Contribution of a lectin, LecM, to the quorum sensing signalling pathway of Ralstonia solanacearum strain OE1-1

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SUMMARY

The soil-borne bacterium Ralstonia solanacearum invades the roots and colonizes the intercellular spaces and then the xylem. The expression of lecM, encoding a lectin LecM, is induced by an OmpR family response regulator HrpG in R. solanacearum strain OE1-1. LecM contributes to the attachment of strain OE1-1 to the host cells of intercellular spaces. OE1-1 produces methyl 3-hydroxyarabinose (3-OH MAME) through a methyltransferase (PhcB) and extracellularly secretes the chemical as a quorum sensing (QS) signal, which activates QS. The expression of lecM is also induced by the PhcA virulence regulator functioning through QS, and the resulting LecM is implicated in the QS-dependent production of major exopolysaccharide EPS I and the aggregation of OE1-1 cells. To investigate the function of LecM in QS, we analysed the transcriptome of R. solanacearum strains generated by RNA sequencing technology. In the lecM mutant, the expression of positively QS-regulated genes and negatively QS-regulated genes was down-regulated (by >90%) and up-regulated (by ~60%), respectively. However, phcB and phcA in the lecM mutant were expressed at levels similar to those in strain OE1-1. The lecM mutant produced significantly less ralfuranone and exhibited a significantly greater swimming motility, which were positively and negatively regulated by QS, respectively. In addition, the extracellular 3-OH MAME content of the lecM mutant was significantly lower than that of OE1-1. The application of 3-OH MAME more strongly increased EPS I production in the phcB-deleted mutant and strain OE1-1 than in the lecM mutant. Thus, the QS-dependent production of LecM contributes to the QS signalling pathway.

Keywords: LecM, quorum sensing, Ralstonia solanacearum.

INTRODUCTION

Many bacteria regulate their cooperative activities and physiological processes through quorum sensing (QS), in which bacterial cells communicate with each other by releasing, sensing and responding to small diffusible signal molecules, called QS signals (Ham, 2013). QS signals accumulate in the environment as the density of the bacterial population increases, and the bacteria monitor this information to track changes in their cell numbers. QS controls the expression of genes involved in activities that are beneficial when performed by groups of bacteria acting in synchrony (Rutherford and Bassler, 2012). Importantly, many pathogenic bacteria use cell–cell signalling to regulate the expression of factors that contribute to virulence (Ham, 2013).

Bacterial wilt caused by the soil-borne, plant-pathogenic, Gram-negative bacterium Ralstonia solanacearum is a devastating bacterial plant disease in tropical, subtropical and warm-temperature regions worldwide (Mansfield et al., 2012). Ralstoniasolanacearum normally invades plant roots from the soil through wounds or via natural openings from which secondary roots emerge (Araud-Razou et al., 1998), and colonizes the intercellular spaces of the root cortex and vascular parenchyma (Hikichi, 2016; Vasse et al., 1995). After invasion into the intercellular cells, cells of R. solanacearum strain OE1-1 attach to the surfaces of host plant cells (Hikichi et al., 2017). Ralstonia solanacearum produces a lectin, LecM (R5-III), which is encoded by lecM and exhibits mannose, fructose, fucose, galactose and arabino affinities (Sudakevitz et al., 2002, 2004). Meng et al. (2015) reported that R. solanacearum strain UWS51 at cooler temperatures up-regulates the expression of lecM, which is required for its full virulence, and LecM functions inside the plant, not only during attachment to root surfaces. lecM expression is positively regulated by a transcriptional regulator of the hrp regulon, HrpG (Mori et al., 2016; Valls et al., 2006), and LecM is involved in the attachment of R. solanacearum cells to the surfaces of plant cells after invasion into intercellular spaces (Mori et al., 2016). OE1-1 then expresses hrp genes, constructs the type III secretion machinery and translocates effectors into host cells (Hikichi et al., 2017), inducing the production of the phosphatidic
acid phosphatase of host plants, which dephosphorylates phosphatic acid into diacylglycerol in the chloroplast membranes of the host plant (Nakano et al., 2013). This leads to the suppression of jasmonic acid- and reactive oxygen-mediated defences, which are induced through Sec14P-mediated phospholipid signalling to produce phosphatic acid in chloroplast membranes (Kiba et al., 2014; Nakano et al., 2013). This allows OE1-1 to escape the innate immunity of host plants and to vigorously multiply on host plant cells (Hikichi et al., 2017).

The vigorous growth of R. solanacearum after escaping the innate immunity of host plants leads to QS (phc QS). Ralstonia solanacearum strains AW1 and K60 produce methyl 3-hydroxypalmitate as a QS signal (Flavier et al., 1997; Kai et al., 2015). In addition, R. solanacearum strains OE1-1 and GMI1000 produce methyl 3-hydroxymyristate (3-OH MAME) as a QS signal (Kai et al., 2015). These QS signals are synthesized by the methyltransferase PhcB (Fig. S1, see Supporting Information; Flavier et al., 1997; Kai et al., 2015). When the QS signals reach a threshold level, they reduce the ability of the histidine kinase PhcS to phosphorylate the response regulator PhcR, resulting in elevated levels of functional PhcA, an LysR-type transcriptional regulator, which plays a central role as a global virulence regulator in phc QS (Genin and Denny, 2012; Hikichi et al., 2017).

