Identification and characterization of chemosensors for D-malate, unnatural enantiomer of malate, in *Ralstonia pseudosolanacearum*

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Abstract

*Ralstonia pseudosolanacearum* Ps29 is attracted by nonmetabolizable D-malate, an unnatural enantiomer. Screening of a complete collection of single-mcp-gene deletion mutants of Ps29 revealed that the RSc1156 homologue is a chemosensor for D-malate. An RSc1156 homologue deletion mutant of Ps29 showed decreased but significant responses to D-malate, suggesting the existence of another D-malate chemosensor. McpM previously had been identified as a chemosensor for L-malate. We constructed an RSc1156 homologue mcpM double deletion mutant and noted that this mutant failed to respond to D-malate; thus, the RSc1156 homologue and McpM are the major chemosensors for D-malate in this organism. To further characterize the ligand specificities of the RSc1156 homologue and McpM, we constructed a Ps29 derivative (designated K18) harbouring deletions in 18 individual mcp genes, including mcpM and RSc1156. K18 harbouring the RSc1156 homologue responded strongly to L-tartrate and D-malate and moderately to D-tartrate, but not to L-malate or succinate. K18 harbouring mcpM responded strongly to L-malate and D-tartrate and moderately to succinate, fumarate and D-malate. Ps29 utilizes L-malate and L-tartrate, but not D-malate. We therefore concluded that L-tartrate and L-malate are natural ligands of the RSc1156 homologue and McpM, respectively, and that chemotaxis toward D-malate is a fortuitous response by the RSc1156 homologue and McpM in Ps29. We propose re-designation of the RSc1156 homologue as McpT. In tomato plant infection assays, the mcpT deletion mutant of highly virulent *R. pseudosolanacearum* MAFF106611 was as infectious as wild-type MAFF106611, suggesting that McpT-mediated chemotaxis does not play an important role in tomato plant infection.

INTRODUCTION

Chemotaxis is one of the most important behaviours in bacteria, allowing these organisms to sense environmental changes and appropriately respond to such changes [1]. Because most chemotactic attractants are growth substrates [2–5], chemotaxis is believed to assist bacteria in efficiently moving toward environments suitable for growth. Bacterial chemotaxis can also be viewed as an important prelude to ecological interactions such as symbiosis, infection, root colonization and metabolism [6]. The molecular mechanisms that underlie bacterial chemotaxis have been studied intensively in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium [7, 8]. Chemotactic ligands are detected by cell surface chemoreceptors called methyl-accepting chemotaxis proteins (MCPs). Upon binding a chemotactic ligand, an MCP generates chemotaxis signals that are communicated to the flagellar motor via a series of chemotaxis (Che) proteins. *E. coli* possesses five MCPs and six Che proteins (CheA, CheB, CheR, CheW, CheY and CheZ).

*Ralstonia solanacearum* is a Gram-negative and motile plant pathogenic bacterium that causes bacterial wilt in economically important crops, including tomato, potato, eggplant, tobacco and banana [9, 10]. This soil-borne bacterium usually enters plant roots through wounds, root tips and secondary root emergence points, from which the organism invades the xylem vessels and spreads to the aerial parts [11]. *R. solanacearum* is a heterogeneous species and termed as ‘the *R. solanacearum* species complex’ [12, 13]. The *R. solanacearum* species complex can be subdivided into four phylogenotypes [14]. Safni et al. [15] have proposed to emend the description of *R. solanacearum* and reclassify current *R. solanacearum* phytype IV strains as *Ralstonia syzygii* subsp. *indonesiensis* and current *R. solanacearum* phytype I and III strains as *Ralstonia pseudosolanacearum*. By this
recognition, \textit{R. solanacearum} consists of strains of current \textit{R. solanacearum} phylotype II only. In this study, we follow the proposed new nomenclature of the \textit{R. solanacearum} species complex.

