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Involvement of ralfuranones in the quorum sensing signalling pathway and virulence of *Ralstonia solanacearum* strain OE1-1

YUKA MORI[1], SHIHO ISHIKAWA[1], HIDEYUKI OHNISHI[2], MIKA SHIMATANI[2], YUKINO MORIKAWA[1], KAZUSA HAYASHI[1], KOUHEI OHNISHI[3], AKINORI KIBA[1], KENJI KAI[2] AND YASUFUMI HIKICHI[1,*]

[1]*Laboratory of Plant Pathology and Biotechnology, Kochi University, Nankoku, Kochi 783-8502, Japan*
[2]*Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599-8531, Japan*
[3]*Research Institute of Molecular Genetics, Kochi University, Nankoku, Kochi 783-8502, Japan*

SUMMARY

The soil-borne, plant-pathogenic *Ralstonia solanacearum* strain OE1-1 produces and secretes methyl 3-hydroxymyristate (3-OH MAME) as a quorum sensing (QS) signal, which contributes to its virulence. A global virulence regulator, PhcA, functioning through the QS system, positively regulates the expression of *ralA*, which encodes furanone synthase, to produce aryl-furanone secondary metabolites, ralfuranones. A ralfuranone-deficient mutant (Δ*ralA*) is weakly virulent when directly inoculated into tomato xylem vessels. To investigate the functions of ralfuranones, we analysed *R. solanacearum* transcriptome data generated by RNA sequencing technology. Δ*ralA* expressed *phcB*, which is associated with 3-OH MAME production, and *phcA* at levels similar to those in strain OE1-1. In addition, Δ*ralA* exhibited down-regulated expression of more than 90% of the QS positively regulated genes, and up-regulated expression of more than 75% of the QS negatively regulated genes. These results suggest that ralfuranones affect the QS feedback loop. Ralfuranone supplementation restored the ability of Δ*ralA* cells to aggregate. In addition, ralfuranones A and B restored the swimming motility of Δ*ralA* to wild-type levels. However, the application of exogenous ralfuranones did not affect the production of the major exopolysaccharide, EPS I, in Δ*ralA*. Quantitative real-time polymerase chain reaction assays revealed that the deletion of *ralA* results in the down-regulated expression of *vsrAD* and *vsrBC*, which encode a sensor kinase and a response regulator, respectively, in the two-component regulatory systems that influence EPS I production. The application of ralfuranone B restored the expression of these two genes. Overall, our findings indicate that integrated signalling via ralfuranones influences the QS and virulence of *R. solanacearum*.

Keywords: quorum sensing, ralfuranones, *Ralstonia solanacearum*, soil-borne, plant-pathogenic bacterium, virulence.

* *Correspondence*: Email: yhikichi@kochi-u.ac.jp

INTRODUCTION

Cells of many bacteria communicate with each other by releasing, sensing and responding to small diffusible signalling molecules, allowing them to regulate their cooperative activities and physiological processes through quorum sensing (QS) (Ham, 2013). These QS-controlled activities influence the virulence and pathogenic potential of bacteria. Many pathogenic bacteria use cell–cell signalling to regulate the expression of virulence factors. The signalling molecule known as diffusible signalling factor (DSF) is a *cis*-unsaturated fatty acid and belongs to a novel class of QS signals in *Xanthomonas campestris* pv. *campestris* (Ham, 2013; Mills *et al.*, 2011; Ryan and Dow, 2011; Tang *et al.*, 1991). DSF appears to be widely conserved in diverse bacterial species. The DSF-type QS signalling system includes a novel secondary messenger, cyclic-di-guanosine monophosphate (cyclic-di-GMP), which facilitates the coupling of QS to the bacterial intracellular regulatory networks. This system is implicated in the regulation of a wide range of bacterial functions. However, intercellular signalling between bacterial cells that are associated with QS remains unclear.

The soil-borne, plant-pathogenic *Ralstonia solanacearum* bacterial strains AW1 and K60 produce methyl 3-hydroxypalmitate (3-OH PAME) as a QS signal that mediates the *phc* QS system (Flavier *et al.*, 1997; Kai *et al.*, 2015). 3-OH PAME is synthesized by PhcB, which is a methyltransferase. When the abundance of 3-OH PAME reaches a threshold level, it decreases the ability of the histidine kinase PhcS to phosphorylate the response regulator PhcR. This results in elevated levels of functional PhcA, which is a LysR-type transcriptional regulator (Clough *et al.*, 1997; Flavier *et al.*, 1997; Genin and Denny, 2012). In addition, strains OE1-1 and GMI1000 produce methyl 3-hydroxymyristate (3-OH MAME) as a QS signal (Kai *et al.*, 2015). The deduced PhcB and PhcS amino acid sequences among *R. solanacearum* strains are related to the productivity of QS signals. The global virulence regulator PhcA plays a central role in the *phc* QS system (Fig. S1, see Supporting Information) (Brumbley and Denny, 1990; Clough *et al.*, 1994; Genin and Denny, 2012).

Ralstonia solanacearum synthesizes aryl-furanone secondary metabolites, known as ralfuranones A, B, I, J, K and L, which are

extracellularly secreted (Kai *et al.*, 2014; Pauly *et al.*, 2013). Ralfuranone I is a precursor of the other ralfuranones (Fig. S1). The production of transaminase and furanone synthase, which are encoded by *ralD* and *ralA*, respectively, depends on PhcA functions via the *phc* QS system, and they are involved in the biosynthesis of ralfuranones (Kai *et al.*, 2014; Schneider *et al.*, 2009; Wackler *et al.*, 2011). Thus, ralfuranone production is dependent on the *phc* QS system. Kai *et al.* (2014) revealed that a ralfuranone-deficient mutant (i.e. Δ*ralA*) produces considerably less exopolysaccharide (EPS) and is less virulent than OE1-1 on tomato plants following direct inoculations of xylem vessels. These results suggest that ralfuranones are required for the full virulence of strain OE1-1. Furthermore, non-phytotoxic ralfuranones were detected in the xylem fluids of tomato plants inoculated with strain OE1-1. Thus, ralfuranones may be linked to the intercellular signalling between OE1-1 cells required for virulence. However, the exact effects of ralfuranones on OE1-1 virulence remain unclear.

To elucidate the exact role of ralfuranones in OE1-1 virulence, we first examined the transcriptome profiles of the Δ*ralA* and *phc* QS-deficient mutants, as well as the wild-type (WT) OE1-1 strain, using RNA sequencing (RNA-seq) technology. We also analysed the involvement of ralfuranones in *phc* QS-dependent, virulence-related phenotypes.