Ralstonia solanacearum synthesizes aryl-furanone secondary metabolites, known as ralfuranones A, B, I, J, K and L, which are secreted extracellularly (Kai et al., 2014, 2016; Pauly et al., 2013). Ralfuranone I is a precursor for other ralfuranones. The expression of ralfuranone synthase, encoded by ralA, is dependent on PhcA functioning through phc QS and is involved in the biosynthesis of ralfuranones (Kai et al., 2014, 2016; Schneider et al., 2009; Wackler et al., 2011). Interestingly, ralfuranones are implicated in a positive feedback loop in phc QS (Fig. S1; Hikichi et al., 2017; Mori et al., 2018b).

The production of major exopolysaccharide EPS I, which is required for R. solanacearum virulence, is positively regulated by phc QS (Huang and Schell, 1995). The epsB gene is included in the eps operon, which is involved in EPS I biosynthesis, and its expression is induced by PhcA functioning through phc QS. LeaM production is also induced by PhcA functioning through phc QS (Meng et al., 2015; Mori et al., 2016) and is involved in the production of phc QS-dependent major exopolysaccharide EPS I, which leads to the aggregation of OE1-1 cells (Fig. S1; Mori et al., 2016). In addition, a comparison of the levels of gene expression of wild-type (WT) R. solanacearum strain GMI1000 and the phcA-deleted mutant during tomato colonization revealed that PhcA positively regulates leaM expression in strain GMI1000, which infects xylem vessels of tomato (Khokhani et al., 2017).

In this study, we first assayed the expression of epsB in the leaM mutant (OE1-1-leaM::EZ Tn5; Mori et al., 2016). To elucidate the function of LeaM in phc QS, we examined the transcriptome profile of the leaM mutant compared with the phc QS-deficient mutants, as well as the wild-type (WT) OE1-1 strain (Kanda et al., 2003), using RNA sequencing (RNA-seq) technology. We then analysed the involvement of LeaM in phc QS-dependent virulence-related phenotypes.

RESULTS

The leaM mutation leads to significantly reduced epsB expression

The leaM mutant produces significantly less EPS I than does the parent strain OE1-1 and the native leaM-expressing complemented leaM mutant (leaM-comp; Mori et al., 2016). Incubation
The lecM mutation affects the gene expression regulated by 3-OH MAME perception

Supplementation with 3-OH MAME leads to the recovery of EPS and ralforanur production in ΔphcB (Kai et al., 2015). To determine the concentration of 3-OH MAME for supplementation to ΔphcB, we assessed the phc QS-dependent cell aggregation of ΔphcB supplemented with 3-OH MAME at concentrations of 0.001–1.0 µm. The aggregation level of ΔphcB cells increased significantly as the 3-OH MAME concentration increased from 0.1 to 1.0 µm (P < 0.05, t-test, Fig. 2).

We performed transcriptome analysis using RNA-seq of R. solanacearum strains OE1-1 and the lecM mutant, but also ΔphcB, ΔphcB supplemented with 1.0 µm 3-OH MAME and the phcA-deleted mutant (ΔphcA). For transcriptome analyses using RNA-seq, total RNA was isolated from cells of R. solanacearum strains OE1-1, the lecM mutant, ΔphcB, ΔphcB supplemented with 1.0 µm 3-OH MAME and ΔphcA, and cultured in ¼ × M63 medium [to an optical density at 600 nm (OD 600) = 0.3]. Cytoplasmic ribosomal RNA was removed from total RNA, resulting in a final RNA yield of 400 ng for each sample. The isolated RNA was subjected to Illumina RNA-seq. The RNA samples were fragmented and ligated with adaptors prior to cDNA synthesis and PCR amplifications. Two biologically independent experiments were conducted for each strain. We obtained 41.8 and 46.0, 45.6 and 44.0, 46.5 and 43.9, 44.0 and 47.3, and 45.3 and 44.4 million 100-bp paired-end reads from strains OE1-1, the lecM mutant, ΔphcB, ΔphcB supplemented with 1.0 µm 3-OH MAME and ΔphcA, respectively. By iterative alignment, 41.0 and 45.0, 45.1 and 42.5, 42.5 and 43.5, 42.5 and 45.8, and 41.4 and 43.0 million 100-bp paired-end reads, respectively, were successfully mapped to the R. solanacearum strain GMI1000 reference genome (Salanoubat et al., 2002). The mapping of the OE1-1 RNA-seq reads to the GMI1000 genome resulted in the identification of 4491 protein-coding transcripts.

The normalized gene expression levels for R. solanacearum strain OE1-1 and the other strains were compared to detect differentially expressed transcripts. The read counts obtained for each sample were expressed as the fragments per kilobase of open reading frame per million fragments mapped (FPKM) normalized prior to analysis for differentially expressed genes. Genes were considered to be differentially expressed if they exhibited log2(fold changes) of ≥1 or ≤−1 (fold changes of ≥2 or ≤−2).