The \textit{R. solanacearum} species complex is motile and shows chemotactic responses to a wide variety of chemical compounds, including amino acids, sugars, organic acids and inorganic phosphate [16, 17]. Yao and Allen [17] observed that \textit{cheA} and \textit{cheW} single mutants of \textit{R. solanacearum} K60 (phylotype II), which were nonchemotactic but motile, were less infectious than the wild-type strain in biologically realistic sand-soak virulence assays. When tomato plants were inoculated with a 1:1 mixture of each nonchemotactic mutant and its wild-type parent, the wild-type strain outcompeted these nonchemotactic mutants. From these results, these authors concluded that chemotaxis is required for full virulence in \textit{R. solanacearum} and that this bacterium depends on taxis to locate and colonize plant roots. We have also found that \textit{R. solanacearum} single mutants were more impaired in virulence than the wild-type strain MAFF106611 (phylotype I) and facilitates this species’ motility to tomato roots. We also found that \textit{R. pseudosolanacearum} was less infectious to tomato plants than the wild-type strain \textit{R. pseudosolanacearum} K60, which was motile and showed strong chemotactic responses to amino acids [16]. In that previous study, we identified \textit{McpM} deletion mutant of \textit{R. pseudosolanacearum} was less infectious to tomato plants than the wild-type strain, while the \textit{mcpA}-deletion mutation did not affect plant infection, suggesting that chemotaxis toward \textit{L}-malate but not toward amino acids facilitates \textit{R. pseudosolanacearum} motility to tomato roots. We also found that \textit{R. pseudosolanacearum} exhibited strong chemotactic responses not only to \textit{L}-malate but also to unnatural enantiomer \textit{D}-malate [16]. Although several bacteria (e.g. \textit{Pseudomonas fluorescens}, \textit{Pseudomonas aeruginosa}, \textit{Klebsiella aerogenes}, \textit{Rhodopseudomonas palissoidea}, \textit{Pseudomonas plecoglossis}) [18–21]) can utilize \textit{D}-malate as sole carbon and energy source, \textit{R. pseudosolanacearum} cannot grow on this compound. To our knowledge, there have been no studies reporting the presence of \textit{D}-malate in plant root exudates. We therefore wondered what biological significance chemotaxis to \textit{D}-malate might have in \textit{R. pseudosolanacearum}. We hypothesized that chemotaxis toward \textit{D}-malate might be a fortuitous response by a \textit{R. pseudosolanacearum} MCP that normally senses a natural compound structurally related to \textit{D}-malate. In the present study, we identified and characterized \textit{R. pseudosolanacearum} MCPs for \textit{D}-malate to assess our hypothesis.

METHODS

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. \textit{R. pseudosolanacearum} Ps29 [formerly named \textit{R. solanacearum} Ps29 (phylotype I, race 1, biovar 3); isolated from tobacco] and \textit{R. pseudosolanacearum} MAFF106611 [formerly named \textit{R. solanacearum} MAFF106611 (phylotype I, race 1, biovar 4); isolated from eggplant] were obtained from the Leaf Tobacco Center (Japan Tobacco) and the National Institute of Agrobiological Sciences, Japan, respectively [22]. Highly motile \textit{R. pseudosolanacearum} Ps29 and its derivatives were used for chemotaxis research, and \textit{R. pseudosolanacearum} MAFF106611 and its derivatives were used for tomato plant virulence assays [16]. \textit{E. coli} JM109 [23] and S17-1 [24] were used for plasmid construction and transconjugation, respectively. \textit{R. pseudosolanacearum} strains and their derivatives were cultivated at 28°C in rich CPG medium [25] or in \textit{R. solanacearum} minimal (RSM) medium [16]. \textit{E. coli} strains were grown at 37°C in 2x YT medium [23]. For plasmid selection and maintenance, kanamycin was provided at 50 µg ml$^{-1}$.

