pilA</i> :Gm ^r	This study
RS1263	RS1002 Δ <i>lrpE pilA</i> :Gm ^r	This study
RS1265	RS1085 <i>fliC</i> :pRS845	Mukaihara et al. 2004
RS1266	RS1085 Δ <i>lrpE fliC</i> :pRS845	This study
RS1270	RS1085 Δ <i>lrpE hrcT</i> :Gm ^r <i>fliC</i> :pRS845	This study
RS1272	RS1002 Δ <i>lrpE chr</i> :mini-Tn5 <i>lrpE</i> ^r	This study
RS1317	RS1085 <i>popC</i> :pRS573 (expressing PopC-FLAG)	Tamura et al. 2005
RS1318	RS1204 <i>popC</i> :pRS573 (expressing PopC-FLAG)	Tamura et al. 2005
RS1319	RS1317 <i>hrpB</i> ^c	Tamura et al. 2005
RS1320	RS1318 <i>hrpB</i> ^c	Tamura et al. 2005
RS1321	RS1085 <i>lrpE</i> :pRS846 (expressing LrpE-FLAG)	This study
RS1322	RS1204 <i>lrpE</i> :pRS846 (expressing LrpE-FLAG)	This study
RS1323	RS1321 <i>hrpB</i> ^c	This study
RS1324	RS1322 <i>hrpB</i> ^c	This study
RS1484	RS1085 <i>lrpE</i> :pRS1025 (expressing LrpE ₁₋₂₃₀ '-'Cya)	This study
RS1485	RS1254 <i>lrpE</i> :pRS1025 (expressing LrpE ₁₋₂₃₀ '-'Cya)	This study
RS1486	RS1484 <i>hrpB</i> ^c	This study
RS1487	RS1485 <i>hrpB</i> ^c	This study
RS1524	RS1085 <i>hpx19</i> :pRS1043 (expressing Hpx19'-'Cya)	This study
RS1525	RS1254 <i>hpx19</i> :pRS1043 (expressing Hpx19'-'Cya)	This study
RS1526	RS1524 <i>hrpB</i> ^c	This study
RS1527	RS1525 <i>hrpB</i> ^c	This study
RS1576	RS1085 <i>popC</i> :pRS1067 (expressing PopC ₁₋₂₃₀ '-'Cya)	This study
RS1577	RS1254 <i>popC</i> :pRS1067 (expressing PopC ₁₋₂₃₀ '-'Cya)	This study
RS1578	RS1576 <i>hrpB</i> ^c	This study
RS1579	RS1577 <i>hrpB</i> ^c	This study
RS1584	RS1085 Δ <i>lrpE hpx14</i> :miniTn5 <i>lacZYA</i> -M21	This study
RS1585	RS1085 Δ <i>lrpE hrcV</i> :miniTn5 <i>lacZYA</i> -M23	This study
RS1586	RS1085 Δ <i>lrpE hrpF</i> :miniTn5 <i>lacZYA</i> -M27	This study
RS1587	RS1085 Δ <i>lrpE hrcR</i> :miniTn5 <i>lacZYA</i> -M35	This study
RS1588	RS1085 Δ <i>lrpE hpaZ</i> :miniTn5 <i>lacZYA</i> -M46	This study
RS1589	RS1085 Δ <i>lrpE popA-lacZYA</i>	This study
RS1590	RS1085 Δ <i>lrpE hpx14</i> :miniTn5 <i>lacZYA</i> -M21 chr::mini-Tn5 <i>lrpE</i> ⁺	This study
RS1591	RS1085 Δ <i>lrpE hrcV</i> :miniTn5 <i>lacZYA</i> -M23 chr::mini-Tn5 <i>lrpE</i> ⁺	This study
RS1592	RS1085 Δ <i>lrpE hrpF</i> :miniTn5 <i>lacZYA</i> -M27 chr::mini-Tn5 <i>lrpE</i> ⁺	This study
RS1593	RS1085 Δ <i>lrpE hrcR</i> :miniTn5 <i>lacZYA</i> -M35 chr::mini-Tn5 <i>lrpE</i> ⁺	This study
RS1594	RS1085 Δ <i>lrpE hpaZ</i> :miniTn5 <i>lacZYA</i> -M46 chr::mini-Tn5 <i>lrpE</i> ⁺	This study
RS1595	RS1085 Δ <i>lrpE popA-lacZYA</i> chr::mini-Tn5 <i>lrpE</i> ⁺	This study
M21	RS1085 <i>hpx14</i> :miniTn5 <i>lacZYA</i> -M21	Mukaihara et al. 2004
M23	RS1085 <i>hrcV</i> :miniTn5 <i>lacZYA</i> -M23	Mukaihara et al. 2004
M27	RS1085 <i>hrpF</i> :miniTn5 <i>lacZYA</i> -M27	Mukaihara et al. 2004
M35	RS1085 <i>hrcR</i> :miniTn5 <i>lacZYA</i> -M35	Mukaihara et al. 2004
M46	RS1085 <i>hpaZ</i> :miniTn5 <i>lacZYA</i> -M46	Mukaihara et al. 2004
M175	RS1085 <i>hrpY</i> :miniTn5' <i>lacZY</i> -M175	Mukaihara et al. 2004
M190	RS1085 <i>lrpE</i> :miniTn5' <i>lacZY</i> -M190	Mukaihara et al. 2004
M204	RS1085 <i>lrpE</i> :miniTn5' <i>lacZY</i> -M204	Mukaihara et al. 2004
<i>E. coli</i>		
JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1</i> Δ (<i>lac-proAB</i>) F' [<i>traD36 proAB</i> ⁺ <i>lacI</i> ^r <i>lacZ</i> Δ M15]	Yanisch-Perron et al. 1985
S17-1	<i>thi pro hsdR</i> ⁺ <i>hsdM</i> ⁺ <i>recA</i> [chr::RP4-2-Tc::Mu-Km::Tn7]	Simon et al. 1983