RESULTS

ralA deletion affects the expression of a large set of genes also regulated by the QS system components *phcA* and *phcB*

For transcriptome analyses using RNA-seq, total RNA was isolated from OE1-1, *phcB*-deleted mutant (Δ*phcB*), *phcA*-deleted mutant (Δ*phcA*) and Δ*ralA* mutant *R. solanacearum* cells cultured in one-quarter-strength M63 medium [to an optical density at 600 nm (OD_{600}) = 0.3]. Cytoplasmic ribosomal RNA was removed from the total RNA, for a final RNA yield of 400 ng for each sample. The isolated RNA was subjected to Illumina RNA sequencing. The RNA samples were fragmented and ligated with adaptors prior to cDNA synthesis and polymerase chain reaction (PCR) amplifications. We obtained 41.0, 46.5, 45.3 and 44.8 million 100-bp paired-end reads from OE1-1, Δ*phcB*, Δ*phcA* and Δ*ralA*, respectively. By iterative alignment, 41.8, 42.5, 42.1 and 41.4 million 100-bp paired-end reads 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 4493 protein-coding transcripts.

The normalized gene expression levels of OE1-1, Δ*phcB*, Δ*phcA* and Δ*ralA* were compared to detect differentially expressed transcripts. Read counts obtained for each sample were FPKM (fragments per kilobase of exon per million fragments mapped) normalized prior to being analysed for differentially expressed genes. Genes were considered to be differentially expressed if they exhibited fold changes of $\geq$2 or $\leq$–2. We detected 688, 983 and 772 genes that were expressed at significantly lower levels in Δ*phcB*, Δ*phcA* and Δ*ralA* than in OE1-1, respectively (Table S1, see Supporting Information, Fig. 1a). In addition, 606 genes were expressed at lower levels in both Δ*phcB* and Δ*phcA*, suggesting that the expression of these genes is positively regulated by PhcA functioning through the *phc* QS system (Fig. S2, see Supporting Information). Of these, 556 genes, including *lecM*, 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*, *egl* and *pehC*), two-component system-related genes (i.e. *solI* and *solR*) and some effector genes secreted through the type III secretion systems (i.e. *RSc1800*, *ripG4*; *RSc1801*, *ripG5*; *Rsp0323*, *ripO1*; *RSp0731*, *ripTP5*; *Rsp1281*, *ripS*; and *RSp1460*, *ripAU*) were expressed at lower levels in Δ*ralA* than in OE1-1 (Table S2, see Supporting Information).

We also detected 298, 320 and 240 genes that were expressed at higher levels in Δ*phcB*, Δ*phcA* and Δ*ralA* than in OE1-1, respectively (Table S2, Fig. 1b). There were 171 genes that were expressed at higher levels in both Δ*phcB* and Δ*phcA*, suggesting that the expression of these genes is negatively regulated by PhcA functioning through the *phc* QS system (Fig. S2). Included among these genes were 135 genes that were more highly expressed in Δ*ralA* than in OE1-1, including flagellar motility-related genes, such as *fliC*, type III secretion-related genes and some type III effector genes (i.e. *RSc3290*, *ripAX1*; *RSp0099*, *ripA2*; *RSp0822*, *ripAF1*; *RSp0877*, *ripX*; *RSp0876*, *ripAB*; *RSp0875*, *ripAC*; *RSp1374*, *ripS2*; *RSp0930*, *ripS3*; *RSp1277*, *ripQ*; *RSp1582*, *ripAZ1*; and *RSp1601*, *ripAD*), and chemotaxis-related genes (Table S3, see Supporting Information).

Expression analysis of the *phc* QS-related genes and 3-OH MAME production

The expression of *ralA* in strain OE1-1 depends on the activation of PhcA in the *phc* QS system, with 3-OH MAME acting as a QS signal (Kai *et al.*, 2015). If ralfuranones control 3-OH MAME production by feedback regulation, deletion of *ralA* might result in systemically decreased QS-dependent gene expression levels. We first analysed the expression of the *phc* QS-related genes, *phcB* and *phcA*, in *R. solanacearum* strains grown in one-quarter-strength M63 medium (to OD_{600} = 0.3) using quantitative real-time PCR (qRT-PCR) assays. There were no significant differences between Δ*ralA* and OE1-1 with regard to *phcB* and *phcA* expression levels ($P > 0.05$) (Fig. 2a). However, Δ*ralA* produced slightly less 3-OH MAME than OE1-1 (Fig. 2b).

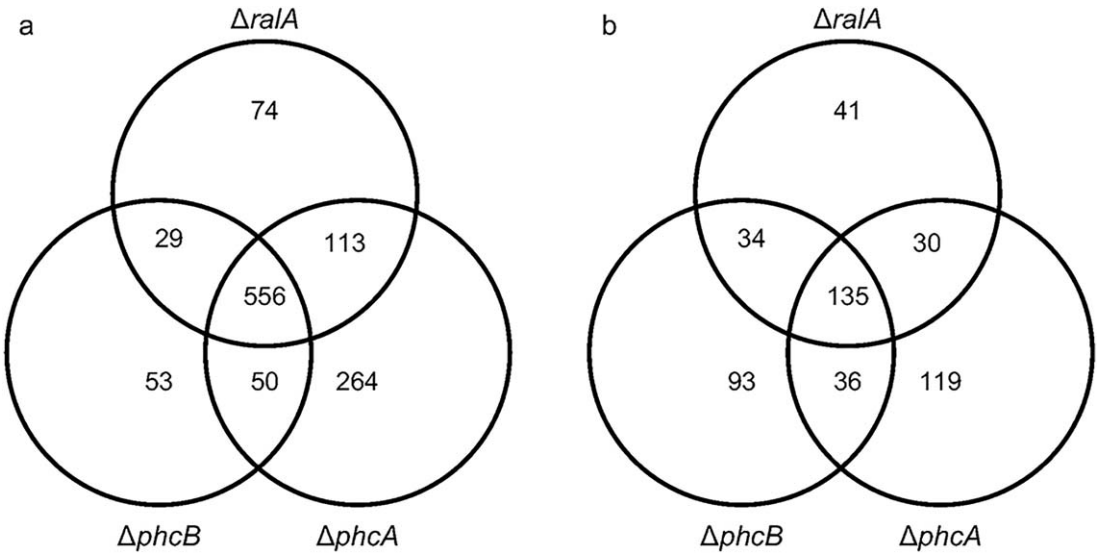

Fig. 1 Number of genes exhibiting expression level fold changes of $\leq$–2 (a) or $\geq$2 (b) in the *Ralstonia solanacearum phcB*-deleted mutant (Δ*phcB*), *phcA*-deleted mutant (Δ*phcA*) and *ralA*-deleted mutant (Δ*ralA*), relative to the expression levels of strain OE1-1. The FPKM (fragments per kilobase of exon per million fragments mapped) values of strains OE1-1, Δ*phcB*, Δ*phcA* and Δ*ralA* were normalized prior to the analyses of differentially expressed genes.