Quantitative chemotaxis assay

Computer-assisted capillary assays were carried out as described previously [26]. Cell movement was observed under an inverted microscope. Cells in a 10 µl suspension were placed on a coverslip, and the assay was started by placing the coverslip upside down on the U-shaped spacer to fill the chemotaxis chamber in the presence of a capillary containing a known concentration of an attractant plus 1% (w/v) agarose. Cells were videotaped, and digital image processing was used to count the number of bacteria accumulating toward the mouth of a capillary at the initial time ($N_0$) and at each given time interval ($N_t$). The strength of the chemotactic response was determined and reported in terms of normalized cell number per frame ($N_t/N_0$). Unless stated otherwise, yeast extract at 0.1% (w/v) was used as a positive control. The chemotaxis buffer was 10 mM HEPES buffer (pH 7.0).

DNA manipulation

Standard techniques were used for plasmid DNA preparations, restriction enzyme digests, ligations, transformations and agarose gel electrophoresis [23]. PCR was carried out using KOD Plus Neo polymerase (Toyobo) according to the manufacturer’s instructions. Plasmids were introduced into \textit{R. pseudosolanacearum} strain by transconjugation using \textit{E. coli} S17-1 or by electroporation as described previously [16].

Construction of plasmids for complementation

pRCII [16] was used for plasmid vector for complementation analysis of \textit{R. pseudosolanacearum} mutants. To construct pPS03 and pPS12, primer pairs 5’-CAGATC
TAGAGATGCCGACTGGGAAACCTTCTG-3'5'-CTGG
ACGTGCTCTACCGGAACATG-3' and 5'-CAGATCTAGATGTGATCGATTTCGCGCTGTTCC-3' were used to amplify
2.1 and 2.4 kb regions containing the RSc1156 and
RSc0671 homologues of R. pseudosolanacearum Ps29,
respectively. The amplified fragments were digested with
XbaI and cloned between the XbaI and HincII sites of
pRCII.

### Table 1. Bacterial strains and plasmids used in this study

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**Construction of unmarked multiple deletion mutant**

Eighteen mcp genes in R. pseudosolanacearum Ps29 were sequentially deleted by an unmarked-gene-deletion technique as described previously [16]. Derivatives of suicide plasmid pK18mobsacB [27], which had been used for construction of an mcp single deletion mutant library of R. pseudosolanacearum Ps29 in the previous study [16], were used for unmarked multiple deletion of mcpA, mcpM and the RSc1155, RSc1156, RSc1234, RSc1460, RSc1894, RSc2799, RSc3136, RSc3307,
Virulence assay
We tested plant infection by *R. pseudosolanacearum* strains using the sand-soak inoculation method [16]. Briefly, sterile tomato (*Solanum lycopersicum* cv. Oogata-fukuju) seeds were kept overnight at 4°C in the dark in order to synchronize germination. Seeds then were placed onto Petri dishes containing plant nutrient solution (PNS) [28] solidified with 1.5% (w/v) agar and allowed to grow in a climate-controlled growth chamber (28°C, 16:8 h light:dark cycle). Bacterial cells grown in RSM medium for 20 h were collected (3300 g for 2 min), washed twice with sterile PNS and adjusted to a final density of approximately 10^6 c.f.u. ml^-1. Seven-day-old tomato roots were wounded by cutting 1 cm away from the base of the stem. The wounded seedling was immediately transferred to a gnotobiotic sand system (35 mm inner diameter and 120 mm length glass tube containing 50 g quartz sand and 12.5 ml PNS) and planted near one wall of the tube, while 50 µl of freshly prepared cell suspension was inoculated near the opposite wall. The plants were maintained in a climate-controlled growth chamber at 28°C with 16:8 h light:dark cycle for 12 days and observed daily. All virulence assays included at least 10 plants per treatment, and each experiment was repeated at least three times.

