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Ralfuranones contribute to mushroom-type biofilm formation by *Ralstonia solanacearum* strain OE1-1

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SUMMARY

After invasion into intercellular spaces of tomato plants, the soil-borne, plant-pathogenic *Ralstonia solanacearum* strain OE1-1 forms mushroom-shaped biofilms (mushroom-type biofilms, mBFs) on tomato cells, leading to its virulence. The strain OE1-1 produces aryl-furanone secondary metabolites, ralfuranones (A, B, J, K and L), dependent on the quorum sensing (QS) system, with methyl 3-hydroxymyristate (3-OH MAME) synthesized by PhcB as a QS signal. Ralfuranones are associated with the feedback loop of the QS system. A ralfuranone productivity-deficient mutant (Δ*ralA*) exhibited significantly reduced growth in intercellular spaces compared with strain OE1-1, losing its virulence. To analyse the function of ralfuranones in mBF formation by OE1-1 cells, we observed cell aggregates of *R. solanacearum* strains statically incubated in tomato apoplast fluids on filters under a scanning electron microscope. The Δ*ralA* strain formed significantly fewer microcolonies and mBFs than strain OE1-1. Supplementation of ralfuranones A, B, J and K, but not L, significantly enhanced the development of mBF formation by Δ*ralA*. Furthermore, a *phcB*- and *ralA*-deleted mutant (Δ*phcB*/*ralA*) exhibited less formation of mBFs than OE1-1, although a QS-deficient, *phcB*-deleted mutant formed mBFs similar to OE1-1. Supplementation with 3-OH MAME significantly reduced the formation of mBFs by Δ*phcB*/*ralA*. The application of each ralfuranone significantly increased the formation of mBFs by Δ*phcB*/*ralA* supplied with 3-OH MAME. Together, our findings indicate that ralfuranones are implicated not only in the development of mBFs by strain OE1-1, but also in the suppression of QS-mediated negative regulation of mBF formation.

Keywords: mushroom-type biofilm, ralfuranones, Ralstonia solanacearum, virulence.

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INTRODUCTION

Bacterial wilt caused by *Ralstonia solanacearum* is one of the most devastating bacterial plant diseases in the tropics, subtropics and warm temperature regions worldwide (Mansfield *et al.*, 2012). *Ralstonia solanacearum* is a soil-borne bacterium that normally invades plant roots from the soil through wounds or natural openings where secondary roots emerge (Araud-Razou *et al.*, 1998), colonizes intercellular spaces of the root cortex and vascular parenchyma, and eventually enters xylem vessels and spreads up into the stems and leaves through the xylem (Hikichi, 2016; Hikichi *et al.*, 2017; Vasse *et al.*, 1995). This colonization in intercellular spaces is required for the virulence of the wild-type strain of *R. solanacearum* (OE1–1) (Hikichi, 2016; Hikichi *et al.*, 1999, 2017; Kanda *et al.*, 2003, 2008; Mori *et al.*, 2016; Shinohara *et al.*, 2005). Following the invasion of the intercellular spaces of tomato plants, strain OE1–1 forms mushroom-shaped biofilms (mushroom-type biofilms, mBFs) on the surfaces of tomato cells adjacent to intercellular spaces, leading to the colonization of intercellular spaces (Hikichi, 2016; Hikichi *et al.*, 2017; Mori *et al.*, 2016). Therefore, mBF formation on tomato cells after invasion of the intercellular spaces is essential for the virulence of *R. solanacearum* on tomato plants. Interestingly, the observation of cell aggregates of *R. solanacearum* strains incubated on filters under a scanning electron microscope (SEM) suggests that the apoplast fluids from tomato plants are better than xylem fluids for mBF formation by strain OE1–1 (Mori *et al.*, 2016).

Cells of many bacteria communicate with each other by releasing, sensing and responding to small diffusible signal molecules, allowing them to regulate their cooperative activities and physiological processes through quorum sensing (QS; Li and Tian, 2012). The ability of bacteria to communicate and behave as a group for social interactions, similar to a multicellular organism, provides significant benefits to bacteria in terms of host colonization, formation of biofilms, defence against competitors and adaptation to changing environments. Bacterial biofilms often consist of mushroom-shaped complex multicellular structures (Klausen *et al.*, 2003), similar to mBFs produced by *R. solanacearum* strain OE1–1 (Mori *et al.*, 2016). Bacterial surface components and extracellular compounds, such as flagella, lipopolysaccharides and exopolysaccharides (EPSs), in combination with environmental and QS signals, are crucial for cell aggregation and biofilm development in most bacterial species (Schembri *et al.*, 2001). Furthermore, many pathogenic bacteria use QS-controlled cell–cell signalling to regulate the expression of factors contributing to virulence (Ham, 2013).

Ralstonia solanacearum strains AW1 and K60 produce methyl 3-hydroxypalmitate (3-OH PAME) as a QS signal in *phc* QS (Flavier *et al.*, 1997; Kai *et al.*, 2015). However, strains OE1–1 and GMI1000 produce methyl 3-hydroxymyristate (3-OH MAME), but not 3-OH PAME, as a QS signal (Kai *et al.*, 2015). Both QS signals are synthesized by PhcB, a putative methyltransferase (Flavier *et al.*, 1997; Kai *et al.*, 2015). When 3-OH PAME or 3-OH MAME reaches a threshold level, it induces the ability of the histidine kinase PhcS to phosphorylate the response regulator PhcR, resulting in elevated levels of functional PhcA, a LysR-type transcriptional regulator, which plays a central role in *phc* QS as the global virulence regulator (Genin and Denny, 2012). Differences of the deduced amino acid sequences of PhcB and PhcS among *R. solanacearum* strains correspond to the productivity of these QS signals (Kai *et al.*, 2015).

Ralstonia solanacearum synthesizes aryl-furanone secondary metabolites, known as ralfuranones A, B, I, J, K and L (Fig. S1, see Supporting Information), which are secreted extracellularly (Kai *et al.*, 2014, 2016; Pauly *et al.*, 2013). Ralfuranone I is a precursor of the other ralfuranones. The expression of the ralfuranone synthase, encoded by *ralA*, is dependent on activated PhcA, and is involved in the biosynthesis of ralfuranones (Kai *et al.*, 2014, 2016; Schneider *et al.*, 2009; Wackler *et al.*, 2011). Ralfuranones are implicated in the feedback loop of *phc* QS (Mori *et al.*, 2017). Furthermore, ralfuranones are implicated in the expression of *vsrAD* and *vsrBC* encoding the two-component regulatory system, which is involved in the adaptation of the bacterium to a specific niche or environmental condition during the pathogen life cycle. From this evidence, it has been proposed that the integrated intracellular/intercellular signalling of OE1–1 cells via each of the ralfuranones, coupled with *phc* QS, contributes to the elaborate and tunable regulation of its virulence. Therefore, ralfuranones are needed for the full virulence of *R. solanacearum* when directly inoculated into xylem vessels of tomato plants (Kai *et al.*, 2014). Although ralfuranones are implicated in the productivity of the major EPS, EPS I, and cell aggregation by strain OE1–1 (Mori *et al.*, 2017), the involvement of ralfuranones in mBF formation after invasion of intercellular spaces, which is required for *R. solanacearum* virulence, remains unknown.