We detected 744 genes that were expressed at significantly lower levels in ΔphcB than in OE1-1 (Table S1, see Supporting Information). The application of 3-OH MAME led to significantly enhanced expression levels of 410 of these genes, suggesting that their expression is positively regulated by 3-OH MAME perception (3-OH MAME-positively regulated genes) (Fig. 3a). Among the 886 genes (PhcA-positively regulated genes) that were expressed at significantly lower levels in ΔphcA than in strain OE1-1, 396 genes were 3-OH MAME-positively regulated genes, suggesting that their expression is positively regulated by phc QS (phc QS-positively regulated genes). Among the PhcA-positively regulated genes, more than 400 genes were not 3-OH MAME-positively regulated genes, suggesting that positive regulation by PhcA may be independent of 3-OH MAME production.

We also detected 770 genes (LecM-positively regulated genes) that were expressed at significantly lower levels in the lecM mutant than in strain OE1-1. Among the LecM-positively regulated genes, 385 genes, including the major EPS (i.e. EPS I) production-related genes, such as those in the eps operon (i.e. epsR and xpsR), the type VI secretion system-related genes, plant cell wall degradation enzyme genes (i.e. pme and egl), two-component system-related genes (i.e. solI and solR) and some effector genes secreted through the type III secretion systems (i.e. RSc1723 and Rsop0323, rip01), were included in the phc QS-positively regulated genes (Fig. 3a; Table S2, see Supporting Information). In

Fig. 2 Cell aggregation by Ralstonia solanacearum OE1-1, the phcB-deleted mutant (ΔphcB) and ΔphcB supplemented with methyl 3-hydroxymyristate (3-OH MAME). The OE1-1 and ΔphcB cells were incubated in ¼ × M63 medium in the wells of polyvinylchloride microtitre plates. ΔphcB cells were also incubated in ¼ × M63 medium supplemented with 3-OH MAME at concentrations of 0.001–1.0 µm. The cells were stained with crystal violet. The experiment was repeated three times, with seven technical replicates in each experiment. Asterisks indicate values significantly different from those of strain OE1-1 (P < 0.05, t-test).
Implication of LecM in quorum sensing

Fig. 3  Number of genes for which expression was regulated by methyl 3-hydroxymyristate (3-OH MAME), PhcA and LecM encoded in lecM in Ralstonia solanacearum strain OE1-1. The transcriptome analyses using RNA sequencing (RNA-seq) were performed on RNA from the R. solanacearum phcB-deleted mutant (ΔphcB), ΔphcB supplemented with 1.0 µm methyl 3-hydroxymyristate (3-OH MAME), phcA-deleted mutant (ΔphcA) and lecM mutant (OE1-1-lecM::EZ Tn5). Numbers of genes that exhibited expression level log₂(fold changes) of ≤−1 (a) or ≥1 (b) in ΔphcB supplemented with 1.0 µm 3-OH MAME relative to the expression levels in ΔphcB, and expression level log₂(fold changes) of ≤−1 (a) or ≥1 (b) in ΔphcA and lecM mutant relative to the expression levels of strain OE1-1. The fragments per kilobase of open reading frame per million fragments mapped (FPKM) values of OE1-1, ΔphcB, ΔphcA and lecM mutant strains were normalized prior to the analysis of differentially expressed genes.

Fig. 4  Influence of the lecM mutation on the production of ralfuranones by Ralstonia solanacearum strains. High-performance liquid chromatography (HPLC) analysis of culture extracts from R. solanacearum strain OE1-1 (a) and lecM mutant (OE1-1-lecM::EZ Tn5, b). The peaks of ralfuranones are marked as A, B, J and K.
addition, 345 LecM-positively regulated genes were also positively regulated by PhcA, but not by 3-OH MAME (Fig. 3a).

We also detected 348 genes that were expressed at higher levels in ΔphcB than in OE1-1 (Table S1). Among them, 3-OH MAME application increased the expression levels of 128 genes (3-OH MAME-negatively regulated genes) in ΔphcB (Fig. 3b). Among the 384 genes (PhcA-negatively regulated genes) that were expressed at significantly greater levels in ΔphcA than in strain OE1-1, 108 genes were 3-OH MAME-negatively regulated genes, suggesting that their expression is negatively regulated by phc QS (phc QS-negatively regulated genes). However, among the PhcA-negatively regulated genes, more than 250 genes were not 3-OH MAME-negatively regulated genes, suggesting that the negative regulation by PhcA may be independent of 3-OH MAME production. The lecM mutation resulted in the significantly reduced expression of 345 genes (LecM-negatively regulated genes). Among these, 104 genes, including flagellar motility-related genes, such as fiIC, type III secretion-related genes and chemotaxis-related genes, were phc QS-negatively regulated genes (Fig. 3b; Table S3, see Supporting Information).

In addition, 192 genes were negatively regulated by PhcA, but not by 3-OH MAME (Fig. 3b).