^a Nal^r and Gm^r, = respectively, nalidixic acid and gentamycin resistant.

inserted into pRS175 to yield pRS176. Using pRS175 and pRS176, the $\Delta(hrpB-hrcT)$ and $\Delta(hrpB-hrcT)::Gm^r$ mutations, respectively, were introduced into *R. solanacearum* strains by the marker-exchange method.

For the construction of *hrcT* mutants, a 4.0-kb *EcoRI* fragment including the *hrpB-hrcT-hrpD-hrcN-hrpF* region was subcloned from pRS103 (Mukaihara et al. 2004) into pHSG398 (Takara, Otsu, Japan) to yield pRS108. A 2.9-kb *BamHI* *Gm^r* gene cassette was inserted into the *Bgl/II* site in the *hrcT* gene in pRS108 to produce pRS387. A 6.9-kb *EcoRI* fragment was inserted into pK18*mobsacB* to yield pRS388. Using pRS388, the *hrcT::Gm^r* mutation was introduced into *R. solanacearum* strains by the marker-exchange method.

For the construction of *hrpY* mutants, the chromosomal DNA of the *hrpY* Tn insertion mutant (M175) was purified. The *hrpY::Tn*-M175 mutation was introduced into *R. solanacearum* strains by electroporation (Mukaihara et al. 2004), and kanamycin-resistance (*Km^r*) recombinants were selected. The construction of $\Delta hrpY$ mutants has been described elsewhere (Mukaihara et al. 2004).

R. solanacearum *fliC* and *pilA* strains were constructed as described previously (Mukaihara et al. 2004).

Complementation of *R. solanacearum* $\Delta lrpE$ mutants.

For the complementation of $\Delta lrpE$ mutants, a *Km^r* transposon vector was used. A 3.1-kb *EcoT22I*-*BamHI* fragment containing the entire *lrpE* gene was subcloned from pRS340 into the *PstI*-*BamHI* site of pPCR-Script Cam (Stratagene, La Jolla, CA, U.S.A.) to yield pRS778. pRS778 was linearized by *BamHI*-*NotI* double digestion and was inserted into pBSL118 (Alexeyev et al. 1995). The resulting pRS796 carrying a mini-Tn5 transposon containing the *lrpE* gene was used in complementation experiments.

Plant assays.

All the plants used in this study were grown individually in plastic pots containing commercial soil at 30°C in a greenhouse.