To elucidate how ralfuranones influence the colonization of OE1–1 after the invasion of intercellular spaces, we first analysed the colonization of the ralfuranone-deficient mutant Δ*ralA* in roots and its virulence on tomato plants inoculated using the root dipping method. Because these analyses implicated ralfuranones in the colonization of OE1–1 in intercellular spaces, we then analysed mBF formation by *R. solanacearum* strains incubated in tomato apoplast fluids on filters using a SEM.

RESULTS

Δ*ralA* loses its virulence on tomato plants

We inoculated 5-week-old tomato plants with *R. solanacearum* strain OE1–1, the ralfuranone productivity-deficient Δ*ralA* mutant (Kai *et al.*, 2014) and the native *ralA*-complemented Δ*ralA* strain *ralA*-comp (Kai *et al.*, 2014) by root dipping. The number of Δ*ralA* mutants in roots at 1 day post-inoculation was not significantly different from that of OE1–1 ($P > 0.05$, Fig. 1a). However, the population of Δ*ralA* at days 2 and 3 was significantly smaller than that of OE1–1 ($P < 0.05$). The population of *ralA*-comp was not significantly different from that of OE1–1 ($P > 0.05$).

In a plate-printing assay, OE1–1 and *ralA*-comp were detected in both inoculated roots and stems of tomato plants, whereas Δ*ralA* was not detected beyond the inoculated roots (Fig. 1b).

Δ*ralA* lost its virulence on tomato plants inoculated by root dipping, whereas the complemented mutant *ralA*-comp showed virulence levels similar to those of OE1–1 (Fig. 1c).

In vitro mBF formation by *R. solanacearum* strain OE1–1

Previously, we have observed mBFs produced by *R. solanacearum* strain OE1–1 incubated in tomato apoplast fluids on filters under a SEM (Mori *et al.*, 2016). Using this system, we first analysed cell aggregates produced by *R. solanacearum* strain OE1–1 incubated for 24 h (Fig. S2a, see Supporting Information). *Ralstonia solanacearum* strain OE1–1 produced microcolonies (Fig. S2b), immature mBFs (Fig. S2c) and mature mBFs (Fig. S2d). We then compared the numbers of microcolonies, immature mBFs and mature mBFs of OE1–1 cells incubated in tomato apoplast fluid on filters for 24 or 32 h. The diameters of the cell aggregates of *R. solanacearum* strains were assayed using Nodame (Tanabata *et al.*, 2014). The number of microcolonies of OE1–1 cells incubated for 32 h was increased significantly compared with that for 24 h ($P < 0.05$, Fig. 2). Although the numbers of immature mBFs and mature mBFs of OE1–1 cells incubated for 32 h were increased slightly compared with those for 24 h, there was no significant difference between them ($P > 0.05$). Although we observed collapsing mBFs when incubated for 42 h, no collapsing mBFs were observed when incubated for both 24 and 32 h (data not shown). As a result of this

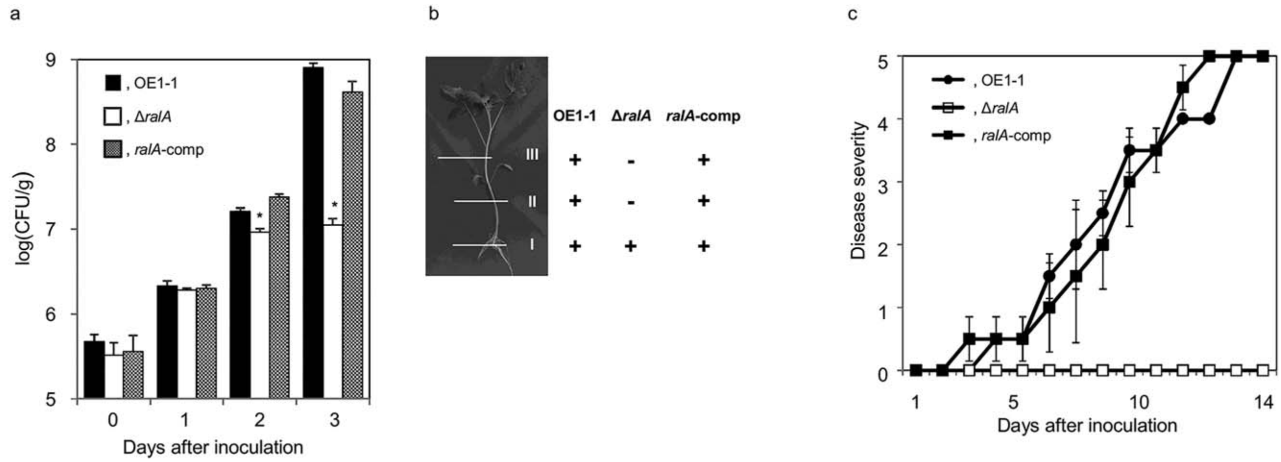

Fig. 1 Behaviour and virulence of *Ralstonia solanacearum* strain OE1–1, the ralfuranone-deficient mutant Δ*ralA* and the complemented Δ*ralA* mutant *ralA*-comp in tomato plants. (a) The population of *R. solanacearum* strains in the roots of tomato plants inoculated by root dipping was analysed using Hara–Ono medium. CFU, colony-forming unit. (b) *Ralstonia solanacearum* strains in roots (I) and stems (II and III) of tomato plants at 10 days after inoculation by root dipping were detected using the plate-printing assay. (c) Bacterial wilt on tomato plants inoculated with *R. solanacearum* strains by root dipping was assayed. Plants were rated on a 0–5 disease index scale: 0, no wilting; 1, 1%–25% wilting; 2, 26%–50% wilting; 3, 51%–75% wilting; 4, 76%–99% wilting; 5, dead. Bars indicate standard errors. Asterisks indicate a significant difference from the wild-type ($P < 0.05$, *t*-test).

work, we chose to investigate mBF formation in apoplast fluids on filters after 24 h.

Δ*ralA* exhibits attachment ability, similar to OE1–1

During biofilm formation, cells of strain OE1–1 first attach to the surface and then produce flat microcolonies followed by mBFs (Mori *et al*., 2016). We observed the attachment ability of strains incubated in tomato apoplast fluids under a phase contrast microscope. OE1–1 cells attached to glass slides (Fig. S3a, see Supporting Information). The attachment of the Δ*ralA* mutant to glass slides (Fig. S3b) was similar to that of OE1–1 and *ralA*-comp (Fig. S3c).