The expression levels of all transcripts in the lecM mutant were positively correlated with those in ΔphcB and ΔphcA (Fig. S3, see Supporting Information).

The lecM mutation leads to reduced ralfuranone production

To analyse the influence of LecM on phc QS-positive regulation, we assessed the phc QS-dependent production of ralfuranones by the R. solanacearum lecM mutant compared with strain OE1-1. The lecM mutant produced less ralfuranones than OE1-1 (Fig. 4).

We then analysed the ralA expression level in R. solanacearum strains grown on ¼ × M63 medium using qRT-PCR assays. We observed a significantly lower ralA expression level in the lecM mutant than in the WT and lecM-comp strains (P < 0.05, t-test, Fig. 1).

LECm mutant cells exhibit greater swimming motility than OE1-1 cells

Flagella biogenesis is negatively regulated by phc QS, leading to the suppression of the swimming motility of R. solanacearum during phc QS activation (Tans-Kersten et al., 2001). To analyse the influence of LecM on phc QS-negative regulation, we analysed the swimming motilities of the R. solanacearum strains. The lecM mutant exhibited a significantly greater swimming motility than WT strain OE1-1 and the lecM-comp mutant strain on ¼ × M63 medium solidified with 0.25% agar (P < 0.05, t-test, Fig. 5).

We analysed the expression level of fliC, encoding flagellin, in R. solanacearum strains grown on ¼ × M63 medium using qRT-PCR assays. We observed significantly greater fliC expression levels in the lecM mutant than in the OE1-1 strain and lecM-comp strain (P < 0.05, t-test, Fig. 5).

Expression analysis of phc QS-related genes

We analysed the expression of the phc QS-related genes phcB and phcA in R. solanacearum strains grown in ¼ × M63 medium (to OD600 = 0.3) using qRT-PCR assays. There were no significant differences among the lecM mutant, lecM-comp and OE1-1 strains.
with regard to phcB and phcA expression levels ($P > 0.05$, t-test, Fig. 1).

**The lecM mutation leads to a significantly reduced 3-OH MAME content**

The ralfuranone production-deficient mutant (ΔralA; Kai et al., 2014) exhibits a lower 3-OH MAME content than the WT strain OE1-1, although ΔralA expresses phcB and phcA, similar to OE1-1 (Mori et al., 2016), suggesting the involvement of ralfuranones in the 3-OH MAME content. Because the lecM mutation leads to significantly reduced ralfuranone production, we determined the 3-OH MAME contents of *R. solanacearum* strains. The lecM mutant exhibited a significantly lower 3-OH MAME content than OE1-1 ($P < 0.05$, Fig. 6). The lecM-comp strain produced significantly more 3-OH MAME than the lecM mutant ($P < 0.05$, t-test, Fig. 6).

**Supplementation with 3-OH MAME affects EPS I production of the lecM mutant**

We analysed the phc QS-dependent EPS I production of *R. solanacearum* strains. Similar to ΔphcB, the lecM mutant produced significantly reduced EPS I compared with strain OE1-1 ($P < 0.05$, t-test; Fig. 7). The application of 1.0 µM 3-OH MAME significantly enhanced the EPS I production of the OE1-1 strain (Table S4, see Supporting Information). The EPS I production of the ΔphcB strain increased significantly with the application of 1.0 µM 3-OH MAME. However, the increased EPS I production by the lecM mutant supplemented with 1.0 µM 3-OH MAME was significantly less than that in 3-OH MAME-supplemented OE1-1 and ΔphcB.

**Invasion of the lecM mutant into xylem vessels**

Previously, we have reported a loss in virulence for the lecM mutant when inoculated through roots by root dipping (Mori et al., 2016). Therefore, we analysed the infection of *R. solanacearum* strains into xylem vessels using the plate-printing assay. The lecM mutant was detected beyond the inoculated roots and in both inoculated roots and stems of tomato plants, similar to OE1-1 and lecM-comp (Fig. S4, see Supporting Information).

**DISCUSSION**

The QS system in *R. solanacearum* consists of the phc regulatory elements, and PhcA functioning through the phc QS system plays a central role, leading to the virulence of this bacterial species...
functions in both the attachment of bacteria to host plant cells
methyl ester hydrolases suppressed extracellular polysaccharide
al., 1994). Because luxI expression is also activated by the QS
(Shinohara et al., 2007). Furthermore, soil metagenome-derived
1984; Engebrecht et al., 1983). When the QS signal accumulates,
is involved in the expression of genes regulated by phc QS (Fig. 3;
Table S1). Previously, we have reported the positive feedback regulation of phc QS by ralfuranones (Hikichi et al., 2017; Mori et al., 2018b). Furthermore, LecM is involved in the expression of genes regulated by phc QS (Fig. 3; Table S1). HrpG, a transcriptome regulator of the hrp regulon, positively regulates lecM expression, producing and extracellularly secreting LecM (Mori et al., 2016; Valls et al., 2006). LecM functions in both the attachment of bacteria to host plant cells and in bacterial cell–cell binding in biofilms (Meng et al., 2015). Furthermore, LecM is involved in the attachment of R. solanacearum cells to surfaces of host cells after invasion of intercellular spaces (Mori et al., 2016). This is followed by the construction of the type III secretion machinery and the secretion of effectors into host cells (Hikichi et al., 2017). OE1-1 thus evades host innate immunity and grows vigorously on host cells, inducing phc QS. Functional PhcA also induces the production of LecM (Meng et al., 2015; Mori et al., 2016). Thus, LecM produced during phc QS is involved in the expression of genes regulated by phc QS.