Ralfuranones affect the aggregation of *R. solanacearum* OE1-1 cells

The aggregation of *R. solanacearum* cells depends on *lecM*, which encodes the lectin, RS-IIL. The expression of *lecM* is positively regulated by the *phc* QS system (Meng *et al.*, 2015; Mori *et al.*, 2016). *Ralstonia solanacearum* strains incubated in one-quarter-strength M63 medium for 24 h were analysed using crystal violet staining. There were significantly fewer cell aggregates in the Δ*ralA* mutant than in the OE1-1 or complemented Δ*ralA* mutant strain (*ralA*-comp) ($P < 0.05$, Fig. 3).

The Δ*ralA* mutant was then grown in one-quarter-strength M63 medium containing 0.2–20 μM of each ralfuranone. The Δ*ralA* cell aggregation level increased with increasing concentrations of ralfuranones A, K and L. When the growth medium was supplemented with 20 μM ralfuranones A or K, more Δ*ralA* cells than OE1-1 cells aggregated (Fig. 3). In addition, ralfuranone L restored the aggregation of Δ*ralA* cells to approximately 75.5% of that of WT cells. Supplementation with ralfuranone B restored the aggregation of Δ*ralA* cells to >80% of the OE1-1 levels, regardless of concentration. In contrast, supplementation with 20 μM ralfuranone J resulted in only a slight increase in the aggregation of Δ*ralA* cells. These results suggest that ralfuranones, especially ralfuranones A and K, influence the aggregation of OE1-1 cells. Based on these assay results, we used 20 μM ralfuranones in subsequent experiments.

Δ*ralA* produces significantly less EPS I than does OE1-1

We quantified the EPS I produced by *R. solanacearum* strains growing on one-quarter-strength M63 solid medium using an enzyme-linked immunosorbent assay. The Δ*ralA* mutant produced significantly less EPS I than did the OE1-1 and *ralA*-comp strains ($P < 0.05$, Fig. 4a). Supplementation with individual ralfuranones did not affect EPS I production by Δ*ralA*.

We then analysed the expression of *epsB* in *R. solanacearum* strains grown in one-quarter-strength M63 medium using qRT-PCR assays. This gene is included in the *eps* operon and is thought to be important for EPS I biosynthesis (Huang and Schell, 1995). The *epsB* expression level was significantly lower in Δ*ralA* than in OE1-1 or *ralA*-comp ($P < 0.05$, Fig. 4b). Supplementation with ralfuranones A, J, K or L resulted in slight increases in *epsB* expression in the Δ*ralA* mutant, whereas ralfuranone B supplementation increased *epsB* expression in the mutant to 76.0% of the level in OE1-1.

Δ*ralA* cells exhibit greater swimming motility than OE1-1 cells

Flagella biogenesis is negatively regulated by the *phc* QS system, and swimming motility is essential for biofilm formation by *R. solanacearum* (Tans-Kersten *et al.*, 2001). Thus, we analysed the swimming motility of the *R. solanacearum* strains. The Δ*ralA* mutant exhibited greater swimming motility than the WT strain OE1-1 on one-quarter-strength M63 medium solidified with 0.25% agar, similar to the *phcA*-deleted mutant. The swimming motility of the *ralA*-comp mutant strain was similar to that of the WT strain (Fig. 5a).

When Δ*ralA* was supplemented with ralfuranones A or B, the observed swimming motility was similar to that of the OE1-1 and *ralA*-comp strains (Fig. 5a). Supplementation with ralfuranone L resulted in slightly decreased Δ*ralA* swimming motility, whereas ralfuranones J and K had no effect.