Ralfuranones are involved in mBF formation by *R. solanacearum* strain OE1–1 incubated in tomato apoplast fluids

The Δ*ralA* mutant formed significantly fewer microcolonies (Fig. 3a) and immature mBFs (Fig. 3b) than OE1–1 and *ralA*-comp ($P < 0.05$; Table S1, see Supporting Information). The Δ*ralA* mutant formed few mature mBFs (Fig. 3c). However, the numbers of mature mBFs produced by the *ralA*-comp strain did not differ significantly from those produced by OE1–1 ($P > 0.05$, Fig. 3a, Table S1).

Ralfuranones A, B, J, K and L at a concentration of 20 μM lead to the recovery of cell aggregation by the Δ*ralA* mutant (Mori *et al*., 2017). We analysed mBF formation by Δ*ralA* supplemented with ralfuranones A, B, J, K or L at a concentration of 20 μM. The application of ralfuranones A, B, J, K or L did not lead to significantly increased microcolony formation by Δ*ralA* ($P > 0.05$, Fig. 3a, Table S1). However, the application of ralfuranones A, B, J or K resulted in significantly increased formation of both immature mBFs ($P < 0.05$, Fig. 3b, Table S1) and mature mBFs ($P < 0.05$, Fig. 3c, Table S1) by the Δ*ralA* strain, whereas 20 μM of ralfuranone L increased slightly the formation of mature mBFs by the Δ*ralA* strain (Fig. 3c, Table S1).

A *phc* QS-deficient mutant (*phcB*-deleted mutant Δ*phcB*) retains its ability to produce mBFs

Ralfuranones are implicated in the feedback loop of *phc* QS (Mori *et al*., 2017). We therefore analysed the formation of mBFs by a

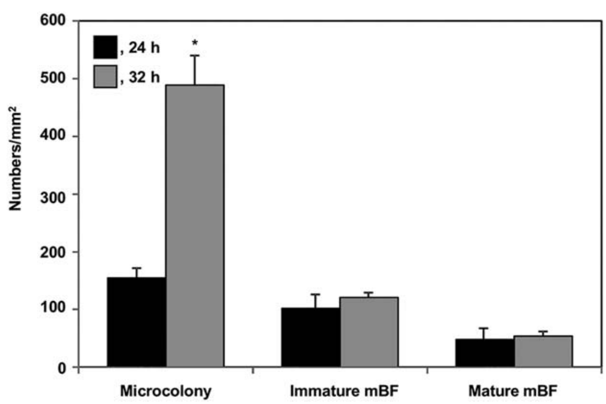

Fig. 2 The numbers of microcolonies of 1–5 μm in diameter, immature mushroom-type biofilms (mBFs) of 5–10 μm in diameter and mature mBFs of >10 μm in diameter produced by cells of *Ralstonia solanacearum* strain OE1–1 incubated in tomato apoplast fluid on nano-percolators for 24 or 32 h. Bars indicate the standard errors. Asterisks indicate a significant difference from OE1–1 incubated for 24 h ($P < 0.05$, *t*-test).

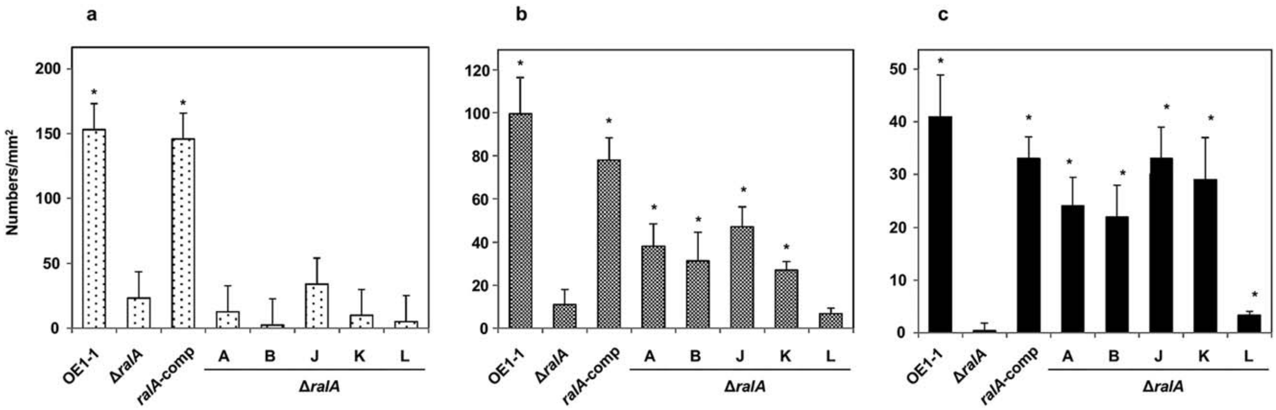

Fig. 3 Influence of ralfuranones on the formation of microcolonies of 1–5 μm in diameter (a), immature mushroom-type biofilms (mBFs) of 5–10 μm in diameter (b) and mature mBFs of >10 μm in diameter (c) by *Ralstonia solanacearum* strains. Cells of *R. solanacearum* strain OE1–1, the ralfuranone-deficient mutant (Δ*ralA*), with or without application of ralfuranones A, B, J, K or L at concentrations of 20 μM, and the complemented Δ*ralA* mutant (*ralA*-comp) incubated in tomato apoplast fluid on nano-percolators were observed under a scanning electron microscope. Bars indicate the standard errors. Asterisks indicate a significant difference from Δ*ralA* ($P < 0.05$, *t*-test).

phc QS-deficient mutant (*phcB*-deleted mutant Δ*phcB*) (Kai *et al*., 2015). The numbers of microcolonies (Fig. 4a), immature mBFs (Fig. 4b) and mature mBFs (Fig. 4c) produced by the Δ*phcB* mutant did not differ significantly from those produced by OE1–1 ($P > 0.05$; Table S2, see Supporting Information). However, a *phcB*- and *ralA*-deleted mutant (Δ*phcB*/*ralA*) produced significantly fewer microcolonies and mature mBFs than strains OE1–1 and Δ*phcB* ($P < 0.05$).

The application of 3-OH MAME at a concentration of 100 nM leads to the recovery of the activity of *phc* QS by Δ*phcB* (Kai *et al*., 2015). We analysed the formation of mBFs by the Δ*phcB*/*ralA* mutant supplied with 3-OH MAME at a concentration of 100 nM. The application of 3-OH MAME resulted in a significantly reduced formation of microcolonies (Fig. 4a), immature mBFs (Fig. 4b) and mature mBFs (Fig. 4c) by Δ*phcB*/*ralA* ($P < 0.05$, Table S2).

