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Two citrate chemoreceptors involved in chemotaxis to citrate and/or citrate-metal complexes in *Ralstonia pseudosolanacearum*

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The bacterial wilt pathogen *Ralstonia pseudosolanacearum* Ps29 exhibited chemotactic responses to citrate. This pathogen expresses 22 putative chemoreceptors. In screening a complete collection of *mcp* single-gene deletion mutants of Ps29, none showed a significant decrease in response to citrate compared with the wild-type strain. Analysis of a collection of stepwise- and multiple-deletion mutants of Ps29 revealed that the RS_RS07350 homolog (designated McpC) and McpP (chemoreceptor mediating both positive chemotaxis to phosphate and negative chemotaxis to maleate) are chemoreceptors for citrate. Double deletion of *mcpC* and *mcpP* markedly reduced the response to citrate, indicating that McpC and McpP are major chemoreceptors for citrate. Wild-type Ps29 was attracted to both free citrate and citrate complexed with divalent metal cations such as magnesium and calcium. The *mcpC mcpP* double-deletion mutant also showed significant reduction in chemotaxis to Mg^{2+}- and Ca^{2+}-citrate complexes. Introduction of a plasmid harboring the *mcpC* gene (but not the *mcpP* gene) restored the ability to respond to these citrate-metal complexes, demonstrating that McpC can sense complexes of citrate and metal ions such as Mg^{2+} and Ca^{2+} as well as free citrate. Thus, *R. pseudosolanacearum* Ps29 expresses two chemoreceptors for citrate. In plant infection assays using tomato seedlings, the *mcpC* and *mcpP* single- and double-deletion mutants of the highly virulent *R. pseudosolanacearum* MAFF106611 strain were as infectious as the wild-type strain, suggesting that citrate chemotaxis does not play an important role in infection of tomato plants in this assay system.

[**Key words:** Chemotaxis; Chemoreceptor; Citrate; *Ralstonia pseudosolanacearum*; Plant pathogen]

The *Ralstonia solanacearum* species complex, composed of *R. solanacearum* (formerly *R. solanacearum* phylotype II), *Ralstonia pseudosolanacearum* (formerly *R. solanacearum* phylotypes I and III), and *Ralstonia syzygii* subsp. *indonesiensis* (formerly *R. solanacearum* phylotype IV) (1,2), is a group of gram-negative bacterial plant pathogens that cause bacterial wilt disease in more than 200 plant species in over 50 families, including economically important crops such as tomato, tobacco, potato, and eggplant (3,4). This soil-borne bacterium usually enters plant roots through wounds, the root tips, and secondary root emergence points, eventually invading the xylem vessels and spreading to the aerial parts of the plant (5). Many factors contribute to bacterial wilt disease, such as the type III secretion system, exopolysaccharide, and plant cell wall-degrading enzymes (6). Yao and Allen (7) reported that chemotaxis is also required for virulence of the *R. solanacearum* species complex.

Chemotaxis is the ability of motile cells to migrate toward favorable conditions in response to a chemical gradient (8). Chemotactic bacteria can locate better environments for growth or ecological behavior by moving toward favorable compounds and away from unfavorable compounds. Chemotactic ligands are detected by chemoreceptors called methyl-accepting chemotaxis proteins (MCPs). The number of MCPs varies among different bacteria. While the enteric bacterium *Escherichia coli* expresses only four MCPs (Tsr, Tar, Trg, and Tap) and one MCP-like receptor (Aer) (9), free-living environmental bacteria express a large number of MCPs, for example, 26 putative MCPs in *Pseudomonas aeruginosa* PAO1 (10), 27 putative MCPs in *Pseudomonas putida* F1 (11), and 37 putative MCPs in *Pseudomonas fluorescens* Pf0-1 (12). Genomic analyses of strains in the *R. solanacearum* species complex suggested the presence of 21–23 putative *mcp* genes.

The *R. solanacearum* species complex shows chemotactic responses to a wide variety of compounds, including amino acids, organic acids, sugars, inorganic phosphate, and boric acid (7,13). We previously identified MCPs for amino acids (McpA) (13), L-malate (McpM) (13), L-tartrate/D-malate (McpT) (14), boric acid (McpB) (15), and phosphate/maleate (McpP) (16). Sand-soak virulence assays using *mcp* single-deletion mutants of *R. pseudosolanacearum* MAFF106611 revealed that McpM-mediated chemotaxis, possibly L-malate chemotaxis, contributes to the migration of this pathogen to plant roots. However, the nonchemotactic but motile *cheA* mutant was less virulent than the *mcpM* mutant, suggesting that root exudate component(s) other than L-malate are involved in plant infection by *R. pseudosolanacearum* (13).