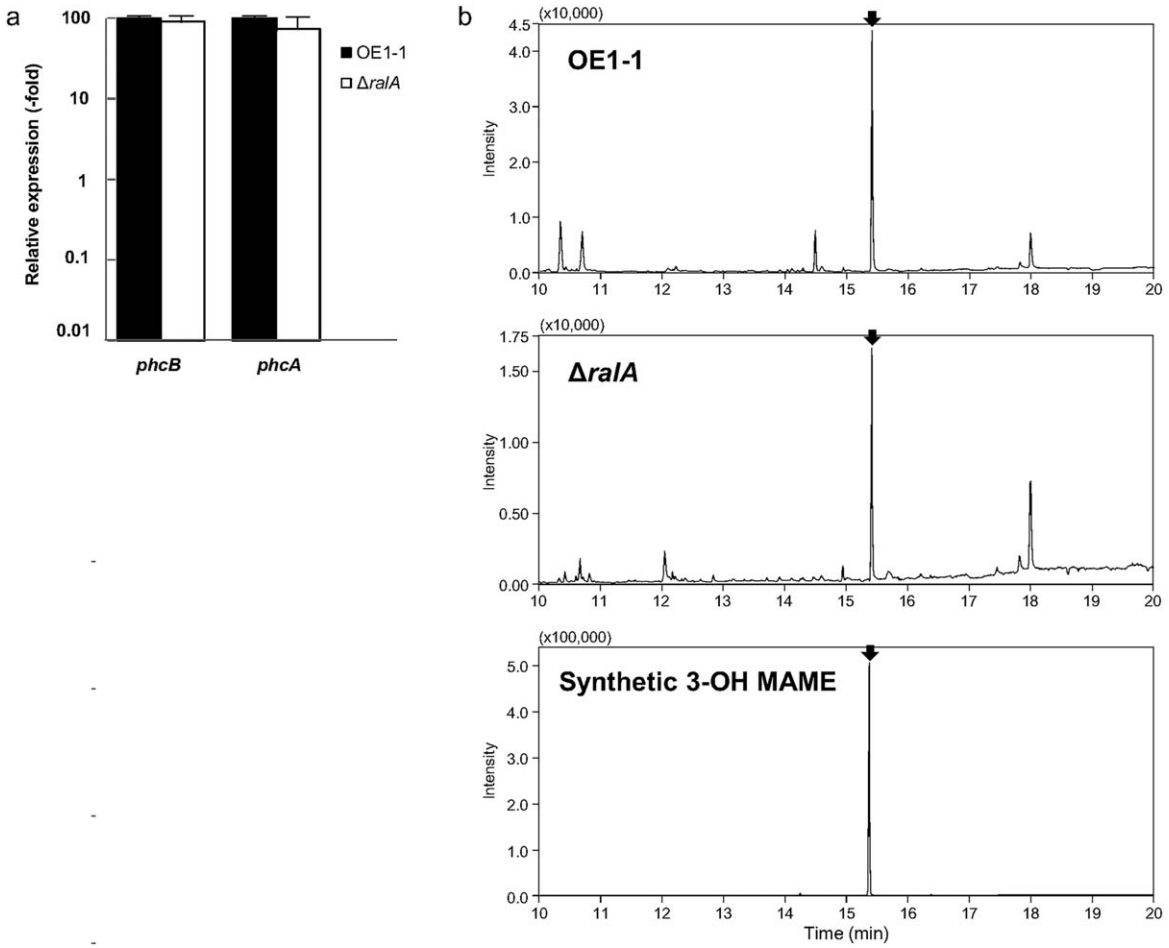

Fig. 2 Influence of *ralA* deletion on the quorum sensing of *Ralstonia solanacearum*. Expression of *phcB* and *phcA* in *R. solanacearum* OE1-1 and *ralA*-deleted mutant (Δ*ralA*) strains (a) and methyl 3-hydroxymyristate (3-OH MAME) purified from the *R. solanacearum* OE1-1 and Δ*ralA* strains (b). The *R. solanacearum* strains were grown in one-quarter-strength M63 medium (to $OD_{600} = 0.3$). Total RNA was then extracted from the bacterial cells. The *rpoD* gene was used as an internal control for quantitative real-time polymerase chain reaction. The gene expression levels are presented relative to the *rpoD* expression level. The experiment was conducted at least twice using independent samples, with similar results. Results for a single representative sample are provided. Values are presented as the mean ± standard deviation of three replicates. Asterisks indicate values that are significantly different from those of OE1-1 cells ($P < 0.05$, *t*-test). Synthetic 3-OH MAME was used as a positive control. The arrows indicate the peaks corresponding to 3-OH MAME.

We analysed the *fliC* expression level in *R. solanacearum* strains grown on one-quarter-strength M63 medium using qRT-PCR assays. We observed higher *fliC* expression levels in the Δ*ralA* mutant than in the WT and *ralA*-comp strains (Fig. 5b). When grown on one-quarter-strength M63 medium containing ralfuranones A or B, the *fliC* expression level in strain Δ*ralA* decreased to a level similar to that of the WT OE1-1 strain. Supplementation with ralfuranone L resulted in a slightly decreased *fliC* expression level in Δ*ralA*, whereas ralfuranones J and K had the opposite effect.

Ralfuranones affect the expression of two-component, system-encoding *vsrA/vsrD* and *vsrB/vsrC*

Although supplementation with ralfuranone B restored *epsB* expression in the Δ*ralA* mutant strain, EPS I production was unaffected. The VsrAD and VsrBC two-component sensor kinase/response regulatory systems are reportedly involved in the regulation of EPS I production dependent on the *phc* QS (Garg *et al.*, 2000; Huang *et al.*, 1998). To elucidate the role of ralfuranones in these two-component systems, we analysed the expression of *vsrA/vsrD* and *vsrB/vsrC* in the *R. solanacearum* strains using qRT-PCR assays. The expression levels of all four genes were significantly lower in the Δ*ralA* mutant than in the WT and *ralA*-comp strains ($P < 0.05$, Fig. 6). When grown in one-quarter-strength M63 medium containing ralfuranone B, the expression of *vsrA/vsrD* and *vsrB/vsrC* was restored to WT levels in the Δ*ralA* mutant. Supplementation with ralfuranone J resulted in partially recovered gene expression levels (Fig. 6), whereas ralfuranones K and L did not influence gene expression in Δ*ralA*. Supplementation with

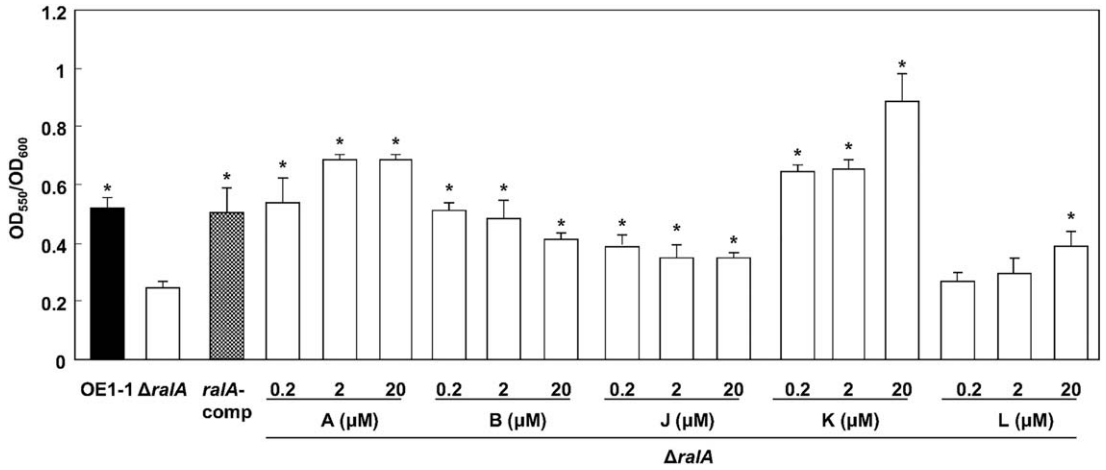

Fig. 3 Cell aggregation results for *Ralstonia solanacearum* OE1-1, the ralfuranone-deficient mutant (Δ*ralA*) and the complemented Δ*ralA* mutant (*ralA*-comp) strains. OE1-1, Δ*ralA* and *ralA*-comp cells were incubated in one-quarter-strength M63 medium in wells of polyvinylchloride microtitre plates. Δ*ralA* cells were also incubated in one-quarter-strength M63 medium supplemented with ralfuranones A, B, J, K or L at concentrations of 0.2–20 μM. The wells were stained with crystal violet. Asterisks indicate values that are significantly different from those of the Δ*ralA* strain in apoplast fluid ($P < 0.05$, *t*-test). OD, optical density.

ralfuranone A resulted in slightly decreased *vsrA*, *vsrD* and *vsrB* expression levels in the Δ*ralA* mutant.