Acyl homoserine lactones are a major class of autoinducer signal used by Gram-negative proteobacteria for intraspecies QS (Ng and Bassler, 2009). LuxI and LuxR are essential for the QS control of bioluminescence in Vibrio fischeri. LuxI is the synthase in the QS signal N-(3-oxohexanoyl)homoserine lactone (Engebrecht and Silverman, 1984; Schaefer et al., 1996). LuxR catalyses the acylation and lactonization reactions between the substrates S-adenosylmethionine and 3-oxohexanoyl-ACP (More et al., 1996; Schaefer et al., 1996). LuxR is the cytoplasmic receptor for the QS signal, as well as the transcriptional activator of the luciferase luxICDABE operon (Engebrecht and Silverman, 1984; Engebrecht et al., 1983). When the QS signal accumulates, it is bound by LuxR, and the LuxR–acyl homoserine lactone complex recognizes a consensus binding sequence upstream of the luxICDABE operon, activating its expression (Stevens et al., 1994). Because luxI expression is also activated by the QS signal-bound LuxR, when the QS circuit engages, QS signal production is induced, and the surrounding environment is flooded with the signal molecule. Interestingly, lipolytic enzymes from culture-based sources, capable of the hydrolysis of 3-OH PAME, suppressed EPS production in R. solanacearum strain AW1-3 (Shinohara et al., 2007). Furthermore, soil metagenome-derived methyl ester hydrolases suppressed extracellular polysaccharide production in R. solanacearum strain GM1000 (Lee et al., 2017). These results demonstrate that reduced content of extracellular 3-OH MAME/3-OH PAME results in reduced activity of phc QS. The lecM mutation led to lower 3-OH MAME contents (Fig. 6), and the extracellular application of 3-OH MAME induced phc QS-dependent EPS I production by the lecM mutant to a lesser extent than the OE1-1 and ΔphcB strains (Fig. 7). Interestingly, the lecM mutation did not affect phcB and phcA expression (Fig. 1). Thus, LecM may affect the activation of phc QS through the instability of extracellularly secreted 3-OH MAME (Fig. S1).

PhcA functioning through phc QS-induced expression of the ralfuranone production-related genes ralA and ralD stimulated the production and extracellular secretion of ralfuranones (Kai et al., 2014; Schneider et al., 2009). Ralfuranones are involved in the positive feedback regulation of phc QS (Hikichi et al., 2017; Mori et al., 2018b). Because PhcA functioning through phc QS induces the production of LecM (Meng et al., 2015; Mori et al., 2016), this feedback regulation by ralfuranones leads to the induction of lecM expression during phc QS. Results in this study suggest that LecM may affect the activation of phc QS, involved in the regulation of phc QS-dependent gene expression. Thus, the ‘autoinduction’–positive feedback loops of phc QS mediated by ralfuranones and LecM in a population of OE1-1 cells switched from a low-cell-density mode to a high-cell-density mode, leading to the induction of phc QS, enhancing phc QS (Fig. S1).

As in phc QS, both LecM and ralfuranones are required for OE1-1 virulence (Hikichi et al., 2017; Kai et al., 2014; Mori et al., 2018a, 2018b). Thus, both the stability of 3-OH MAME through LecM and the phc QS feedback regulated by ralfuranones may contribute to the full virulence of strain OE1-1. Zuluaga et al. (2013) reported that the tomato apoplast is significantly richer in sugars, such as sucrose, fructose, glucose, galactose and mannose, than the xylem, and R. solanacearum probably catabolizes the abundant apoplast sugars. LecM exhibits mannose, fructose, fucose, galactose and arabinose affinity (Sudakevitz et al., 2004). Sudakevitz et al. (2004) reported that extracts of R. solanacearum strain GM1000 had fructose/mannose-specific haemagglutinating activities that could be attributed to LecM. Furthermore, OE1-1 cells incubated in apoplast fluids produce mushroom-type biofilms (Mori et al., 2016). On the contrary, OE1-1 cells incubated in xylem fluids do not produce a mushroom-type biofilm and aggregate formlessly. This suggests that intercellular spaces provide favourable conditions for mushroom-type biofilm formation by OE1-1 cells (Hikichi et al., 2017). Therefore, it is hypothesized that the LecM-mediated regulation of strain OE1-1, when invading intercellular spaces and xylem vessels, leads to mushroom-type biofilm formation and formless cell aggregation, respectively, contributing to its virulence. Interestingly, when inoculated using root dipping, the lecM strain invaded xylem vessels (Fig. S3). However, the lecM mutant loses its virulence (Mori et al., 2016). Thus, LecM-mediated regulation in OE1-1 cells during mushroom-type biofilm
formation may be involved in the priming of OE1-1 virulence after invasion of xylem vessels.