The *R. solanacearum* species complex shows strong chemotaxis to dicarboxylic acids such as malate and tartrate as well as to the tricarboxylic acid citrate (7,13). Like L-malate, citrate is a major component of plant root exudate, with several studies reporting that citrate is the most abundant organic acid in root exudate (17,18). However, the potential role of chemotaxis to citrate in plant

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infection by members of the *R. solanacearum* complex is unclear because MCP(s) for citrate have not been identified. Here, we report the identification of two MCPs for citrate and their relationship to plant infection by *R. pseudosolanacearum*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions The bacterial strains and plasmids used in this study are listed in Table 1. Highly motile *R. pseudosolanacearum* Ps29 and its derivatives were used for chemotaxis analyses, and *R. pseudosolanacearum* MAFF106611 and its derivatives were used for tomato plant infection assays. All derivatives showed motility comparable to wild-type strain. *E. coli* JM109 and S17-1 were used for plasmid construction and transconjugation, respectively. Both *R. pseudosolanacearum* Ps29 and MAFF106611 were grown at 28°C in *R. solanacearum* minimal (RSM) medium (13) with or without preculture in CPG medium (19), respectively, whereas *E. coli* strains were cultured in Luria–Bertani medium. When necessary, 40 μg/ml of kanamycin was added.

Chemotaxis assay Computer-assisted capillary assays were carried out as described previously (20). Cell movement was observed under an inverted microscope. Cells in a 10-μl suspension were placed on a coverslip, and the assay was initiated by placing the coverslip upside down on a U-shaped spacer to fill the chemotaxis chamber in the presence of a glass capillary containing test compound plus 1% (wt/vol) agarose. Cell responses were videotaped. Digital image processing was used to count the number of bacteria accumulating toward the mouth of the capillary at the initial time (N_0) and 1 min (Ps29 strains) or 2 min (MAFF106611 strains) after initiation (N_1 or N_2). The strength of the chemotactic response was based on the normalized cell number per frame (N_1/N_0 or N_2/N_0). The chemotaxis buffer was 10 mM HEPES (pH 7.0).

DNA manipulation Standard procedures were used for plasmid DNA preparation, restriction enzyme digestion, ligation, transformation, and agarose gel electrophoresis (21). PCR was carried out using KOD FX Neo polymerase (Toyobo, Japan) according to the manufacturer's instructions. All PCR primers were designed based on the genome sequence of *R. pseudosolanacearum* GMI1000. Plasmids were introduced into *R. pseudosolanacearum* strains by transconjugation using *E. coli* S17-1 or by electroporation (2.5 kV, 250 Ω, and 25 μF) using a 2-mm cuvette.

Construction of unmarked deletion mutants The *mcp* genes were deleted using an unmarked gene-deletion technique as described previously (13). For construction of the RS_RS07350 (old locus tag RSc1460) homolog gene (*mcpC*) and *mcpP* double-deletion mutant of *R. pseudosolanacearum* Ps29, the *mcpC* single-deletion mutant (DPS05) and pNMPS16 (13) were used. The *R. pseudosolanacearum* MAFF106611 *mcpC* and *mcpP* single- and double-deletion mutants were constructed in a manner similar to those of Ps29 using MAFF106611 genomic DNA.

Construction of complementation plasmid pRCII (13) was used as the plasmid vector for complementation analysis of *R. pseudosolanacearum* Ps29 mutants. To construct pPS05, a 1.7-kb region encoding *mcpC* of *R. pseudosolanacearum* Ps29 was amplified by PCR using primer pair 5′-CTATGAATTCGGCTCAAGTTTGAGA-ACGGGCTACC-3′/5′-CATAGGATCCCATATCGCGCAGGCGTACTGGAAC-3′. The amplified fragments were digested with *Eco*RI and *Bam*HI and cloned between the *Eco*RI and *Bam*HI sites of pRCII.

Virulence assay Plant infection by *R. pseudosolanacearum* strains was assessed using the sand-soak inoculation method as described previously (13). Bacterial cells grown in RSM medium for 20 h were collected by centrifugation (3300 g for 2 min), washed twice with sterile deionized water, and adjusted to a final density of approximately 10^6 CFU/ml in sterile deionized water. Roots of 7-day-old tomato seedlings were wounded by cutting 1 cm away from the base of the stem. The wounded seedling was transferred to a gnotobiotic sand system (35 mm inner diameter and 120 mm length glass tube containing 50 g of quartz sand and 12.5 ml of PNS (22)) and planted near one wall of the tube. Fifty microliters of the cell suspension was inoculated near the opposite wall of the tube (the distance between the seedling and the inoculation spot was 30 mm). The plants were maintained in a climate-controlled growth chamber at 28°C with a 16:8 h light:dark cycle for 12 days and observed daily.