Ralfuranones affect the expression of genes encoding sigma factors

RpoN (i.e. sigma factor σ^{54}) regulates flagellar motility, EPS biosynthesis and biofilm formation in plant-pathogenic bacteria (Dong and Mekalanos, 2012; Hao *et al.*, 2013; Kazmierczak *et al.*, 2005; O'Toole *et al.*, 1997; Reitzer and Schneider, 2001; da Silva Neto *et al.*, 2008). *Ralstonia solanacearum* has two genes encoding σ^{54} (i.e. *rpoN1* and *rpoN2*). *rpoN1* also influences the virulence of strain GMI1000 (Ray *et al.*, 2015). Furthermore, the expression of *solI* and *solR*, which are associated with the SolI/R acyl-homoserine lactone QS system, depends on PhcA and RpoS (Flavier *et al.*, 1997). To clarify the involvement of ralfuranones in the regulation of sigma factors, we compared the expression of *rpoN1*, *rpoN2* and *rpoS* in strains Δ*ralA* and OE1-1 incubated in one-quarter-strength M63 medium. We observed significantly decreased *rpoN2* expression levels in the Δ*ralA* mutant, whereas the expression of *rpoN1* was not significantly affected ($P < 0.05$, Fig. 7a). The *rpoS* expression level was 2.5 times higher in Δ*ralA* than in OE1-1. We subsequently analysed the effects of ralfuranones on the expression of *rpoN2*. Although supplementation with ralfuranones A, K and L did not influence the expression of *rpoN2* in Δ*ralA*, ralfuranones B and J partially restored *rpoN2* expression (Fig. 7b).

DISCUSSION

The QS system in *R. solanacearum* consists of *phc* regulatory elements, and PhcA functioning via the *phc* QS system plays a central role, leading to the virulence of this bacterial species (Clough *et al.*, 1997; Flavier *et al.*, 1997; Genin and Denny, 2012). The results of our transcriptome analysis using RNA-seq (Fig. 1, Tables S1–S3) and qRT-PCR assays revealed that the deletion of *ralA* leads to significant changes in the expression levels of the majority of the *phc* QS-dependent genes, but not *phcB* and *phcA* (Fig. 2a). These results suggest that a lack of *ralA* may have implications for transcriptional regulation by the *phc* QS system. It is likely that the ralfuranones influence the *phc* QS-mediated functionalization of PhcA. It is tempting to speculate that ralfuranones affect the regulation of 3-OH MAME production at the post-transcriptional level based on the decreased production of 3-OH MAME in the Δ*ralA* mutant. The expression of ralfuranone production-related genes (i.e. *ralA* and *ralD*) is positively regulated by PhcA functions through the *phc* QS system (Kai *et al.*, 2014; Schneider *et al.*, 2009; Wackler *et al.*, 2011). These results suggest that ralfuranones may be associated with the feedback loop of the *phc* QS system (Fig. S3, see Supporting Information).

Kai *et al.* (2014) reported that the Δ*ralA* mutant exhibits significantly decreased virulence following direct inoculations of tomato xylem vessels. This implies that ralfuranones contribute to the full virulence of strain OE1-1. Furthermore, Kai *et al.* (2014) also detected ralfuranones in the xylem fluids of tomato plants following the direct inoculation of xylem vessels with strain OE1-1 using the wounded petiole inoculation method. These observations indicate that ralfuranones, produced by strain OE1-1 infecting xylem vessels, affect the expression of *phc* QS-regulated genes, leading to the full virulence of strain OE1-1.

The production of EPS I by OE1-1 is positively regulated by the *phc* QS system (Brumbley and Denny, 1990; Clough *et al.*, 1994; Genin and Denny, 2012). Although all of the ralfuranones used in

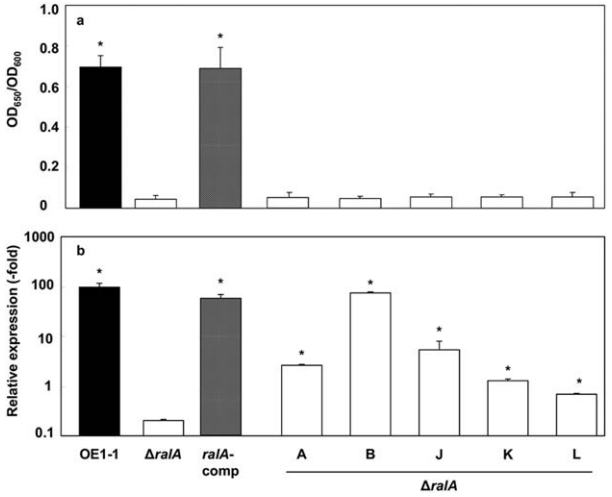

Fig. 4 Influence of *ralA* deletion on exopolysaccharide I (EPS I) production by *Ralstonia solanacearum*. (a) Quantification of EPS I in supernatants using an enzyme-linked immunosorbent assay with anti-*R. solanacearum* EPS I antibodies. The *R. solanacearum* OE1-1, ralfuranone-deficient mutant (Δ*ralA*) and Δ*ralA* complemented mutant (*ralA*-comp) strains were incubated on one-quarter-strength M63 medium in plates. The Δ*ralA* mutant was also incubated on one-quarter-strength M63 medium containing 20 μM ralfuranones A, B, J, K or L. (b) *epsB* expression levels in three *R. solanacearum* strains. Strains OE1-1, Δ*ralA* and *ralA*-comp were cultured in one-quarter-strength M63 medium. The Δ*ralA* mutant was also cultured in one-quarter-strength M63 medium containing 20 μM ralfuranones A, B, J, K or L. Total RNA was extracted from a bacterial culture [optical density at 600 nm (OD_{600}) = 0.3]. The *rpoD* gene was used as an internal control for quantitative real-time polymerase chain reaction. The gene expression levels are presented relative to the *rpoD* expression level. The experiment was conducted at least twice using independent samples, with similar results. Results for a single representative sample are provided. Values are presented as the mean ± standard deviation of three replicates. Asterisks indicate values that are significantly different from those of the Δ*ralA* cells ($P < 0.05$, *t*-test).