Competitive plant colonization assay
Twenty grams of quartz sand was put in each glass tube (22 mm inner diameter, 25 mm outer diameter, 120 mm length). The open end of the tube was plugged with a silicone resin stopper. The tube was then autoclaved for 15 min at 121°C. Sterile PNS (5 ml) then was added to each autoclaved sand column. Tomato (*S. lycopersicum* cv. Oogata-fukuju) seeds were sterilized as described in Virulence assay section. After storing sterile seeds at 4°C in the dark, seeds were placed on Petri dishes containing PNS solidified with 1.5% (w/v) agar and incubated in a climate-controlled growth chamber (28°C, 16:8 h light:dark cycle) for 3 days to allow germination. A germinated seed was aseptically placed at the centre of each growth tube at 5 mm below the surface of the quartz sand and then grown in a climate-controlled growth chamber (28°C, 16:8 h light:dark cycle) for another 3 days. Bacterial cells were grown for 20 h in RSM medium, centrifuged (3300 g, 2 min), washed twice with sterile deionized water and adjusted to 10^7 c.f.u. ml^-1 in sterile deionized water. For the competitive colonization assay, 50 µl of 1:1 (v/v) mixture of the tested strain and the competitor (the Km' strain of *R. solanacearum* MAFF106611) was mixed and inoculated to the edge of each plant growth tube. The plant growth tubes were incubated in a climate-controlled growth chamber (28°C, 16:8 h light:dark cycle). After 2, 4 and 6 days of incubation, each tomato seedling was homogenized and shaken vigorously in 0.5 ml sterile deionized water to suspend the bacteria. The bacterial suspension was diluted, and 50 µl was plated on CPG agar plates with and without kanamycin.

Statistical analysis
All data are presented as means±SD. Chemotactic response data and plant infection data were evaluated using Student’s *t*-test or Fisher’s LSD test. *P*<0.05 was considered statistically significant.

Nucleotide sequence accession numbers
The nucleotide sequence of the *mcpT* gene in *R. pseudosolanacearum* MAFF106611 has been deposited in the DDBJ, EMBL and GenBank nucleotide sequence databases under accession number KX537646.

RESULTS
Chemotactic responses to d-malate by *R. pseudosolanacearum*
Computer-assisted capillary assays were conducted to measure chemotactic responses to d-malate by *R. pseudosolanacearum* Ps29. Cell number around a glass capillary increased with time when the glass capillary contained 5 mM d-malate, while cell number did not vary when the glass capillary contained HEPES buffer (control) (Fig. 1a). This result confirmed that *R. pseudosolanacearum* Ps29 is attracted by d-malate. Responses to d-malate were dependent on concentrations of d-malate in the glass capillary (Fig. 1b). Notably, however, responses to d-malate were not as strong as those to l-malate, a known strong attractant [16].

Identification of genes encoding chemosensory proteins for d-malate
Chemotactic ligands are detected by cell surface MCPs. Upon binding a chemotactic ligand, an MCP generates chemotaxis signals that are communicated to the flagellar motor via a series of Che proteins [7, 8]. The genome sequence of *R. pseudosolanacearum* GMI1000 has been determined [29] and was found to possess 22 putative *mcp* genes. Out of the 22 putative *mcp* genes, only 4 MCPs including 2 aerotaxis MCPs and MCPP for l-malate and amino acids have been functionally characterized in *R. solanacearum* species complex [16, 17].

We demonstrated that *R. pseudosolanacearum* Ps29 possesses homologues of 22 *R. pseudosolanacearum* GMI1000 *mcp* genes [16]. In that previous study, we constructed a library of *R. pseudosolanacearum* Ps29 mutants harbouring unmarked deletions in each of the 22 *mcp* genes. To identify the gene(s) encoding an MCP for d-malate, we examined mutant strains of that library for responses to d-malate and found that two mutants (DPS03 and DPS12) showed significantly lower responses to d-malate than did wild-type Ps29 (Student’s *t*-test, *P*<0.05) (Fig. 2a). DPS03 and DPS12 harbour deletions in the homologues of *R. pseudosolanacearum* GMI1000 RSc1156 and RSc0671, respectively. To assess whether d-malate-specific chemotaxis was indeed impaired in these strains, we examined these mutants for their responses to yeast extract. The responses of DPS03 to yeast...
extract were comparable to those of wild-type Ps29, while DPS12 showed decreased responses to yeast extract (Fig. 2b), suggesting that DPS12 carries a more general defect in chemotaxis. Therefore, we selected the RSc1156 homologue for further study.