Competitive plant colonization assay The competitive colonization assay was carried out as described previously (13) with some modification. Bacterial cells and gnotobiotic sand system containing the wounded tomato seedling were prepared as described in the virulence assay section. For the competitive colonization assay, 50 μl of 1:1 (v/v) mixture of the tested strain and the Km^r strain (as competitor) of *R. pseudosolanacearum* MAFF106611 (13) was inoculated near the opposite side of the seedling in the gnotobiotic system. The plants were maintained in a climate-controlled growth chamber at 28°C with 16:8 h light:dark cycle for 12 days. After 2, 4, and 6 days of incubation, each tomato seedling was homogenized and shaken vigorously in 0.5 ml of sterile deionized water to suspend the bacteria. The bacterial suspension was diluted and plated onto CPG agar plates with and without kanamycin.

RESULTS

Identification of citrate MCPs Computer-assisted capillary assays showed that citrate chemotaxis by *R. pseudosolanacearum* Ps29 was concentration dependent (Fig. 1). In our previous study,

TABLE 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)[a]	Reference
Bacterial strain		
Ralstonia pseudosolanacearum		
Ps29	Wild type strain race 1, biovar 3, phylotype I	43
POC10	Ps29 derivative; Δ*mcpA* (LC005226) Δ*mcp02* (LC005227) Δ*mcp09* (LC005234) Δ*mcp10* (LC005235) Δ*mcp12* (LC005237) Δ*mcpM* (LC005239) Δ*mcp15* (LC005240) Δ*mcp17* (LC 005242) Δ*mcp18* (LC005243) Δ*mcp19* (LC005244)	23
POC11	Ps29 derivative; POC10 Δ*mcpC* (LC005230)	23
POC12	Ps29 derivative; POC11 Δ*mcpP* (LC005241)	23
POC22	Ps29 derivative; 22 putative *mcp* genes deletion mutant	23
DPS05	Ps29 derivative; Δ*mcpC*	13
DPS16	Ps29 derivative; Δ*mcpP*	13
DPS0516	Ps29 derivative; Δ*mcpC*Δ*mcpP*	This study
MAFF106611	Wild type strain race 1, biovar 4, phylotype I	43
DMF05	MAFF106611 derivative; Δ*mcpC* (LC381281)	This study
DMF16	MAFF106611 derivative; Δ*mcpP* (MF138068)	This study
DMF0516	MAFF106611 derivative; Δ*mcpC*Δ*mcpP*	This study
MFK	MAFF106611 derivative; Km^r	13
Escherichia coli		
JM109	*recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* ($r_k^-m_k^+$), *e14*$^-$ (*mcrA*$^-$), *supE44*, *relA1*, Δ(*lac-proAB*)/F' [*traD36*, *proAB*$^+$, *lacI*^{q}, *lacZ* ΔM15]	21
S17-1	MM294 derivative, RP4-2 Tc::Mu-Km::Tn7; chromosomally integrated	44
Plasmid		
pK18*mobsacB*	Km^r pUC18 derivative; *lacZα mob* site *sacB*	45
pNMPS16	pK18*mobsacB* with a 0.3–kb PCR fragment upstream of *mcpP* and a 0.8–kb PCR fragment downstream of *mcpP* from the Ps29 genome	13
pRCII	*E. coli-Ralstonia* shuttle vector derived from pKZ27; IncQ, *lac* promoter; Km^r	13
pPS05	pRCII with a 1.7-kb PCR fragment including *mcpC* of Ps29	This study
pPS16	pRCII with a 1.7-kb PCR fragment including *mcpP* of Ps29	16

[a] LC005226 to LC005244, LC381281 and MF138068 in parenthesis indicate the accession number of the *mcp* genes.

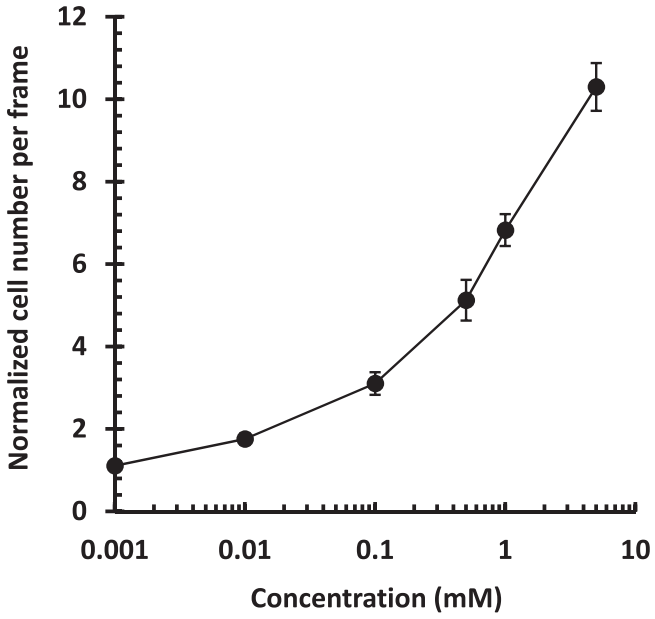

FIG. 1. Concentration-dependent chemotaxis toward citrate by wild-type *R. pseudosolanacearum* Ps29. Vertical bars represent the standard error of measurement for experiments performed at least nine times.