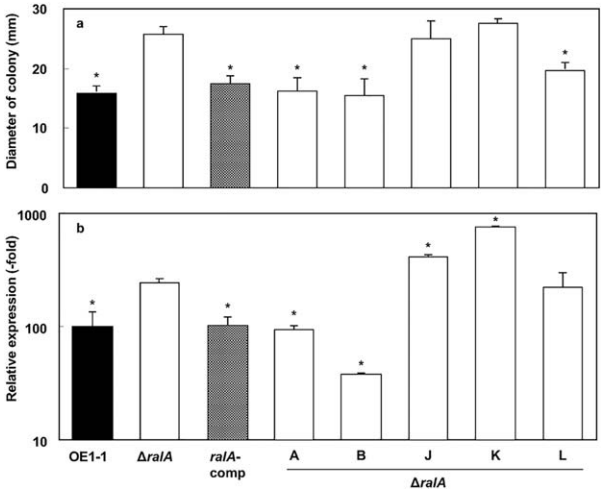

Fig. 5 Swimming motility and *fliC* expression in *Ralstonia solanacearum* strains. (a) To analyse swimming motility, *R. solanacearum* OE1-1, ralfuranone-deficient mutant (Δ*ralA*) and complemented Δ*ralA* mutant (*ralA*-comp) strains were grown on one-quarter-strength M63 medium solidified with 0.25% agar. The Δ*ralA* mutant was also incubated on agar-solidified, one-quarter-strength M63 medium containing 20 μM ralfuranones A, B, J, K or L. (b) To examine *fliC* expression, *R. solanacearum* strains OE1-1, Δ*ralA* and *ralA*-comp were cultured in one-quarter-strength M63 medium. The Δ*ralA* mutant was also cultured in one-quarter-strength M63 medium containing 20 μM ralfuranones A, B, J, K or L. Total RNA was extracted from a bacterial culture [optical density at 600 nm (OD_{600}) = 0.3]. The *rpoD* gene was used as an internal control for quantitative real-time polymerase chain reaction. The gene expression levels are presented relative to the *rpoD* expression level. The experiment was conducted at least twice using independent samples, with similar results. Results for a single representative sample are provided. Values are presented as the mean ± standard deviation of three replicates. Asterisks indicate values that are significantly different from those of the Δ*ralA* strain ($P < 0.05$, *t*-test).

this study positively regulated *epsB* expression in the Δ*ralA* mutant (Fig. 4b), EPS I production was not affected by any of the ralfuranones (Fig. 4a). Ralfuranones may thus affect the expression of unknown additional crucial EPS I biosynthesis-related genes. Furthermore, ralfuranones B and J were involved in the positive regulation of *vsrA*, *vsrD*, *vsrB* and *vsrC* expression (Fig. 6). In contrast, ralfuranone A may help to negatively regulate the expression of these genes. The PhcA and VsrAD two-component sensor/response regulatory systems are necessary for the full activation of the transcription of *xpsR*. In addition, both a transcriptional regulator XpsR and a response regulator VsrC up-regulate the expression of the *eps* operon (Garg *et al.*, 2000; Huang *et al.*, 1998). EPS I production is thus influenced by the VsrAD and VsrBC two-component systems. Therefore, the integrated regulation of *vsrAD* and *vsrBC* expression by ralfuranones A, B and J may also contribute to EPS I production (Fig. S3).

Flagella biogenesis in *R. solanacearum* is negatively regulated by the *phc* QS system (Tans-Kersten *et al.*, 2001). Interestingly, ralfuranones A and B negatively regulated *fliC* expression (Fig. 5b) and swimming motility (Fig. 5a) in the current study. Furthermore, the VsrAD and VsrBC two-component systems affect the regulation of flagella biogenesis (Genin and Denny, 2012). Therefore, ralfuranones A, B and J may also influence flagellar motility by regulating the expression of *vsrAD* and *vsrBC*. Furthermore, Schneider *et al.* (2009) reported that VsrAD is upstream of PhcA and is involved in the biosynthesis of ralfuranones. These observations suggest that the expression of *vsrAD* may be feedback regulated through ralfuranones A, B and J, leading to the regulation of PhcA function (Fig. S3).

σ54 (*rpoN*) is involved in the regulation of nitrogen metabolism, and subsequently affects many other biological activities in diverse Proteobacteria (Buck *et al.*, 2000). In *R. solanacearum*, RpoN1, but not RpoN2, helps to regulate the transcription of twitching motility-related genes, and affects the nitrate assimilation pathway and virulence (Ray *et al.*, 2015). Furthermore, *rpoN2*

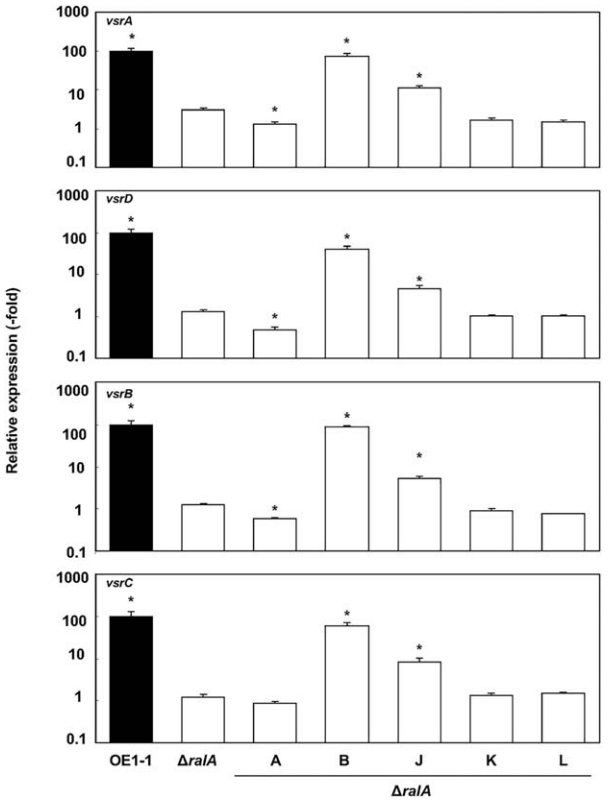

Fig. 6 Expression of *vsrA*/*vsrD* and *vsrB*/*vsrC* two-component, system-encoding genes in *Ralstonia solanacearum*. Strain OE1-1 and the ralfuranone-deficient mutant Δ*ralA* were cultured in one-quarter-strength M63 medium. The Δ*ralA* mutant was also cultured in one-quarter-strength M63 medium containing 20 μM ralfuranones A, B, J, K or L. Total RNA was extracted from a bacterial culture [optical density at 600 nm (OD_{600}) = 0.3]. The *rpoD* gene was used as an internal control for quantitative real-time polymerase chain reaction. The gene expression levels are presented relative to the *rpoD* expression level. The experiment was conducted at least twice using independent samples, with similar results. Results for a single representative sample are provided. Values are presented as the mean ± standard deviation of three replicates. Asterisks indicate values that are significantly different from those of the Δ*ralA* mutant ($P < 0.05$, *t*-test).