We then analysed the formation of mBFs by the Δ*phcB*/*ralA* mutant supplied with 3-OH MAME at a concentration of 100 nM and ralfuranones A, B, J, K or L at a concentration of 20 μM. The additional application of each ralfuranone significantly enhanced the formation of microcolonies (Fig. 5a), immature mBFs (Fig. 5b) and mature mBFs (Fig. 5c) by Δ*phcB*/*ralA* supplied with 3-OH MAME ($P < 0.05$; Table S3, see Supporting Information). In particular, Δ*phcB*/*ralA* supplied with both 3-OH MAME and ralfuranones J or K formed significantly more mBFs than strain OE1–1 ($P < 0.05$).

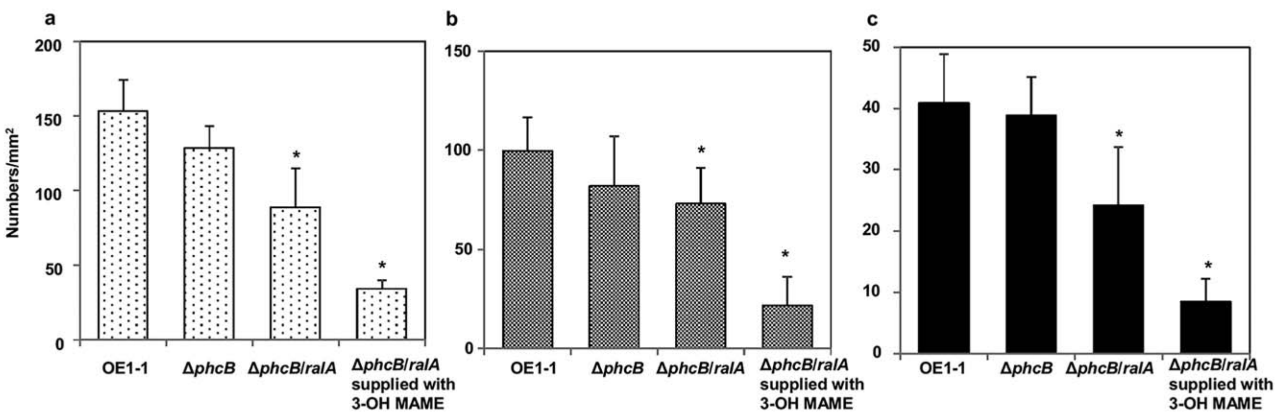

Fig. 4 Influence of *phc* quorum sensing (QS) on the formation of microcolonies of 1–5 μm in diameter (a), immature mushroom-type biofilms (mBFs) of 5–10 μm in diameter (b) and mature mBFs of >10 μm in diameter (c) by *Ralstonia solanacearum* strains. Cells of *R. solanacearum* strain OE1–1, the *phc* QS-deficient mutant (Δ*phcB*), and the *phcB*- and *ralA*-deficient mutant (Δ*phcB*/*ralA*), with or without application of methyl 3-hydroxymyristate (3-OH MAME) at a concentration of 100 nM, incubated in tomato apoplast fluid on nano-percolators were observed under a scanning electron microscope. Bars indicate the standard errors. Asterisks indicate a significant difference from Δ*ralA* ($P < 0.05$, *t*-test).

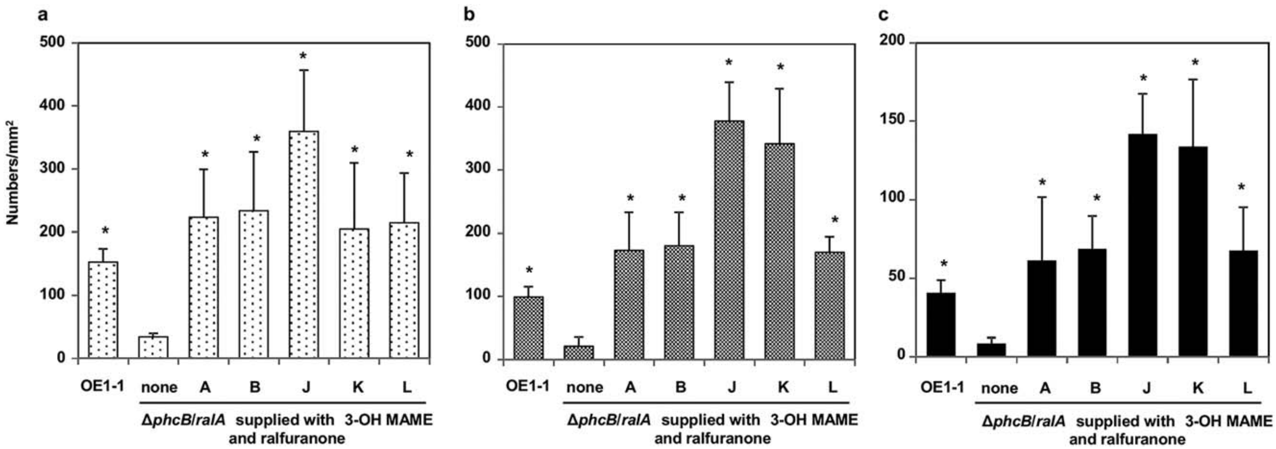

Fig. 5 Effect of *phc* quorum sensing and ralfuranones on the formation of microcolonies of 1–5 μm in diameter (a), immature mushroom-type biofilms (mBFs) of 5–10 μm in diameter (b) and mature mBFs of >10 μm in diameter (c) by *Ralstonia solanacearum* strains. Cells of *R. solanacearum* strain OE1–1 and the *phcB*- and *ralA*-deficient mutant Δ*phcB*/*ralA*, with application of methyl 3-hydroxymyristate (3-OH MAME) at a concentration of 100 nM and with or without application of ralfuranones A, B, J, K or L at a concentration of 20 μM, incubated in apoplast fluids from tomato plants on nano-percolators were observed under a scanning electron microscope. Bars indicate the standard errors. Asterisks indicate a significant difference from Δ*phcB*/*ralA* supplemented with 3-OH MAME ($P < 0.05$, *t*-test).

EPS I productivity-deficient mutant retains its ability to produce mBFs, but not cell aggregation

It has been proposed that EPS I is involved in biofilm formation by *R. solanacearum* (Genin and Denny, 2012). We assayed cell aggregation by the Δ*phcB* mutant and an EPS I productivity-deficient mutant (Δ*epsB*), which exhibited significantly less EPS I productivity ($P < 0.05$, Fig. 6a). Both Δ*epsB* and Δ*phcB* exhibited significantly reduced cell aggregation compared with strain OE1–1 ($P < 0.05$, Fig. 6b). The means of the optical density at 600 nm (OD_{600}) of strain OE1–1, Δ*epsB* and Δ*phcB* cultures were 0.75, 0.69 and 0.72, respectively, and there was no significant difference between them ($P > 0.05$).

We then analysed the formation of mBFs by Δ*epsB*. Δ*epsB* produced significantly more microcolonies (Fig. 7a) and immature mBFs (Fig. 7b) than strain OE1–1 ($P < 0.05$). However, Δ*epsB* produced mature mBFs at a similar level to strain OE1–1 (Fig. 7c).