we constructed a library of 22 R. *pseudosolanacearum* Ps29 *mcp* single-deletion mutants (13). In screening of the library, none of the mutants showed a significant decrease in response to citrate ($P < 0.01$ by Student's *t*-test) (Fig. S1), suggesting that citrate chemotaxis is mediated by multiple receptors or a receptor(s) other than the 22 putative MCPs. To examine the latter possibility, response to citrate by POC22, a 22-*mcp*-deletion mutant of *R. pseudosolanacearum* Ps29, was assayed. The POC22 mutant was unable to respond to citrate (Fig. 2A), excluding the possibility that citrate chemotaxis is mediated by one or more chemoreceptors other than the 22 MCPs and indicating that these 22 MCPs include chemoreceptor(s) for citrate. We therefore attempted to identify MCPs for citrate by screening a library of multiple-*mcp*-deletion mutants (designated POC*n*, where *n* is the number of deleted *mcp* genes) (23) obtained in the course of construction of the 22-*mcp*-deletion mutant POC22. Although the response to citrate by strain POC10 (a mutant with deletion of 10 m*cp*s) was comparable to that of the wild-type strain, POC11 (the POC10 with the additional deletion of the homolog of *R. pseudosolanacearum* GMI1000 RS_RS07350) showed a significantly lower-level response to citrate than did POC10 ($P < 0.05$ by Student's *t*-test) (Fig. 2B). Deletion of the *mcpP* gene encoding a phosphate/maleate sensor (16) in POC11 to create POC12 resulted in loss of the ability to respond to citrate (Fig. 2B). These results suggested that the RS_RS07350 homolog and *mcpP* are involved in citrate chemotaxis.

Careful review of the responses by the RS_RS07350 homolog single-deletion mutant (DPS05) and the *mcpP* single-deletion mutant (DPS16) revealed that both mutants showed a slight but statistically significant decrease in chemotaxis to 5 mM citrate compared with the wild-type strain ($P < 0.05$ by Student's *t*- test) (Fig. 2C). Double deletion of these *mcp* genes resulted in a marked reduction in the response to citrate. Introduction of pPS05 (harboring the RS_RS07350 homolog) and pPS16 (harboring *mcpP*) restored the ability of the double mutant DPS0516 to respond to citrate (Fig. 2C), demonstrating that the RS_RS07350 homolog and McpP are MCPs for citrate in *R. pseudosolanacearum* Ps29. We designated the RS_RS07350 homolog as *mcpC* (MCP for citrate).

Ligand specificity of McpC and McpP We next investigated the ligand specificities of McpC and McpP. We had already reported that McpP senses not only phosphate as an attractant but also maleate as a repellent (16). To assess whether McpC and McpP are involved in chemotaxis to other organic acids, we examined an *mcpC mcpP* double-deletion mutant (DPS0516) for chemotactic responses to organic acids identified as *R. pseudosolanacearum* Ps29 chemoattractants, including L-malate, D-malate, succinate, fumarate, L-tartrate, and D-tartrate. The DPS0516 strain showed responses to each compound comparable to those of wild-type strain Ps29 (Fig. S2), suggesting that McpC and McpP are not involved in chemotaxis to these organic acids. Although this result does not completely rule out the possibility that McpC and McpP are capable of sensing these compounds, it does clearly indicate that McpC and McpP are primarily chemoreceptors for citrate in *R. pseudosolanacearum* Ps29.

Citrate is known to form complexes with divalent metal cations such as magnesium and calcium (24). As several citrate chemoreceptors are known to also recognize metal cation–citrate complexes (25,26), the involvement of McpC and McpP in chemotaxis to metal cation–citrate complexes was investigated. Wild-type *R. pseudosolanacearum* Ps29 showed chemotactic responses to Mg^{2+}- and Ca^{2+}-citrate complexes, although these responses were not as strong as the response to free citrate (Fig. 3A). This reduction in the strength of the chemotactic response in the presence of metal ions was not observed in the analysis of chemotaxis to L-alanine, which does not form complexes with metal ions (Fig. 3A). These results suggest that metal cation–citrate complexes elicit weaker chemotactic responses than free citrate in *R. pseudosolanacearum* Ps29. In the *mcpC mcpP* double-deletion mutant (DPS0516), there were no significant differences between the responses to Mg^{2+}- and Ca^{2+}-citrate complexes versus the control buffer. Introduction of plasmid pPS05, which harbors the Ps29 *mcpC* gene, restored the ability of strain DPS0516 to respond to both Mg^{2+}- and Ca^{2+}-citrate complexes (Fig. 3B), demonstrating that McpC senses metal cation–citrate complexes as well as free citrate. Conversely, introduction of plasmid pPS16, which harbors the Ps29 *mcpP* gene, did not enable strain DPS0516 to respond to metal cation–citrate complexes (Fig. 3B), suggesting that McpP is specific for free citrate.