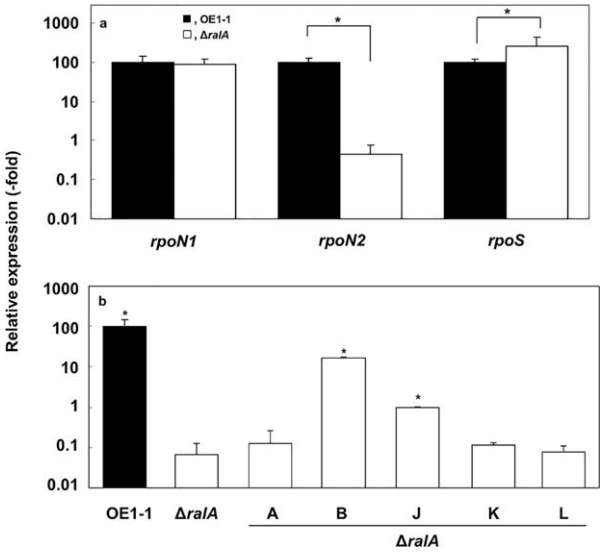

Fig. 7 Expression of *rpoN1*, *rpoN2* and *rpoS* in *Ralstonia solanacearum* strain OE1-1 and the ralfuranone-deficient mutant (Δ*ralA*). Total RNA was extracted from cells grown in one-quarter-strength M63 medium [optical density at 600 nm (OD_{600}) = 0.3] (a) or in one-quarter-strength M63 medium containing 20 μM ralfuranones A, B, J, K or L (b). The *rpoD* gene was used as an internal control for quantitative real-time polymerase chain reaction. The gene expression levels are presented relative to the *rpoD* expression level. The experiment was conducted at least twice using independent samples, with similar results. Results for a single representative sample are provided. Values are presented as the mean ± standard deviation of three replicates. Asterisks indicate values that are significantly different from those of OE1-1 (a) and Δ*ralA* (b) ($P < 0.05$, *t*-test).

expression is dependent on *rpoN1*. In the current study, the inability to produce ralfuranones resulted in significantly decreased *rpoN2* expression levels, whereas *rpoN1* expression was relatively unaffected (Fig. 7a). Ralfuranones B and J positively regulated *rpoN2* expression (Fig. 7b), suggesting that these two ralfuranones may directly influence *rpoN2* expression. Ray *et al.* (2015) revealed the broad conservation and stability of *rpoN2* in the *R. solanacearum* species complex. They speculated that *rpoN2* might be involved in the adaptation of the bacterium to a specific niche or environmental condition during its life cycle. Based on these observations, we propose that ralfuranones also regulate the expression of virulence-related genes via the regulation of *rpoN2* expression.

A novel transcriptional regulator (i.e. EfpR) in *R. solanacearum* strain GMI1000 acts as a global catabolic repressor that directly or indirectly down-regulates the expression of multiple metabolic pathway genes (Perrier *et al.*, 2016). Furthermore, EfpR also controls virulence traits, such as EPS production and motility (i.e. swimming or twitching). However, the expression of *efpR* is not significantly altered in the *phcA* mutant. In addition, *phcA* does not appear to be differentially regulated in the *efpR* mutant background. Our transcriptome analyses revealed differences between the Δ*phcA* (Table S2) and Δ*efpR* (Perrier *et al.*, 2016) mutants with regard to the expression profiles of genes related to EPS I production and flagella motility. Interestingly, EfpR suppressed *ralD* expression, which regulates ralfuranone production. Furthermore, the *phcA* mutant was observed to exhibit increased metabolic versatility, with the ability to metabolize a wider repertoire of metabolic substrates than the *efpR* mutant (Peyraud *et al.*, 2016). Thus, there is probably some interplay between the two central regulators (i.e. PhcA and EfpR) that is mediated through ralfuranones. EPS biosynthesis represents a significant cost for *R. solanacearum* (Peyraud *et al.*, 2016) and PhcA controls multiple virulence functions encoded by hundreds of genes, including the *eps* gene cluster (Genin and Denny, 2012). The expression of *efpR*

in the *phc* QS-deficient mutants, Δ*phcB* and Δ*phcA*, not only Δ*ralA*, did not differ significantly from that in strain OE1-1 (Table S1). These results may lead to the complementation of *epsB* expression in the Δ*ralA* mutant, but not EPS I production by the Δ*ralA* strain, supplemented with individual ralfuranones.

Ralstonia solanacearum produces and extracellularly secretes ralfuranones. Ralfuranone I is a precursor for the other ralfuranones (Fig. S1) (Kai *et al.*, 2016; Pauly *et al.*, 2013), and is non-enzymatically converted into ralfuranone B in the supernatant (Kai *et al.*, 2016). The non-enzymatic elimination of benzaldehyde from ralfuranone B produces ralfuranone A, whereas ralfuranones J and K are the products of the enzymatic oxidation of ralfuranone B. Ralfuranone L is enzymatically synthesized from ralfuranone I. One obvious question is why does *R. solanacearum* produce so many types of ralfuranone. It is thought that the extracellular secretion of each ralfuranone by OE1-1 changes over time (Fig. S1; Kai *et al.*, 2016). Interestingly, the application of ralfuranone B and other ralfuranones led to recovered phenotypes of Δ*ralA*. Therefore, during the early stages of infection, 3-OH MAME-mediated intercellular signalling activates *phc* QS, leading to the production and secretion of ralfuranones. Each ralfuranone may then mediate intercellular signalling between OE1-1 cells in association with the feedback loop of the *phc* QS system. Overall, the integrated intracellular/intercellular signalling of OE1-1 cells via each ralfuranone, coupled with *phc* QS, may contribute to the elaborate and tunable regulation of *R. solanacearum* virulence (Fig. S3). This may enable *R. solanacearum* to infect many plant species and remain virulent during infections.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

We used the following *R. solanacearum* strains: OE1-1 (Kanda *et al.*, 2003), Δ*ralA* (i.e. ralfuranone-deficient mutant) (Kai *et al.*, 2014), *ralA*-comp (i.e. native *ralA*-expressing complemented Δ*ralA* mutant) (Kai *et al.*, 2014) and the *phc* QS-deficient mutants, Δ*phcB* (Kai *et al.*, 2015) and Δ*phcA* (Mori *et al.*, 2016). The *R. solanacearum* strains were routinely grown in one-quarter-strength M63 medium (Cohen and Rickenberg, 1956) 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.