Ailloud et al. (2016) performed transcriptome analyses of R. solanacearum strains infecting host plants using RNA-seq, and many virulence-related genes were differentially expressed during infection, compared with in vitro growth, such as growth on a rich medium. A large majority of the PhcA controlled genes followed the same regulation pattern in both rich medium in vitro and after in planta growth, except for a set of HrpG-HrpB regulated genes, including the type III secretion machinery and type III effectors, whose genes appeared to be specifically induced by PhcA in the plant environment, whereas this regulator repressed their expression in rich medium (Perrier et al., 2018). PhcA mediates a second strategic switch between the initial attachment of strain GMI1000 and its subsequent dispersal inside the host, and helps it to optimally invest resources and correctly sequence multiple steps in the bacterial wilt disease cycle (Khokhani et al., 2017). Our previous (Mori et al., 2016) and present studies on transcriptome analyses using RNA-seq of R. solanacearum strains incubated in a poor medium, ¼ × M63, showed that the phc QS signalling pathway was regulated through LecM and ralfuranones in strain OE1-1 (Figs 3 and S1). LecM- and ralfuranone-mediated regulation was involved in mushroom-type formation and invasion into xylem vessels, and contributed to full virulence. Strain OE1-1 produces mushroom-type biofilms when incubated in apoplastic fluids from tomato plants, but not in xylem fluids (Mori et al., 2016). Therefore, there is a difference in the regulation of gene expression levels in R. solanacearum strains during early root colonization and in infected tomato xylem vessels. Taken together, further transcriptome analyses of R. solanacearum strains infecting host plants, focusing on LecM- and ralfuranone-mediated regulation, will help to uncover the key regulatory mechanisms of R. solanacearum virulence.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids and growth conditions**

We used the following R. solanacearum strains: OE1-1 (Kanda et al., 2003), the lecM mutant (Mori et al., 2016), lecM-comp (native lecM-expressing complemented lecM mutant; Mori et al., 2016), ΔphcB (Kai et al., 2015), ΔphcA (Mori et al., 2016) and ΔralA (Kai et al., 2014). The R. solanacearum strains were routinely grown in ¼ × M63 medium at 30 °C. Escherichia coli strains were grown in Luria–Bertani medium (Hanahan, 1983) at 37 °C. Gentamycin (50 µg/mL) was used in selective media.

**Analysis of ralfuranones produced by R. solanacearum strains**

*Ralstonia solanacearum* strains were grown in 100 mL of MGRLS medium (MGRL medium supplemented with 3% sucrose; Kai et al., 2014) in 300-mL Erlenmeyer flasks at 30 °C with rotation (130 rpm) for 4 days. Following growth, the bacterial cultures were extracted three times with an equal volume of ethyl acetate. The combined extracts were dried over Na2SO4 and evaporated to dryness. The residues were dissolved in methanol (500 µL) and subjected to high-performance liquid chromatography (HPLC) analysis: column, Inertsil ODS-3 (250 mm × 4.6 mm, 5 µm; GL Sciences, Tokyo, Japan); column oven, 40 °C; flow rate, 1 mL/min; eluent, acetonitrile–H2O (20%–95% linear gradient in 40 min, then 95% acetonitrile for 10 min); injection volume, 10 µL. The experiment was conducted at least twice using independent samples, with similar results. Results for a single representative sample are provided.

**Analysis of 3-OH MAME produced by R. solanacearum strains**

*Ralstonia solanacearum* strains grown in B medium at 30 °C for 4–6 h were diluted to an OD600 of 1.0 with new medium. A sample (50 µL) of the cell suspension was pipetted onto a BA agar plate (90 mm, 25 mL; Kai et al., 2015), and the plate was incubated for 24 h at 30 °C. The BA agar was cut into small pieces and soaked in ethyl acetate (50 mL) for 2 h twice. The combined extracts were dried over Na2SO4 and concentrated. The residue was dissolved in ethyl acetate (2 mL) and subjected to gas chromatography–mass spectrometry (GC-MS) analysis. GC-MS data were recorded with a GCMS-QP2010 Plus (9, Kyoto, Japan) and an InertCap 5MS/NP column (25 m × 0.25 mm, 0.25 µm film, GL Sciences). The conditions used were as follows: injection, 1 mL (splitless; valve time, 60 s); injector temperature, 20 °C; carrier gas, He (at 0.8 mL/min); transfer line temperature, 300 °C; ion source temperature, 230 °C; electron energy, 70 eV. The temperature of the column oven was programmed as follows: 50 °C for 5 min, followed by an increase to 300 °C at 20 °C/min, with the temperature then being maintained at 300 °C for 5 min (Kai et al., 2015); data reflect three replicates each.