Distribution of McpC and McpP homologous proteins The predicted structures of McpC and McpP are typical of MCPs. Dense alignment surface analysis (27) identified the ligand-binding domain (LBD) of McpC and McpP as a region spanning 159 amino acids (residues 31 to 189) and 168 amino acids (residues 29 to 187), respectively. Protein BLAST analyses using the putative LBD sequences of McpC and McpP as queries revealed that MCPs with LBDs similar to those of McpC and McpP are distributed among members of the *R. solanacearum* species complex and that those LBDs are highly similar (90–100% identity) to the *R. pseudosolanacearum* Ps29 McpP and McpC LBDs. Homologous proteins with LBDs similar to that of McpC are also distributed among other *Ralstonia* species, including *Ralstonia insidiosa*, *Ralstonia pickettii*, and *Ralstonia mannitolilytica*, *Mumia flava*, *Capriavidus* sp., and *Burkholderiaceae* sp. However, these LBDs are less similar to that of Ps29 McpC (53–80% identity). Both *R. pickettii* and *R. mannitolilytica* as well as the blood disease bacterium were found to possess MCPs with LBDs similar to that of McpP (31–75% identity). These results suggest that proteins homologous to McpC and McpP are differentially distributed among *Ralstonia* species and related genera.

Relationship between citrate chemotaxis and plant infection The role of citrate chemotaxis in bacterial wilt disease of tomato was investigated. In these experiments, the highly virulent *R. pseudosolanacearum* MAFF106611 strain was used