For pathogenicity tests on susceptible eggplants (*Solanum melongena* cultivar senryo-nigou), a stem-cutting method was applied as follows. The *R. solanacearum* inoculum was prepared by washing a pellet of bacterial cells, which were cultured overnight in the BG medium, with distilled water (DW) and then diluting with DW to an appropriate concentration. Eight-week-old eggplants were cut at the stem above the sixth leaf from the cotyledon; generally, a one-third portion of the stem was cut off and three leaves, namely, the sixth, fifth, and fourth leaves from the cotyledon, were retained. Then, a 10- μ l droplet containing 5×10^5 *R. solanacearum* cells in DW was deposited on the cut surface of the stem. The inoculated eggplants were grown at 30°C in a greenhouse. In our assay system, the disease symptoms of the inoculated plants are first observed as an unusual yellowing of leaves. The yellowing progresses from the lower leaves to the upper leaves, and wilting begins to occur following the yellowing. Wilting also progresses from the lower leaves to the upper leaves. The severity of wilting was evaluated daily using a disease index scale ranging from 0 to 4, according to the appearance of disease symptoms in the sixth and fifth leaves, as follows: none of the leaves are wilted, 0; the fifth leaf begins to yellow, 1; the fifth leaf begins to wilt or the sixth leaf begins to yellow, 2; the sixth leaf begins to wilt, 3; all leaves are completely wilted, 4. Each *R. solanacearum* strain was tested, using 12 plants in one experimental round. Under our greenhouse condition, the best tolerance of eggplants to bacterial wilt was exhibited from late spring to mid-autumn (from May to October in Japan), probably due to both longer days and stronger sunlight during plant growth

in this period. Pathogenicity tests on the $\Delta lrpE$ mutants were performed at least five times during this period over three years, and similar results were obtained repeatedly.

For the tests of the recovery of bacterial cells from inoculated plants, 4-week-old eggplants were cut at the stem above the fourth leaf and were inoculated by depositing a 10- μ l droplet containing 1×10^6 *R. solanacearum* cells in DW. Three days after the inoculation, a 1-cm-long sample of the stem 3 cm below the inoculation site was obtained and macerated in 1 ml of sterile DW and was left to stand for 3 h. The samples of bacterial ooze from the vascular system of the stem were mounted on a glass slide and were analyzed by phase-contrast microscopy using an Axiophot2 microscope (Zeiss).

Electron microscopy.

Bacterial cultures were diluted with DW, and the diluent was deposited on collodion membrane-coated carbon grids, was stained three times with 0.4% uranyl acetate, was filtered twice through a 0.2- μ m membrane, was left to dry, and was then examined under a Hitachi H-7500 electron microscope (Hitachi, Tokyo) at 80 KV.

Construction of *R. solanacearum* mutants expressing LrpE-FLAG fusion protein.

To construct the LrpE-FLAG fusion protein, a plasmid-based method (Tamura et al. 2005) was used. In brief, an approximately 1-kb PCR-amplified fragment containing part of the 3' coding sequence of the *lrpE* gene was inserted into pNC123 (Tamura et al. 2005). The resulting pRS846, carrying *lrpE-flag*, was integrated into the genome of the *R. solanacearum* EPS⁻ strain RS1085 and its $\Delta(hrpB-hrcT)$ derivative, RS1204, through conjugation with S17-1. The transformants contained the plasmid insertion, which expressed the LrpE-FLAG fusion protein, into the chromosomal *lrpE* locus.

For the protein secretion assay, to highly express the FLAG fusion protein, the *hrpB^c* mutation was introduced into *R. solanacearum* strains by the electroporation of RS1239 chromosomal DNA (Tamura et al. 2005) and the selection of *Gm^r* recombinants. The resulting *hrpB^c* mutants were used in the assay.

Protein secretion analysis.

Hrp-secreted proteins from *R. solanacearum* strains were purified and immunodetected as previously described (Tamura et al. 2005).

Construction of *R. solanacearum* mutants expressing LrpE₁₋₂₃₀'-Cya or PopC₁₋₂₃₀'-Cya fusion protein.