Although DPS03 showed decreased responses to D-malate, chemotaxis to D-malate was not completely abolished in this mutant (Fig. 3a). This result suggested the presence of an additional MCP for D-malate. In our previous study, we identified McpM as an MCP for L-malate [16]. To assess whether McpM could sense an enantiomer of D-malate, we deleted the mcpM gene in DPS03 to construct an RSc1156 homologue mcpM double mutant (DPS0314).

Computer-assisted capillary assays revealed that DPS0314 failed to respond to D-malate (Fig. 3a). Introduction of pPS03 (harbouring the RSc1156 homologue of Ps29) or pPS14 (harbouring mcpM) restored the ability of DPS0314 to respond to D-malate (Fig. 3b). This result suggested that the RSc1156 homologue and McpM are the major MCPs for D-malate in R. pseudosolanacearum Ps29.

We next investigated the ligand specificity of the RSc1156 homologue and McpM. To facilitate this experiment, we constructed a multi-gene deletion mutant (K18) of R. pseudosolanacearum Ps29 by sequential deletion of 18 mcp genes, including the RSc1156 homologue and mcpM. We introduced (separately) pPS03 and pPS14 into K18 and examined the resulting recombinant strains for chemotactic responses to D-malate-related compounds, including L-malate, succinate, fumarate, maleate, L-tartrate, D-tartrate and butyrate. The multi-gene mutant harbouring an empty plasmid (K18[pCRII]) showed no chemotactic responses to any of these D-malate-related compounds. In contrast, K18 [pPS03] exhibited strong responses to D-malate and to L-

Fig. 1. Quantitative chemotactic responses of R. pseudosolanacearum Ps29 to D-malate and L-malate. (a) Concentration–response curves of responses to D-malate (open squares) and L-malate (open triangles). Digital image processing was used to count the number of bacteria around the mouth of a capillary containing test compound and 1 % (w/v) agarose. Videotape frames were analysed at the initiation of observation and 1 min after the initiation. Normalized cell numbers were calculated by dividing the number of bacterial cells at 1 min by that at the initiation of the observation. (b) Time courses of responses to 0.5 (solid squares), 2.0 (solid circles) and 5.0 mM (solid triangles) D-malate. HEPES buffer (crosses) was used as a negative control. Error bars represent the SD values from the means of measurements done in at least triplicate experiments.

Fig. 2. Chemotactic responses of R. pseudosolanacearum Ps29 and isogenic single-mcp-gene deletion mutants to 2 mM D-malate (a) and to 0.1 % (w/v) yeast extract (b). Normalized cell numbers were calculated by dividing the number of bacterial cells at 1 min by that at the initiation of the observation. Error bars represent the SD values from the means of measurements done in at least triplicate experiments. Asterisks indicate statistically significant differences between the wild-type strain and mutants (Student’s t-test, P<0.05).
tartrate and a moderate response to D-tartrate (Fig. 4a), but did not respond to L-malate, succinate, fumarate, maleate or butyrate. K18[pPS14] exhibited strong responses to D-tartrate as well as to L-malate and moderate responses to succinate, fumarate and D-malate. Butyrate and maleate did not elicit chemotactic responses in K18[pPS14]. Among the attractants that were sensed by McpM and the RSc1156 homologue, L-malate, succinate, fumarate and L-tartrate could be utilized by \( R.\ pseudosolanacearum \) Ps29 as sole carbon and energy source (data not shown). Based on these results, we designated the RSc1156 homologue as McpT (MCP for L-tartrate). It is worth noting that introduction of plasmid containing RSc0671 homologue did not restore the ability of K18 to respond to D-malate (data not shown).

The \( mcpT \) deletion mutant of Ps29 (DPS03) showed a very weak response to L-tartrate; introduction of an \( mcpT \) plasmid (pPS03) restored the ability of this mutant to respond to L-tartrate (Fig. 5). This result indicated that McpT is a major MCP for L-tartrate in \( R.\ pseudosolanacearum \) Ps29.