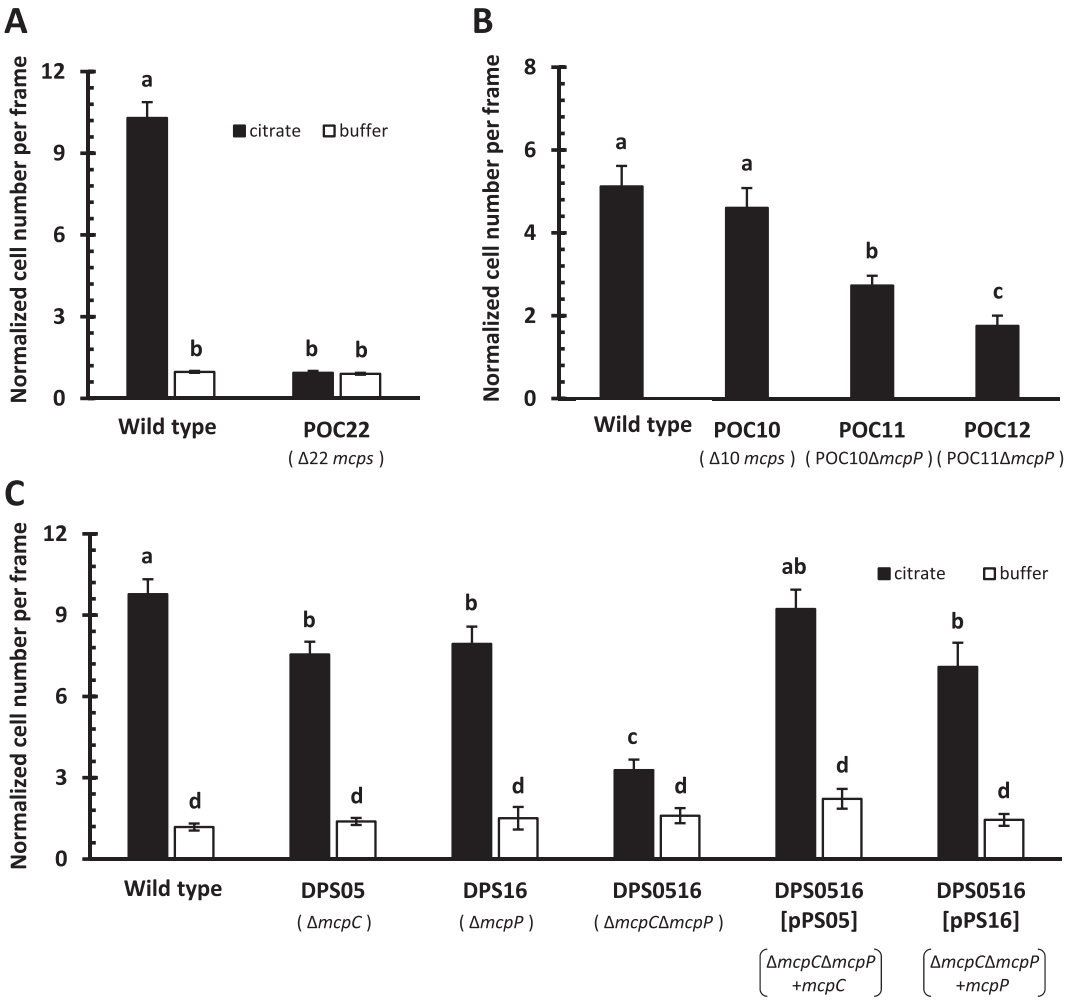

FIG. 2. Identification of MCPs for citrate in *R. pseudosolanacearum* Ps29. (A) Chemotaxis to 5 mM citrate (closed bars) and 10 mM HEPES buffer as a negative control (open bars) by the wild-type strain and total-*mcp*-deletion mutant (POC22). (B) Chemotaxis to 0.5 mM citrate by the wild-type Ps29 and multiple-*mcp*-deletion mutants (POC*n*, where *n* represents the number of deleted *mcps*). (C) Chemotaxis to 5 mM citrate (closed bars) and 10 mM HEPES buffer as a negative control (open bars) by the wild-type strain, DPS05, DPS16, DPS0516, and DPS0516 harboring complementing plasmids. Vertical bars represent the standard error of at least triplicate measurements. Different letters indicate significant differences ($P < 0.05$ by Student's *t*-test).

instead of strain Ps29. PCR and DNA sequencing analyses of strain MAFF106611 demonstrated the presence of *mcpC* and *mcpP*, the respective products of which are 99% identical to their Ps29 counterparts. The *mcpC* deletion mutant (DMF05), *mcpP* deletion mutant (DMF16), and *mcpC mcpP* double-deletion mutant (DMF0516) showed chemotactic phenotypes similar to the strain Ps29 mutants (Fig. S3). We examined plant infection by the mutants using the sand-soak inoculation method (Fig. 4), in which bacteria are inoculated into sand 3 cm away from a tomato seedling. Infection of plants in this assay therefore requires the bacteria to locate the host plants from a distance and move to them to invade. Wild-type strain MAFF106611 started wilting at 4 days postinoculation (dpi) and killed 80% of the tomato plants by 12 dpi. The time line of wilting in response to challenge with strain DMF05, DMF16, or DMF0516 was similar to that of the wild-type parent. We also conducted a competitive tomato colonization assay by sand soak inoculating tomato seedlings with a 1:1 mixture of strain DMF0516 and a kanamycin-resistant strain (MFK) as a competitor that competed fully with the wild-type strain (Fig. S4A). Strain DMF0516 showed the same level of competitive plant colonization with MFK as the wild-type strain (Fig. S4B), consistent with the results of virulence assays. These results suggest that citrate chemotaxis mediated by McpC and McpP does not play a crucial role in initial localization of plant roots by *R. pseudosolanacearum* MAFF106611.

DISCUSSION

In this study, we identified McpC and McpP as chemoreceptors for citrate using a library of *R. pseudosolanacearum* Ps29 multiple-*mcp*-gene deletion mutants. In addition, we showed that McpC can sense citrate in complexes with Mg^{2+} and Ca^{2+} as well as free citrate, although McpP, which has been identified as being involved in both positive chemotaxis to phosphate and negative chemotaxis to maleate (16), cannot sense metal cation–citrate complexes. Analysis of chemotaxis using an *mcpC mcpP* double-deletion mutant (DPS0516) suggested that these two MCPs are not involved in chemotaxis to other organic acids (e.g., malate, tartrate, succinate, and fumarate). Thus, McpC and McpP, both of which act as a major MCP for citrate, have some differences in terms of ligand specificity. The LBDs of bacterial MCPs can be classified according to their sizes into cluster-I (120–210 amino acids) and cluster-II (220–299 amino acids) domains (28). The LBDs of McpC and McpP exhibit sequence similarity of 29%. Both of these LBDs belongs to the cluster-I group and are annotated as