DISCUSSION

After colonization of the intercellular spaces of roots, *R. solanacearum* enters xylem vessels and spreads up into the

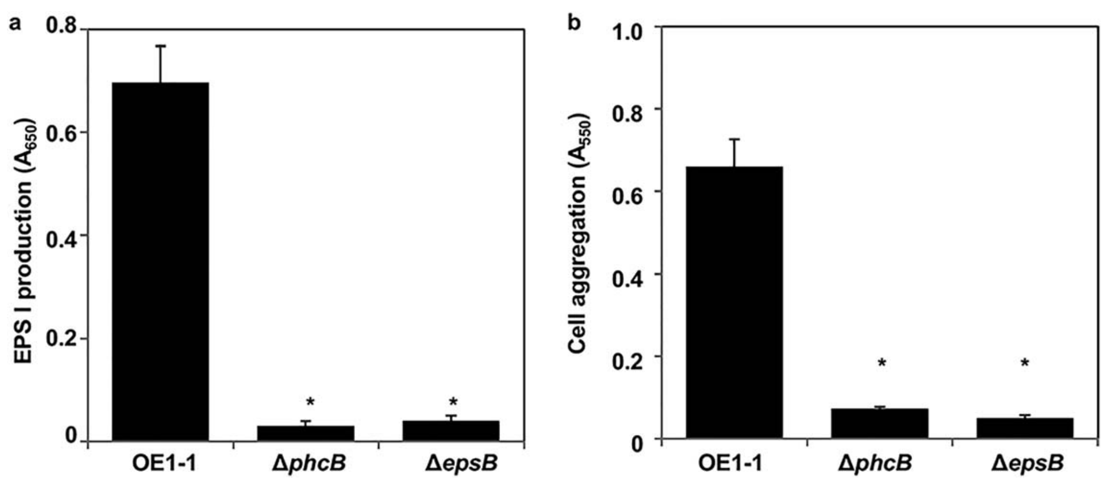

Fig. 6 Production of exopolysaccharide I (EPS I) and cell aggregation by the *epsB*-deleted Δ*epsB* mutant of *Ralstonia solanacearum*. (a) Immunological quantification of EPS I in the supernatants of the *phc* quorum sensing (QS)-deficient mutant Δ*phcB* and Δ*epsB* of *R. solanacearum* was analysed using enzyme-linked immunosorbent assay (ELISA) with anti-*R. solanacearum* EPS I antibodies. EPS I productivity was quantified by the absorbance at 650 nm (A_{650}). (b) Cell aggregation by *R. solanacearum* strains incubated in 1/4 × M63 medium in polyvinylchloride (PVC) plate wells was stained with crystal violet and its absorbance at 550 nm (A_{550}) was assayed. Bars indicate the standard errors. Asterisks indicate significant difference from wild-type strain OE1–1 ($P < 0.05$, *t*-test).

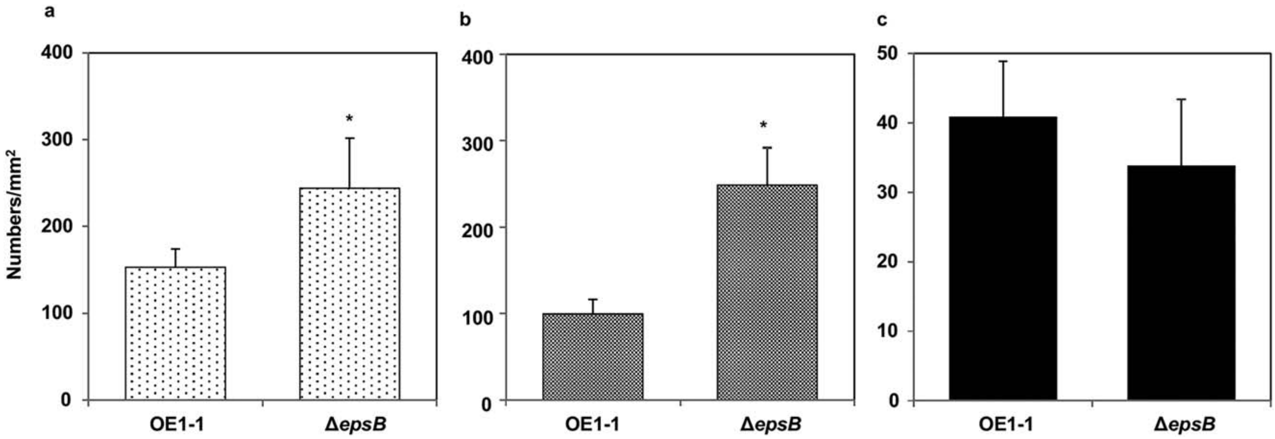

Fig. 7 Influence of exopolysaccharide I (EPS I) on the formation of microcolonies of 1–5 μm in diameter (a), immature mushroom-type biofilms (mBFs) of 5–10 μm in diameter (b) and mature mBFs at >10 μm in diameter (c) by *Ralstonia solanacearum* strains. Cells of *R. solanacearum* strains OE1–1 and the *epsB*-deleted Δ*epsB* mutant incubated in apoplast fluids from tomato plants on nano-percolators were observed under a scanning electron microscope. Bars indicate the standard errors. Asterisks indicate a significant difference from Δ*phcB*/*ralA* supplemented with methyl 3-hydroxymyristate (3-OH MAME) ($P < 0.05$, *t*-test).

stems and leaves through the xylem (Hikichi, 2016; Hikichi *et al.*, 2017; Vasse *et al.*, 1995), where its high level of multiplication leads to wilting symptoms, as a result of the reduced sap flow caused by the presence of a large number of bacterial cells and EPS slime produced by the bacteria in some xylem vessels (Genin and Denny, 2012). Therefore, it has been proposed that EPS is an important virulence factor. The production of the major EPS, EPS I, is positively regulated by *phc* QS. Furthermore, ralfuranones are positively regulated by *phc* QS and are implicated in the feedback loop of *phc* QS (Mori *et al.*, 2017), positively regulating EPS I production. Therefore, when directly inoculated into xylem vessels of tomato plants, ralfuranones contribute to the full virulence of OE1–1 (Kai *et al.*, 2014). In addition, the current study has shown the involvement of extracellularly produced ralfuranones in the colonization of the intercellular spaces of tomato plants by OE1–1 (Fig. 1a), leading to systemic infection (Fig. 1b). Therefore, ralfuranones are required for OE1–1 virulence on tomato plants inoculated through roots (Fig. 1c). Furthermore, the observation of mBF formation by *R. solanacearum* strains using the *in vitro* mBF formation system showed the involvement of ralfuranones in the development of mBFs (Fig. 3), but not in the attachment of strain OE1–1 cells (Fig. S3). Therefore, the many functions of ralfuranones in the infection route of OE1–1 may lead to the formation of mature mBFs on host cells after invasion into intercellular spaces, systemic infectivity and bacterial cell aggregation in xylem vessels, contributing to OE1–1 virulence.