**Virulence assays**

We next investigated the role of \( mcpT \) in bacterial wilt virulence on tomato. For this experiment, we used highly virulent \( R.\ pseudosolanacearum \) MAFF106611 instead of \( R.\ pseudosolanacearum \) Ps29 because \( R.\ pseudosolanacearum \) Ps29 yields weaker virulence on tomato [16]. We confirmed the presence of the \( mcpT \) homologue in \( R.\ pseudosolanacearum \) MAFF106611 by PCR analysis and DNA sequencing. The \( R.\ pseudosolanacearum \) MAFF106611 \( mcpT \) gene encodes a protein that is 100% identical to the \( R.\ pseudosolanacearum \) Ps29 McpT. Like the Ps29 strain, \( R.\ pseudosolanacearum \) MAFF106611 was attracted by D-malate, and the MAFF106611 \( mcpT \) deletion mutant (DMF03) showed a significantly decreased response to D-malate (Fig. S1, available in the online Supplementary Material).

The sand-soak inoculation method was conducted to assess plant infection by \( R.\ pseudosolanacearum \) strains. In this method, cells of test strains are inoculated into sand at 3 cm away from a tomato plant. Plant infection by this assay requires bacterial cells to locate and invade host plants from a distance. When wild-type \( R.\ pseudosolanacearum \) MAFF106611 was tested, tomato plants started wilting at 4 days post-inoculation, and 70% of the plants had been killed at 10 days post-inoculation (Fig. 6). As we noted in our previous report, the \( mcpM \) deletion mutant of \( R.\ pseudosolanacearum \) MAFF106611 (DMF14) was less infectious than wild-type MAFF106611 [16]. In contrast, there was no significant difference between the infectivity of the \( mcpT \) deletion mutant and the isogenic wild-type MAFF106611. This result suggested that McpT-mediated chemotaxis does not have a crucial role in initial location of plant roots by the bacterium in this sand-soak inoculation virulence assay.

**Competitive plant colonization assays**

To investigate if the \( mcpT \) mutation would affect root colonization, we conducted competitive plant colonization assays by inoculating tomato seedlings with a 1:1 mixture of a test and competitor strains. Because the \( R.\ pseudosolanacearum \) MAFF106611 Km\(^r\) mutant (MFK) competed fully with wild-type strain MAFF106611 (Fig. 7a), we used MFK as the competitor strain in competitive plant colonization assays to distinguish the competitor strain from test strains; the Km\(^r\) phenotype facilitated the distinction between the test and competitor strains. The results of the competitive plant colonization assays were consistent with those of virulence assays: strain DMF03 fully competed with MFK (Fig. 7b).

**DISCUSSION**

We identified McpT as an MCP for D-malate and found that McpM, which we previously identified as an MCP...
for L-malate, also senses D-malate. McpT senses L-tartrate as strongly as D-malate, but unlike D-malate, L-tartrate can be utilized by *R. pseudosolanacearum* as sole carbon and energy source. Because L-tartrate occurs in numerous plant species, especially in vitaceous plants [30], this bacterium would have many chances to encounter L-tartrate in natural environments. Additionally, D-malate is an unnatural enantiomer of malate. These observations suggest that L-tartrate is a natural ligand of McpT.
A typical MCP possesses two transmembrane regions in the N-terminal region. A hydrophilic region between the two transmembrane regions constitutes a periplasmic domain [31]. Chemotactic ligands are known to bind to the periplasmic domains [ligand-binding domains (LBDs)] of MCPs, thereby initiating chemotactic signalling (in some cases, MCPs indirectly sense attractants by binding of ligand–periplasmic ligand binding protein complexes). The diverse ligand specificities among MCPs reflect amino acid sequence diversity of the LBDs. Protein BLAST analysis [32] using the putative LBD of McpT as a query sequence revealed the presence of highly homologous (more than 55 % identity) LBDs in McpT-orthologous proteins encoded by Ralstonia pickettii (a member of the R. solanacearum species complex), by Actinobacteria such as Mumia flavus and Streptomyces pluripotens and by Burkholderia species such as Burkholderia plantarii. Protein BLAST did not detect significant similarity between the LBDs of R. pseudosolanacearum McpT and McpM. Other work has shown that the LBDs of MCPs can be classed into cluster I domains (120–210 amino acids) and cluster II domains (220–290 amino acids) based on the sizes of the LBDs [33]. Although there is no significant similarity between the LBDs of McpT and McpM, the LBDs of both these proteins belong to the cluster I group, with predicted LBD sizes of 160 and 153 amino acids, respectively. Based on the Phyre2 structure prediction program [34], the R. pseudosolanacearum Ps29 McpT LBD is predicted to form a four-helix-bundle domain (Fig. S2), as also predicted for McpM [16] and for E. coli Tar and Tsr [33].