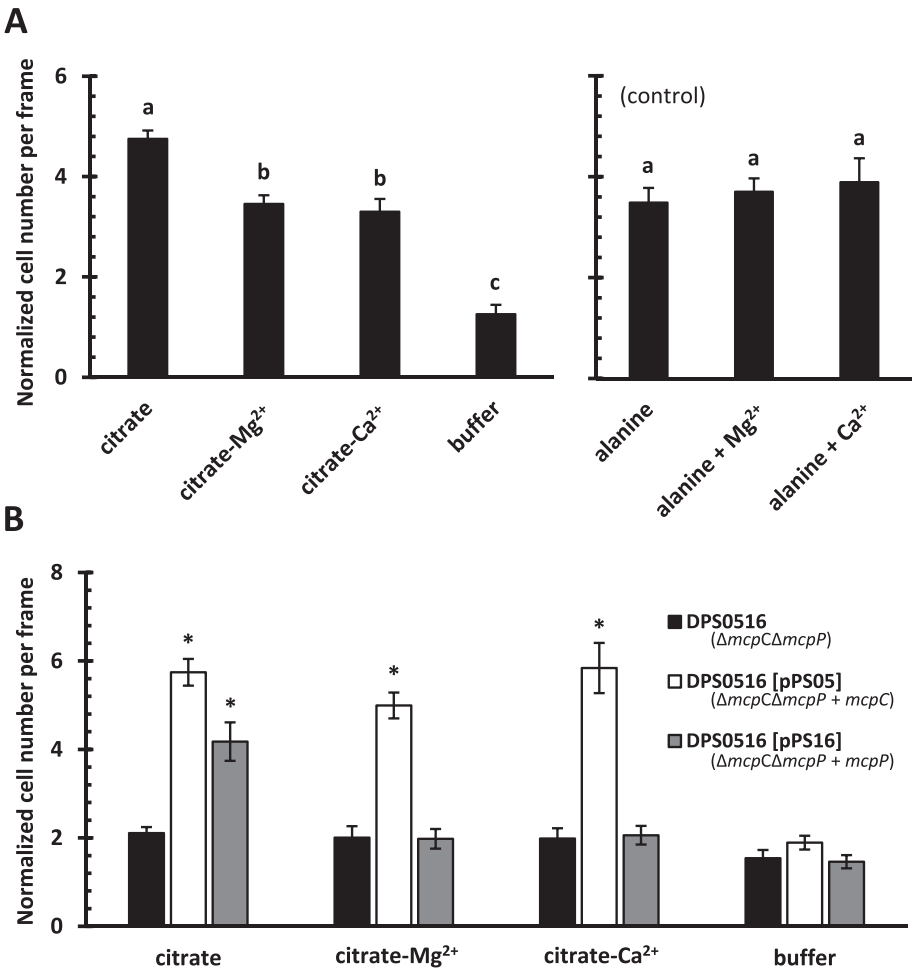

FIG. 3. Chemotactic responses to citrate and metal cation–citrate complexes by *R. pseudosolanacearum* Ps29 strains. Mg^{2+}- and Ca^{2+}-citrate were 0.5 mM citrate with 10 mM $MgCl_2$ or $CaCl_2$, respectively. In measurements of chemotaxis to metal chloride compounds, the cell suspension was supplemented with 1 mM $MgCl_2$ or $CaCl_2$. (A) Chemotaxis of the wild-type strain. As a control, 0.5 mM L-alanine with and without 10 mM metal chloride were used. Different letters indicate significant differences ($P < 0.05$ by Student's *t*- test). (B) Chemotaxis of DPS0516 (closed bars) and DPS0516 strain harboring pPS05 (open bars) and pPS16 (gray bars). Vertical bars represent the standard error of at least six measurements. Asterisks indicate significant differences in the chemotactic responses between DPS0516 and complemented strains ($P < 0.05$ by Student's *t*-test).

forming 4-helix bundles (4HB) in Pfam and InterPro. Protein structure predictions using the Phyre^2 fold recognition server (29) also suggested the presence of a 4HB domain in the *R. pseudosolanacearum* Ps29 McpC and McpP LBDs (Fig. S5).

To date, MCPs for citrate have been reported in several bacteria. McpS of *P. putida* KT2440 (30) and McfS of *P. putida* F1 (31) are citrate receptors with a broad ligand range, and they sense not only citrate but also other TCA cycle intermediates such as malate, succinate, and fumarate. MCP2201 and MCP2901 of *Comamonas testosteroni* CNB-1 also recognize many compounds, such as aromatic compounds and/or TCA cycle intermediates, including citrate (32,33). Conversely, citrate MCPs with a narrow ligand range also have been identified. Citrate chemotaxis of *Salmonella enterica* serovar Typhimurium is mediated by the Tcp receptor, which can sense both free citrate and Mg^{2+}-citrate complexes as attractants (similar to McpC) and phenol as a repellent (similar to McpP). The Tcp receptor belongs to cluster I based on the size of the LBD (160 amino acids), which forms a 4HB, similar to McpC and McpP of *R. pseudosolanacearum* Ps29. Martín-Mora et al. (25) showed that the McpS paralogue McpQ of *P. putida* KT2440 is also chemoreceptor specific to citrate and that it mediates chemotaxis preferentially to citrate in complex with Mg^{2+} or Ca^{2+}, whereas McpS cannot recognize these metal cation–citrate complexes (24). In contrast to the Tcp receptor of *S. enterica* and McpC and McpP of *R. pseudosolanacearum*, McpQ (with an LBD containing 253 amino acids) of *P. putida* KT2440 falls into cluster II, and its LBD assumes a helical-bimodular fold. Thus, there are some similarities and differences between known citrate MCPs and McpC and McpP of *R. pseudosolanacearum*.