**RNA extraction, elimination of ribosomal RNA and sequencing**

Two biologically independent experiments were conducted for each strain. Total RNA was isolated from R. solanacearum strains grown in ¼ × M63 medium (to OD600 = 0.3) using a High Pure RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). Ribosomal RNA was removed from the extracted total RNA using a Ribo-Zero rRNA Removal Kit (Grav-negative bacteria) (Illumina, Madison, WI, USA). Oriented paired-end RNA-seq (2 × 100 bp) was conducted by Hokkaido System Science (Sapporo, Japan) using an Illumina HiSeq 2000 system and the procedures recommended by Illumina. The adaptors and primers were designed by Hokkaido System Science. The selected inserts were 100 bp. We conducted the paired-end sequencing of the libraries.
Mapping and analysis of RNA-seq data

Reads were trimmed using Cutadapt (version 1.1; https://code.google.com/p/cutadapt/) and Trimomatic (version 0.32; https://www.usadel.org/cms/?page=trimmomatic), and then mapped with TopHat (version 2.0.10; https://tophat.ccb.cb.columbia.edu/). Read counts obtained for each of the samples are presented as FPKM, which was calculated with Cufflinks (version 2.2.1; https://cole-trapnell-lab.github.io/cufflinks/).

qRT-PCR

A 500-ng total RNA template sample was reverse transcribed using a PrimeScript RT Reagent Kit (Takara, Otsu, Japan). A qRT-PCR assay was conducted with a 20-µL reaction mixture containing 1 µL cDNA stock and 10 pm primers (Table S5, see Supporting Information) using a SYBR GreenER qPCR Reagent System (Invitrogen, Tokyo, Japan). Reactions were completed in an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA). The cycling parameters for all primers were as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 31 s (Mori et al., 2016). Melting curve runs were completed at the end of each reaction to verify the specificity of the primers (i.e. presence of a single product). Relative quantification of gene expression was carried out according to the instructions for the Applied Biosystems 7300 Real-time PCR system, using the comparative cycle threshold method for the calculation of the quantity value. All values were normalized against the rpoD expression level as an internal standard for each cDNA sample (Mori et al., 2016; Narusaka et al., 2011). There were no significant differences in the rpoD expression levels among R. solanacearum strains. The experiment was conducted at least twice using independent samples, with similar results. Results for a single representative sample are provided.

Bacterial cell aggregation assay

The aggregation of R. solanacearum cells was measured in vitro using a slight modification of the polyvinylchloride microtitre plate assay described by O’Toole and Kolter (1998). Briefly, 5-µL overnight cultures of R. solanacearum grown in 1/4× M63 medium adjusted to OD600 = 0.005 were used to inoculate 95 µL of 1/4× M63 medium in the wells of a polyvinylchloride microtitre plate (Nunc MicroWell plate; Thermo Fisher Scientific Inc., Waltham, MA, USA). Tomato apoplast fluid was added to the wells, and the plate was incubated at 30 °C for 24 h without shaking. To quantify the cell aggregation, 25 µL of 1.0% (w/v) crystal violet solution were added to the wells. After a 15-min incubation, the unbound crystal violet stain was gently removed with a pipette, and the wells were washed with distilled water, 70% ethanol and distilled water again. The remaining crystal violet in each well was solubilized with 100 µL of 100% ethanol, and then quantified by measuring the absorbance at 550 nm. The resulting value was normalized according to the number of cells (OD550/OD600). This value was considered to represent the relative cell aggregation (Mori et al., 2016). The experiment was repeated three times, with seven technical replicates in each experiment.

Swimming motility

Overnight cultures of R. solanacearum strains were washed with distilled water and diluted to a cell density of 1.0 × 108 colony-forming units (CFU)/mL. For the swimming assay, 3-µL aliquots of cell suspensions were added to the centre of 1/4× M63 medium solidified with 0.25% agar. Motility was examined using three plates per strain. All plates were incubated at 30 °C. The diameters of the swimming areas were measured at 48 h post-incubation (Mori et al., 2018b). The experiment was repeated three times, with five technical replicates in each experiment.

EPS I production

Quantitative analyses of EPS I production were conducted using an enzyme-linked immunosorbent assay (Mori et al., 2016). The overnight culture of R. solanacearum strains was rinsed, and diluted to a cell density of 1.0 × 105 CFU/mL. Then, 100 µL of these cell suspensions were spread on plates of 1/4× M63 agar medium and incubated for 2 days at 30 °C. Cells were resuspended to 1.0 × 105 CFU/mL, and the cell density was confirmed through dilution plating. EPS I was quantified using anti-R. solanacearum EPS I antibodies by an enzyme-linked immunosorbent assay per 100 µL volume (1.0 × 105 CFU) of cell suspension according to the manufacturer’s instructions (Agdia Inc., Elkhart, IN, USA). EPS I productivity was quantified by absorbance at 650 nm. EPS I production values were statistically analysed using the Tukey–Kramer honestly significant difference (HSD) test (n = 9) following an analysis of variance (ANOVA) with Easy R software (Saitama Medical Center, Jichi Medical University, Saitama, Japan; Kanda, 2013). The experiment was repeated three times, with five technical replicates in each experiment.