Fig. 5. Chemotactic responses of R. pseudosolanacearum Ps29 strains to 5 mM L-tartrate. WT, Wild-type Ps29; DPS03, mcpT deletion mutant; DPS14, mcpM deletion mutant; DPS03(pPS03), DPS03 harbouring pPS03. Error bars represent the ± values from the means of measurements done in triplicate experiments. Different letters indicate significant differences (Fisher’s LSD test, P<0.05).

Fig. 6. Virulence of R. pseudosolanacearum MAFF106611 wild-type strain and mcpT deletion mutant (DMF03) on tomato seedlings. In each experiment, 10 tomato seedlings were examined; killed plants were counted, and numbers were used to calculate the percentage of dead plants. Means and ± values were calculated from at least three independent experiments. There were no significant differences in percentage of dead plants between wild-type and DMF03 (Student’s t-test, P<0.05).

Fig. 7. Plant colonization assay for competition between the R. pseudosolanacearum MAFF106611 kanamycin-resistant strain (MFK) and wild-type strain MAFF106611 (a) or the mcpT deletion mutant (DMF03) (b). Error bars represent the ± values from the means of measurements done in at least triplicate experiments.
We used a multi-gene deletion mutant of *R. pseudosolanacearum* Ps29 (K18) as a host strain to investigate the ligand specificity of McpM and McpT. Chemotaxis assays of K18 [pPS14] (harbouring *mcpM*) demonstrated that McpM can sense succinate but not butyrate, suggesting that McpM is a generic sensor of C_4_ compounds with two terminal carboxyl groups. K18 [pPS14] responded to fumarate but not to maleate, suggesting that McpM recognizes succinate in the anti-conformation. L-Malate elicited much stronger McpM-mediated chemotaxis in K18 [pPS14] than succinate did, indicating that a C_4_ compound with two terminal carboxyl groups and one hydroxyl group in S-configuration (i.e. L-malate) is a natural ligand of McpM (Fig. 4b). We infer, in the case of D-malate, that McpM senses the compound’s succinate’s structure. K18 [pPS03] (harbouring *mcpT*) showed a strong response to D-malate but not to succinate or fumarate (Fig. 4a). In this strain, the response to L-tartrate was as strong as that to D-malate. These results suggest that McpT recognizes C_4_ compounds with two terminal carboxyl groups and one hydroxyl group in the R-configuration (i.e. D-malate).

In conclusion, we identified McpT and McpM as MCPs for D-malate in *R. pseudosolanacearum* Ps29. Based on the strength of the chemotactic responses and the results of the growth assays, we infer that the natural chemotactic ligands of McpT and McpM are L-tartrate and L-malate, respectively. We hypothesize that *R. pseudosolanacearum* chemotaxis toward D-malate may be due to fortuitous recognition by an L-tartrate chemoreceptor (McpT) and an L-malate chemoreceptor (McpM). McpM-mediated chemotaxis facilitates *R. pseudosolanacearum* motility to tomato roots in sand; in contrast, McpT-mediated chemotaxis is not required for motility toward roots. Therefore, the primary role of McpT-mediated chemotaxis is likely searching for a favourable growth substrate, that is, one that includes L-tartrate.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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