Chemotaxis plays an important role in plant–microbe interactions such as nodulation by *Rhizobium leguminosarum* (34), root colonization by *P. fluorescens* (35) and *Bacillus subtilis* (36), and plant infection by *Dickeya dadantii* (37) and the *R. solanacearum* species complex (7). These soil-borne bacteria are believed to locate plant roots by sensing root exudate. In a previous study, we demonstrated that chemotaxis to L-malate, which is a major component of plant root exudate (17), facilitates migration of *R. pseudosolanacearum* to tomato plants (13). As citrate is also a strong chemoattractant and major component of root exudate, it seems likely that citrate also serves as a chemotactic signaling compound that enables bacteria to locate and interact with plant roots. However, the results of the present virulence assay and competitive plant colonization assay using the sand-soak inoculation method revealed that the infectivity and competitive colonization ability of the *mcpC mcpP* double-deletion mutant of MAFF106611 (DMF0516) did not differ significantly in comparison with the wild-type strain. Several possibilities can be considered from these results. (i) The most likely possibility is that the concentration of citrate released from the tomato roots was

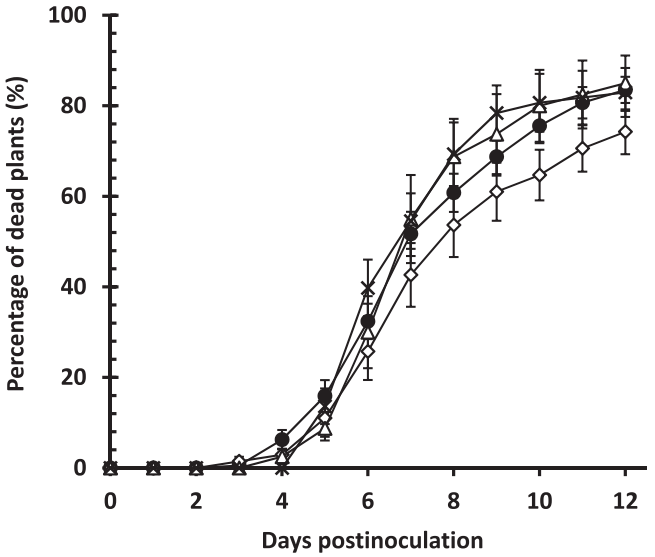

FIG. 4. Sand-soak inoculation virulence assay of *R. pseudosolanacearum* MAFF106611 strains on tomato seedlings. Wild-type strain (closed circles); DMF05, *mcpC* deletion mutant (open diamonds); DMF16, *mcpP* deletion mutant (open triangles); DMF0516, *mcpC mcpP* double-deletion mutant (crosses). In each experiment, eight tomato seedlings were examined and observed to calculate the percentage of dead plants. Means and standard errors were calculated from at least nine independent experiments. There were no significant differences between the wild-type strain and *mcp* mutants ($P < 0.05$ by Student's *t*-test).

too low in this assay system, while tomato exudate reportedly contains 3–110 μg citrate/plant (46–70% of the total pool of organic acids), depending on the stage of plant growth and cultivation conditions (17). (ii) It is possible that citrate chemotaxis of *R. pseudosolanacearum* does not play an important role in the soil environment. Because citrate forms complexes with metal cations such as Mg^{2+} and Ca^{2+}, which are abundant in soil (38,39) and plant root exudate (40,41), citrate could be primarily present as a metal complex in soil environments. The magnitude of responses to both Mg^{2+}- and Ca^{2+}-citrate complexes mediated by McpC were lower than the response to free citrate mediated by both McpC and McpP in wild-type *R. pseudosolanacearum* Ps29. This phenomenon is completely opposite to that of *P. putida* KT2440, which showed a response of much greater magnitude to Mg^{2+}-citrate than free citrate (25). (iii) Alternatively, differences in expression levels of *mcp* genes in plant assays versus chemotaxis assays could cause unexpected results in plant experiments. López-Farfán and co-workers (42) recently demonstrated that environmental conditions determine *mcp* expression levels in *P. putida* KT2440 as a model bacterium with a large number of chemoreceptors. The DPS0516 mutant still exhibited a moderate response to citrate (Fig. 2C), clearly indicating the presence of citrate receptor(s) other than McpC and McpP. In the plant assay system, if the unidentified citrate MCP(s) was/were expressed at much higher levels than in the chemotaxis assay, the DMF0516 mutant could have responded strongly to citrate released from tomato roots. To form a final conclusion regarding the involvement of citrate chemotaxis in plant infection by members of the *R. solanacearum* species complex, identification of all citrate receptors, including minor MCP(s), will be needed.

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