Systemic infectivity assays

Tomato plants (Solanum esculentum cv. Ohgata-Fukuju) were grown in pots containing commercial soil (Tsuchitaro; Sumitomo Forestry Landscaping, Tokyo, Japan) in a growth room at 25 °C under 10 000 lx for 16 h per day, and watered with diluted 1/5 × Hoagland’s solution (Hikichi et al., 1999). The roots of 5-week-old tomato plants were soaked in bacterial suspensions at 1.0 × 108 CFU/mL for 30 min and then washed in running water. Plants were then grown in water culture pots (Yamato Water Culture Pot No. 1, Yamato Plastic Co. Ltd., Yamatotakada, Japan) with fivefold-diluted Hoagland’s solution. At 10 days post-inoculation, after sterilization of plant surfaces with 70% ethanol, the roots and stems of five tomato plants were cut into
three pieces each using razor blades (Kanda et al., 2008). The cut site of each piece (l, roots; II and III, stems; Fig. S4) was pressed onto strain-specific media [Hara–Ono medium (Hara and Ono, 1983) for OE1-1, Hara–Ono medium containing 50 μg/mL kanamycin and 25 μg/mL gentamycin for lecM-comp] and then incubated at 30 °C for 3 days.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1 Model of the regulation of phc quorum sensing (QS) mediated by RS-IIL and ralfuranes in Ralstonia solanacearum strain OE1-1.

Fig. S2 Colonies of Ralstonia solanacearum strains OE1-1, lecM mutant (OE1-1-lecM::EZTn5) and native lecM-expressing complemented lecM mutant (lecM-comp) on Hara–Ono medium (Hara and Ono, 1983). Ralstonia solanacearum strains were incubated on strain-specific media (Hara–Ono medium for OE1-1, Hara–Ono medium containing 50 µg/mL kanamycin for the lecM mutant and Hara–Ono medium containing 50 µg/mL kanamycin and 25 µg/mL gentamycin for lecM-comp) at 30 °C for 2 days.

Fig. S3 Correlations of gene expression level changes in Ralstonia solanacearumphcB-deleted mutant (ΔphcB, a) and phcA-deleted mutant (ΔphcA, b) with the expression level changes (i.e. ≥2 or ≤−2) of genes regulated by RS-IIL encoded in lecM. The FPKM (fragments per kilobase of open reading frame per million fragments mapped) values for R. solanacearum strains OE1-1, ΔphcB, ΔphcA and lecM mutant (OE1-1-lecM::EZTn5) were normalized prior to the analyses of differentially expressed genes.

Fig. S4 Behaviour of Ralstonia solanacearum strain OE1-1, lecM mutant (OE1-1-lecM::EZTn5) and native lecM-expressing complemented lecM mutant (lecM-comp) in tomato plants. Ralstonia solanacearum strains in tomato plants at 10 days after inoculation by root dipping were detected using the plate-printing assay (Kanda et al., 2008). At 10 days post-inoculation with R. solanacearum strains, the surfaces of the roots and stems were sterilized with 70% ethanol. The roots and stems of five tomato plants were then cut into three pieces each using razor blades. The cut site of each piece (I, roots; II and III, stems; Fig. 8b) was pressed onto strain-specific media (Hara–Ono medium (Hara and Ono, 1983) for OE1-1, Hara–Ono medium containing 50 µg/mL kanamycin for the lecM mutant and Hara–Ono medium containing 50 µg/mL kanamycin and 25 µg/mL gentamycin for lecM-comp) at 30 °C for 2 days.

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mutant and Hara–Ono medium containing 50 µg/mL kanamycin and 25 µg/mL gentamycin for lecM-comp] and incubated at 30 °C for 3 days.

Table S1 RNA sequencing data for all transcripts in *Ralstonia solanacearum* strains grown in ¼ × M63 medium.
Table S2 (1) Predicted function of proteins encoded by positively RS-IIL-regulated genes among positively *pha* quorum sensing (QS)-regulated genes in *Ralstonia solanacearum* strain OE1-1 grown in ¼ × M63 medium. (2) Predicted function of proteins encoded by positively RS-IIL-regulated genes among positively *pha* QS-regulated genes in *Ralstonia solanacearum* strain OE1-1 grown in ¼ × M63 medium. (3) Predicted function of proteins encoded by positively RS-IIL-regulated genes among positively *pha* QS-regulated genes in *Ralstonia solanacearum* strain OE1-1 grown in ¼ × M63 medium. (4) Predicted function of proteins encoded by positively RS-IIL-regulated genes among positively *pha* QS-regulated genes in *Ralstonia solanacearum* strain OE1-1 grown in ¼ × M63 medium.

Table S3 Predicted function of proteins encoded by RS-IIL-negatively regulated genes among *pha* quorum sensing (QS)-negatively regulated genes in *Ralstonia solanacearum* strain OE1-1 grown in ¼ × M63 medium.
Table S4 Tukey–Kramer analysis of exopolysaccharide EPS I production by *Ralstonia solanacearum* strains.
Table S5 Primers used in this study.