Ralstonia solanacearum produces a consortium of plant cell wall-degrading enzymes, e.g. β-1,4-endoglucanase, a β-1,4-cellobiohydrolase and a pectin methyl esterase, the production of which is positively regulated through *phc* QS (González and Allen, 2003; Huang and Allen, 1997; Tans-Kersten *et al.*, 1998). The production and extracellular secretion of these enzymes through the type II secretion system are required for invasion into xylem vessels, leading to virulence (Liu *et al.*, 2005; Tsujimoto *et al.*, 2008). The results in this study showed the involvement of ralfuranones in mBF formation (Fig. 3). Furthermore, Δ*ralA* lost its systemic infectivity (Fig. 1b), suggesting that ralfuranone production may be involved in OE1–1 invasion into xylem vessels. Mature mBFs produced by strain OE1–1 can dissolve, releasing cells from the mBF (Mori *et al.*, 2016). It is thus thought that OE1–1 cells released from mature mBFs can invade xylem vessels.

EPSs are required for autoaggregation and biofilm development in most bacterial species (Bogino *et al.*, 2013). The expression of the *eps* operon, including *epsB*, is positively regulated by *phc* QS, producing the major EPS, EPS I, dependent on *phc* QS by *R. solanacearum* (Genin and Denny, 2012). Assays of cell aggregation by *R. solanacearum* strains showed that EPS I produced dependently on *phc* QS is required for cell aggregation by strain OE1–1 (Fig. 6). Interestingly, deficiency of EPS I productivity by *epsB* deletion led to the enhanced formation of microcolonies and immature mBFs, but did not affect significantly the formation of mature mBFs (Fig. 7). It is thus thought that EPS I may be directly involved in cell aggregation but not the development of mature mBFs by strain OE1–1 (Fig. S4, see Supporting Information).

The application of 3-OH MAME led to the recovery of the *phc* QS-dependent phenotypes, such as EPS production and ralfuranone productivity, by Δ*phcB* (Kai *et al.*, 2015). The reduced formation of mBFs by Δ*phcB*/*ralA* relative to strains OE1–1 and Δ*phcB*, and the significantly reduced formation of mBFs by Δ*phcB*/*ralA* with application of 3-OH MAME, suggest that *phc* QS may negatively regulate mBF formation in the absence of ralfuranones (Fig. 4; Table S1). The formation of immature and mature mBFs by Δ*ralA* supplemented with ralfuranone A, B, J or K (Fig. 5b,c) suggests the involvement of these ralfuranones in the development of mBF formation (Fig. S3; Table S2). Furthermore, the co-application of ralfuranone A, B, J, K or L increased the formation of not only mBFs, but also microcolonies, by Δ*phcB*/*ralA* supplemented with 3-OH MAME (Fig. 5; Table S2), suggesting that ralfuranones A, B, J, K and L (especially J and K) may suppress the *phc* QS-mediated negative regulation of mBF formation. Ralfuranone I is non-enzymatically converted into ralfuranone B (Fig. S1; Kai *et al*., 2016). The non-enzymatic elimination of benzaldehyde from ralfuranone B produces ralfuranone A, and ralfuranones J and K are the products of the enzymatic oxidation of ralfuranone B. It is thought that a change in production of ralfuranones J and K from ralfuranone B by OE1–1 may especially contribute to all stages during mBF formation.

QS is the regulation system for gene expression in response to changes in cell population density (Miller and Bassler, 2001). Bacteria produce and release QS signals that increase in concentration as a function of cell density. The detection of a minimal threshold stimulatory concentration of a QS signal leads to a change in gene expression. Ralfuranones, whose production is induced through *phc* QS by strain OE1–1 (Kai *et al*., 2014), affect the *phc* QS feedback loop (Mori *et al*., 2017). Biofilm formation is a temporal process for Gram-negative proteobacteria, in which a transition through distinct stages of multicellular organization is involved. During biofilm formation, planktonic bacteria first attach to surfaces and grow there (Monds and O'Toole, 2009). The clonal growth of attached cells or the active translocation of cells across the surface leads to microcolony formation. Microcolonies grow in size and coalesce, forming biofilms. Mature biofilms are then dissolved, releasing planktonic cells. QS functioning during microcolony formation is required for the formation of mature biofilms (Bogino *et al*., 2013; Rinaudi and Giordano, 2010). In *R. solanacearum*, immediately after invasion into the intercellular spaces, the expression of *lecM* encoding a lectin, RS-IIL, is positively regulated by HrpG (Mori *et al*., 2016; Sudakevitz *et al*., 2004; Valls *et al*., 2006). RS-IIL is required for the attachment ability of strain OE1–1 to the surface of plant cells, leading to the formation of mBFs (Mori *et al*., 2016). In this study, ralfuranones A, B, J and K, whose production is induced through *phc* QS, contributed to both the formation of microcolonies and the development of mBFs (Fig. 3). Furthermore, strain OE1–1 formed mature mBFs during incubation for 24 h (Fig. 2). Therefore, integrated signalling via not only RS-IIL, but also ralfuranones, whose production is dependent on *phc* QS, is implicated in mBF formation by *R. solanacearum* strain OE1–1 (Fig. S4).

Apoplast fluids are preferential for mBF formation by strain OE1–1 (Mori *et al*., 2016). This mBF formation is independent of *phc* QS-dependent aggregation by OE1–1 cells (Fig. 4; Table S1). Interestingly, RS-IIL, whose production is induced by PhcA functioning at an OE1–1 cell density of more than 10^7 colony forming units (CFU)/mL, is also implicated in EPS I production, leading to aggregation by OE1–1 cells (Mori *et al*., 2016). Furthermore, the *lecM*-deleted mutant exhibits reduced virulence when inoculated directly into xylem. RS-IIL is reportedly involved in cell aggregation by *R. solanacearum* strain UW551 in the xylem vessels of tomato plants, leading to its full virulence (Meng *et al*., 2015). Taken together, these results indicate that the integrated intracellular/intercellular signalling of *R. solanacearum* cells via each ralfuranone and RS-IIL, coupled with *phc* QS, is implicated in the regulation of mBF formation after invasion into intercellular spaces and aggregation in xylem vessels by *R. solanacearum* cells (Fig. S4).

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Ralstonia solanacearum* strains were routinely grown in 1/4 × M63 medium (Cohen and Rickenberg, 1956) at 30 °C, and *Escherichia coli* strains were grown in Luria–Bertani (LB) medium (Hanahan, 1983) at 37 °C. The following antibiotics were used in selective media in the amounts indicated (μg/mL): ampicillin (Amp), 75; gentamycin, 25; kanamycin (Km), 50.