To obtain the 'Cya fusion protein, a plasmid-based system was constructed. First, a 1.2-kb *BamHI*-*HindIII* fragment encoding 'Cya₂₋₄₀₀, which includes amino acids 2 to 400 of the *Bordetella* adenylate cyclase toxin (Ladant and Ullmann 1999), was amplified from pMS107 (Sory and Cornelis 1994) using the primers P974 (5'-GGGATCCCAGCAATCGCATCAGG-3') and P975 (5'-GAAGCTTTGTCATAGCCGGAAT-3') with *BamHI* (underlined) and *HindIII* (double underlined) sites, respectively, and was cloned into pARO191 (Parke 1990) to yield pARO-'Cya. The 57-nucleotide HA tag-coding sequence was inserted into pARO-'Cya to yield pARO-HA-'Cya, in which unique *EcoRI*, *XbaI*, *SmaI*, and *XhoI* restriction sites were used to fuse a gene cassette encoding HA-'Cya at the 3' end of the target gene. A 690-bp fragment encoding the N-terminal first 230 amino acids was amplified from the *lrpE* and *popC* genes and respectively inserted into pARO-HA-'Cya to yield pRS1025 and pRS1067. Each plasmid was integrated into the genome of *R. solanacearum* RS1085 and its $\Delta hrpY$ derivative, RS1254, through conjugation with S17-1, to yield

strains expressing the LrpE₁₋₂₃₀'-'Cya or PopC₁₋₂₃₀'-'Cya fusion protein. Strains expressing the full-length-Hpx19'-Cya fusion protein were constructed by a similar method. For in vitro adenylate cyclase activity assay, *hrpB*^c mutants were constructed for each *R. solanacearum* strain.

Adenylate cyclase activity assay and cAMP extraction.

For in vitro adenylate cyclase assay, *R. solanacearum* *hrpB*^c strains expressing the 'Cya fusion protein were grown overnight in BG medium at 28°C. Cells of these strains were centrifuged and washed twice with DW and were resuspended in a secretion medium at an optical density at 600 nm (OD₆₀₀) = 0.5 (approximately 5 × 10⁸ CFU/ml). The cultures were incubated for 16 h, and cells were harvested by centrifugation and were diluted 10-fold with B-PER reagent (Pierce, Rockford, IL, U.S.A.). Then, a clear lysate (soluble protein fraction) was prepared according to the manufacturer's instructions. Total protein concentration was measured with a protein assay kit (Bio-Rad, Hercules, CA, U.S.A.). An aliquot of 10 µl was added to 90 µl of reaction buffer (final concentrations: 50 mM Tris-HCl, pH 8; 6 mM MgCl₂; 0.12 mM CaCl₂; 0.1 mg/ml bovine serum albumin; 2 mM ATP; and 0.1 µM bovine calmodulin, when required). The reaction was allowed to proceed for 10 min at 30°C and was stopped by boiling for 5 min. The samples were stored at -80°C, until assayed for cAMP. The cAMP level was measured with a cAMP enzyme immunoassay kit (Biotrak; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

For in planta adenylate cyclase assay, *R. solanacearum* strains expressing the 'Cya fusion protein were grown overnight in BG medium at 28°C. Cells were centrifuged and washed twice with DW and were resuspended in DW at OD₆₀₀ = 0.5 (approximately 5 × 10⁸ CFU/ml). The bacterial solution was inoculated using a needleless syringe into fully expanded leaves of 6-week-old susceptible eggplants. The inoculated plants were kept at 28°C in a greenhouse, and two leaf disks (0.8 cm² each) were taken 3, 6, 9, 12, and 15 h after inoculation and were frozen in 2-ml Eppendorf tubes in liquid nitrogen. The frozen samples were ground by shaking six times with 5-mm diameter tungsten beads for 30 s in an Eppendorf tube, using a sample smasher SH-48 (Kurabou, Osaka, Japan) at 1,200 rpm. Then, 200 µl of Biotrak assay buffer was added to the samples, which were then boiled for 5 min. Cell debris was removed by centrifugation for 10 min twice. The supernatant was collected in new tubes and was stored at -80°C until assayed for cAMP.

β-Galactosidase assay.

A β-galactosidase assay was performed as described by Miller (1992) with some modifications. Cells of *R. solanacearum* strains were cultured in the BG medium for 16 h at 28°C and were collected by centrifugation. The cell pellets were washed twice in distilled water (DW) and were resuspended in 1:100 1/4 M63 glucose minimal medium. The cultures were incubated for 16 h at 28°C and were used for the assay.

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