Synthesis of ralfuranones

Ralfuranones A, B, J, K and L were synthesized and analysed as described previously (Kai *et al.*, 2014).

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

Ralstonia solanacearum cultures grown in B medium (Clough *et al.*, 1994) at 30 °C for 4–6 h were diluted in fresh medium (until $OD_{600} = 1.0$). A 50-μL aliquot of the cell suspension was transferred onto a Brilliant Green (BG) agar plate (diameter, 90 mm; capacity, 25 mL; Kai *et al.*, 2015) and incubated for 24 h at 30 °C. The BG agar was then cut into small pieces and soaked twice in ethyl acetate (50 mL) for 2 h each. The combined extracts were dried over Na_2SO_4 and concentrated. The residue was dissolved in 2 mL of ethyl acetate and analysed using a gas chromatography–mass spectrometry system (Kai *et al.*, 2015).

RNA extraction, elimination of ribosomal RNA and sequencing

Total RNA was isolated from *R. solanacearum* strains grown in one-quarter-strength M63 medium (until $OD_{600} = 0.3$) 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 (Gram-negative bacteria) (Illumina, Madison, WI, USA). Oriented paired-end RNA sequencing (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 paired-end sequencing of the libraries.

Mapping and analysis of RNA-seq data

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

qRT-PCR assay

A 500-ng total RNA template 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 S4, 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. 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). The relative gene expression quantities were calculated using the comparative cycle threshold method. All values were normalized against the *rpoD* expression level (i.e. internal standard for each cDNA sample). There were no significant differences in the *rpoD* expression level between *R. solanacearum* strains.

Bacterial cell aggregation assay

The aggregation of *R. solanacearum* cells was measured *in vitro* using a slightly modified polyvinylchloride (PVC) microtitre plate assay (O'Toole and Kolter, 1998). Briefly, 5 μL of overnight cultures of *R. solanacearum* adjusted to $OD_{600} = 0.005$ were used to inoculate 95 μL of one-quarter-strength M63 medium in the wells of a PVC 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 was 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 then distilled water. The remaining crystal violet in each well was solubilized with 100 μL of 100% ethanol, and then quantified by measurement of the absorbance at 550 nm. The resulting value was normalized according to the number of cells. This value was considered to represent the relative cell aggregation (OD_{550}/OD_{600}).

Measurement of EPS I production

Overnight cultures of *R. solanacearum* strains were washed with distilled water and then diluted to a cell density of 1.0×10^2 colony-forming units (CFU)/mL. A 100-μL aliquot of these cell suspensions was spread on one-quarter-strength M63 agar plates and incubated for 2 days at 30 °C. Cells were then resuspended to a concentration of 1.0×10^5 CFU/mL, and the cell density was confirmed through dilution plating. EPS was quantified using anti-*R. solanacearum* EPS antibodies (Agdia Inc., Elkhart, IN, USA) in an enzyme-linked immunosorbent assay (Agdia Inc.). The assay was conducted using a 100-μL cell suspension (1.0×10^4 CFU). Three technical replicates were assessed. The production of EPS I was quantified on the basis of the absorbance at 650 nm.

Swimming motility

Overnight cultures of *R. solanacearum* strains were washed with distilled water and then diluted to a cell density of 5.0×10^5 CFU/mL. For the swimming assay, 5-μL aliquots of cell suspensions were added to the centre of one-quarter-strength M63 medium solidified with 0.25% agar. Motility was examined using three plates per strain. All plates were incubated at 30 °C. The diameter of the swimming areas was measured at 48 h post-inoculation.

Nucleotide sequence accession numbers

The nucleotide sequences of the genes analysed by qRT-PCR were deposited in the DDBJ/GenBank/EMBL databases (Table S5, see Supporting Information).

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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 website:

Fig. S1 Schematic diagram of the quorum sensing and conversion of ralfuranone I to various ralfuranones in *Ralstonia solanacearum* strain OE1-1.

Fig. S2 Relationships between gene expression level changes in the *phcB*-deleted (Δ*phcB*, a), *phcA*-deleted (Δ*phcA*, b) and ralfuranone-deficient (Δ*ralA*) *Ralstonia solanacearum* mutants, and between expression level fold changes (i.e. $\geq$2 or $\leq$–2) of genes regulated by PhcB and PhcA in the Δ*phcA* and Δ*ralA* mutants (c). The FPKM (fragments per kilobase of exon per million fragments mapped) values for *R. solanacearum* strains OE1-1, Δ*phcB*, Δ*phcA* and Δ*ralA* were normalized prior to the analyses of differentially expressed genes.

Fig. S3 Model of the regulation of the *phc* quorum sensing (QS) system mediated through ralfuranones in *Ralstonia solanacearum* strain OE1-1.

Table S1 RNA-sequencing data for all transcripts in *Ralstonia solanacearum* strain OE1-1, *phcB*-deleted mutant (Δ*phcB*), *phcA*-deleted mutant (Δ*phcA*), and ralfuranones-deficient mutant (Δ*ralA*) grown in 1/4 × M63 medium.

Table S2 Predicted function of proteins encoded by genes whose expression is positively regulated by both the *phc* quorum sensing systems and ralfuranones in *Ralstonia solanacearum* strain OE1-1 grown in one-quarter-strength M63 medium.

Table S3 Predicted function of proteins encoded by genes whose expression is negatively regulated by both the *phc* quorum sensing systems and ralfuranones in *Ralstonia solanacearum* strain OE1-1 grown in one-quarter-strength M63 medium.

Table S4 Primers used in this study.

Table S5 Genes analysed by quantitative real-time polymerase chain reaction.