General DNA manipulations

The isolation of genomic DNA, plasmid DNA manipulations and polymerase chain reaction (PCR) were performed using standard techniques (Sambrook *et al*., 1989). *Ralstonia solanacearum* was transformed by electroporation as described previously (Mori *et al*., 2016). Double-stranded DNA sequencing templates were prepared with Fast Gene™ Plasmid miniprep kits (NIPPON Genetics, Tokyo, Japan) and sequences were determined using an Automated DNA Sequencer Model 373 (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). DNA sequence data were analysed using DNASYS-Mac software (Hitachi Software Engineering, Yokohama, Japan). Enzymes, including restriction endonucleases (Takara Bio, Ohtsu, Japan), were used according to the manufacturer's instructions.

Creation of an *epsB*-deleted mutant Δ*epsB*

A 716-bp DNA fragment (epsB-1) was PCR amplified from the genomic DNA of OE1–1 with primers epsB-1-FW (5′-TCCGATCCGCGCAATGTC-3′) and epsB-1-RV (5′-GCAGCCGCTACATCAGGGTCGTATTCCGTG-3′). A 745-bp DNA fragment (epsB-2) was PCR amplified from the genomic DNA of OE1–1 with primers epsB-2-FW (5′-GACCCTGATGTAGCGGCTG-CAGCGCGGC-3′) and epsB-2-RV (5′-GCCGGCACGCCTTCATTG-3′). Using epsB-1 and epsB-2 as templates, a 1441-bp DNA fragment was PCR amplified with primers epsB-1-FW and epsB-2-RV, and then cloned into pMD20 (Takara Bio) to create pMD20epsB. The pMD20epsB construct was digested with *Bam*HI and *Hin*dIII to release a 1.4-kbp fragment, which was ligated into the *Bam*HI and *Hin*dIII sites of pK18mobsacB (Kvitko and Collmer, 2011) to create pDepsB. This plasmid was electroporated into OE1–1 cells, and kanamycin-resistant and sucrose-sensitive recombinants were selected. Recombinants were incubated in PY medium [polypeptone (0.5%), yeast extract (0.2%)] for 6 h, and a kanamycin-

Table 1 Strains and plasmids used in this study.

	Relevant characteristics	Source
Plasmids used for cloning		
pMD20	pUC19 derivative, Amp^r	Takara Bio
pMD20epsB	pMD20 derivative carrying a 1441-bp DNA fragment for *epsB* deletion, Amp^r	This study
pK18mobsacB	Km^r, *oriT* (RP4), *sacB*, *lacZα*	Kvitko and Collmer (2011)
pΔralA	pK18mobsacB derivative carrying 1759-bp DNA fragment for *ralA* deletion, Km^r	Kai *et al.* (2014)
pDepsB	pK18mobsacB derivative carrying a 1441-bp DNA fragment for *epsB* deletion	This study
Escherichia coli strain		
DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17supE44* Δ(*lac*)*U169*(ϕ*80lac*ΔM15)	Takara Bio
Ralstonia solanacearum strains		
OE1–1	Wild-type strain, phylotype I, race 1, biovar 4	Hikichi *et al.* (1999)
Δ*ralA*	*ralA*-deleted mutant of OE1–1	Kai *et al.* (2014)
ralA-comp	A transformant of Δ*ralA* with pCralA containing native *ralA*	Kai *et al.* (2014)
Δ*phcB*	*phcB*-deleted mutant of OE1–1	Kai *et al.* (2015)
Δ*phcB*/Δ*ralA*	*ralA*-deleted mutant of Δ*phcB*	This study
Δ*epsB*	*epsB*-deleted mutant of OE1–1	This study

sensitive and sucrose-resistant recombinant, Δ*epsB*, was selected. DNA for sequencing was PCR amplified with primers epsB-SQ-FW3 (5′-CAGCAAGGTCTTCGTGACCG-3′) and epsB-SQ-RV3 (5′-CCGATGCGATACGGGATCAG-3′) to verify the correct substitution of the deleted *epsB* in OE1–1 (data not shown).

Creation of a *phcB*- and *ralA*-deleted Δ*phcB*/*ralA* mutant

The plasmid pΔ*ralA* (Kai *et al.*, 2014) was electroporated into the *phcB*-deleted Δ*phcB* mutant (Kai *et al.*, 2015), and kanamycin-resistant and sucrose-sensitive recombinants were selected. Recombinants were incubated in PY medium for 6 h, and a kanamycin-sensitive and sucrose-resistant recombinant, Δ*phcB*/*ralA*, was selected. DNA for sequencing was PCR amplified with primers ral-SQ-FW3 (5′-TGCGTTAGGCATGGTTTGG-3′) and ral-SQ-RV3 (5′-TTGTAGGCGTTCATGGTCC-3′) to verify the correct substitution of the deleted *ralA* in Δ*phcB* (data not shown).

Synthesis of ralfuranones and 3-OH MAME

Ralfuranones A, B, J, K and L (Kai *et al.*, 2014, 2016) and 3-OH MAME (Kai *et al.*, 2015) were synthesized in previous studies.

Attachment ability assay

Glass slides were soaked in suspensions of *R. solanacearum* strains at 1.0×10^8 CFU/mL in Petri dishes (diameter, 9 cm). After incubation for 24 h at 30 °C, the glass slides were washed twice with deionized water and observed under a phase contrast microscope (FSX-100, Olympus, Tokyo, Japan) (Mori *et al.*, 2016).

Observation of *R. solanacearum* cells incubated on nano-percolators under a SEM

We dropped overnight cultures of *R. solanacearum* strains adjusted to $OD_{600} = 0.005$ (10 μL) onto 190 μL of medium on nano-percolator filters (JEOL, Tokyo, Japan) and incubated them without shaking at 30 °C (Mori *et al.*, 2016). After removal of the supernatant, we added 100 μL of ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS) to the bacteria on the nano-percolator filters and fixed the bacteria at room temperature for 1 h. The fixed bacteria were washed twice with deionized water and then dried at room temperature. The bacteria on the nano-percolator filters were vacuum deposited with gold using an ion sputter coater (Hitachi E-1010, Hitachi High-Technologies, Tokyo, Japan) according to the manufacturer's instructions. The sample on the filter was mounted directly on the specimen holder and examined using a Miniscope® TM-3000 SEM (Hitachi High-Technologies Corporation) with a magnification of 250. The diameter of cell aggregates produced by *R. solanacearum* strains in an area of 0.3267 mm^2 was then assayed using Nodame (Nodule Area Measuring Software, http://phenotyping.image.cooan.jp/; Tanabata *et al.*, 2014). Each assay was repeated in 15 successive trials. The numbers of microcolonies (Fig. S2b) of 1–5 μm in diameter, immature mBFs (Fig. S2c) of 5–10 μm in diameter and mature mBFs (Fig. S2d) of >10 μm in diameter were assessed using Nodame, and were statistically analysed using the Tukey–Kramer honestly significant difference (HSD) test ($n = 15$) following an analysis of variance (ANOVA) with Easy R software (Saitama Medical Center, Jichi Medical University, Saitama, Japan; Kanda, 2013) (Tables S1–S3).

EPS I productivity

Quantitative analyses of EPS I production were conducted using an enzyme-linked immunosorbent assay (ELISA) (Mori *et al.*, 2016). The overnight culture of *R. solanacearum* strains was rinsed, and diluted to a cell density of 1.0×10^2 CFU/mL; 100 μL of this cell suspension were spread on plates of 1/4 × M63 agar medium and incubated for 2 days at 30 °C. The cells were then re-suspended to 1.0×10^5 CFU/mL. Cell density was confirmed through dilution plating. EPS I was quantified using anti-*R. solanacearum* EPS I antibodies with ELISA (Agdia Inc., Elkhart, IN, USA) according to the manufacturer's instructions per 100 μL volume (1.0×10^4 CFU) of cell suspension (three technical replicates were assessed). EPS I productivity was quantified by the absorbance at 650 nm.

Bacterial cell aggregation assay

We inoculated 5 μL of overnight culture of *R. solanacearum* adjusted to $OD_{600} = 0.005$ onto 95 μL of medium in wells of a polyvinylchloride (PVC) microtitre plate (NUNC MICRO WELL PLATE, Thermo Fisher Scientific Inc., Waltham, MA, USA). After incubation without shaking for 24 h at 30 °C, 25 μL of 1.0% crystal violet solution were added to the wells. After 15 min of incubation at room temperature, the unbound crystal violet stain was gently removed with a pipette. The wells were washed with distilled water, 70% ethanol and distilled water in turn. The crystal violet in each well was solubilized by adding 100 μL of 100% ethanol, and was quantified by the absorbance at 550 nm (Mori *et al.*, 2016).

Virulence assays

The near-isogenic line GCR26 of tomato (*Solanum lycopersicum* cv. Craigella) was kindly provided by NIAS GENEbank (http://www.gene.affrc.go.jp/index_en.php). Tomato plants were grown in pots containing a mixture of vermiculite and peat moss (3 : 1) and watered with fivefold-diluted Hoagland's solution in a growth room at 25 °C under 10 000 lx for 16 h per day (Hikichi *et al.*, 1999). The roots of 5-week-old tomato plants were soaked in bacterial suspension at 1.0×10^8 CFU/mL for 30 min and then washed in running water. The inoculated plants were grown in water-culture pots (Yamato Water Culture Pot No. 1, Yamato Plastic Co. Ltd, Yamatotakada, Japan) with fivefold-diluted Hoagland's solution in a growth room at 25 °C under 10 000 lx for 16 h per day. Inoculum concentrations were determined spectrophotometrically and confirmed by dilution plating. Within each trial, 12 plants of each strain were treated, yielding 60 plants per strain. Plants were coded and inspected for wilting symptoms daily after inoculation. Plants were rated on a 0–5 disease index scale: 0, no wilting; 1, 1%–25% wilting; 2, 26%–50% wilting; 3, 51%–75% wilting; 4, 76%–99% wilting; 5, dead. Each assay was repeated in five successive trials.

Bacterial populations and behaviour in tomato plants

Roots were excised at 0, 1, 2 and 3 days post-inoculation from five plants that had been inoculated with bacteria through the roots. Each sample was immersed in 70% ethanol solution for 1 min, followed by twice washing with distilled water, and ground in 1 mL of distilled water using a mortar and pestle. A 0.1-mL sample of the original suspension or a 10-fold serial dilution was spread onto three Hara–Ono medium (Hara and Ono, 1983) plates. For *ralA*-comp, the medium contained gentamycin at 25 μg/mL. The colonies were counted after 2 days of incubation at 30 °C.

A plate-printing assay was performed as follows (Kanda *et al.*, 2008). At 10 days post-inoculation by root dipping, the roots and stems of five tomato plants were cut into three pieces each using razor blades. The cut site of each piece (I, roots; II and III, stems; Fig. 1b) was pressed onto Hara–Ono medium for OE1–1 and Δ*ralA*, and onto Hara–Ono medium containing gentamycin at 25 μg/mL for *ralA*-comp, and then incubated at 30 °C for 3 days.

Apoplast extractions

To extract the apoplast fluid from the leaves of tomato plants, the third leaf down from the apex of 5-week-old tomato plants was cut, washed with distilled water and dried with a paper towel (Mori *et al.*, 2016). One to three leaves were then introduced into a 1-mL syringe with 15 mL of distilled water, and pressure–vacuum cycles were applied until the leaves were completely infiltrated. Following infiltration, the leaves were carefully removed from the syringe and blotted with a paper towel. Each leaf was then introduced into a 5-mL syringe placed inside a 50-mL conical tube containing a 1.5-mL collection tube. Apoplast extract was collected by centrifuging the tubes at 4000 ***g*** for 5 min at room temperature. The fraction collected in the 1.5-mL tube was centrifuged again for 5 min at 4000 ***g*** at room temperature. The supernatant was filtered through a 0.2-μm pore size membrane filter (Minisart CE; Sartorius, Gottingen, Germany) and stored at −20 °C until use.

Accession number of nucleotide sequence

The nucleotide sequence of *epsB* of strain OE1–1 has been deposited in DDBJ/GenBank/EMBL under accession number LC102463.

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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 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 (a) Observation of cells of *Ralstonia solanacearum* strain OE1-1 incubated in tomato apoplast fluid on nanopercolators for 24 h under a scanning electron microscope. OE1-1 cells produced microcolonies of 1–5 μm in diameter (b), immature mushroom-type biofilms (mBFs) of 5–10 μm in diameter (c) and mature mBFs of $>$10 μm in diameter (d).

Fig. S3 Attachment ability of *Ralstonia solanacearum* strains OE1-1 (a), ralfuranone-deficient mutant Δ*ralA* (b) and the complemented Δ*ralA* mutant *ralA*-comp (c). Glass slides were soaked in suspensions of *R. solanacearum* strains.

Fig. S4 Model of the regulation of mushroom-type biofilm (mBF) formation, the production of major exopolysaccharide EPS I and cell aggregation by *Ralstonia solanacearum* strain OE1-1 through *phc* quorum sensing. 3-OH MAME, methyl 3-hydroxymyristate.

Table S1 Tukey–Kramer analysis of the involvement of ralfuranones in the formation of microcolonies, immature mushroom-type biofilms and mature mushroom-type biofilms by *Ralstonia solanacearum* strains.

Table S2 Tukey–Kramer analysis of the involvement of *phc* quorum sensing in the formation of microcolonies, immature mushroom-type biofilms and mature mushroom-type biofilms by *Ralstonia solanacearum* strains.

Table S3 Tukey–Kramer analysis of the relationship between *phc* quorum sensing and ralfuranones in the formation of microcolonies, immature mushroom-type biofilms and mature mushroom-type biofilms by *Ralstonia solanacearum* strains.