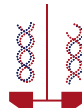

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Negative chemotaxis of *Ralstonia pseudosolanacearum* to maleate and identification of the maleate chemosensory protein

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Ralstonia pseudosolanacearum **Ps29 was repelled by maleate. Screening of a complete collection of Ps29 single-methyl-accepting chemotaxis protein (*mcp*) gene mutants identified the RSp0303 homolog (McpP) as a chemotaxis sensor mediating negative chemotaxis to maleate. Interestingly, the *mcpP*-deletion mutant was attracted to maleate, indicating that this bacterium expresses a MCP(s) for both positive and negative chemotaxis to maleate. We constructed a Ps29 derivative (designated POC14) harboring deletions in 14 individual *mcp* genes, including *mcpP*, to characterize McpP. Introduction of a plasmid harboring the *mcpP* gene (pPS16) restored the ability to negatively respond to maleate, confirming that McpP is a MCP for negative chemotaxis to maleate. We thought that maleate might be applied to controlling plant infection by *R. pseudosolanacearum*. To evaluate this possibility, we measured chemotactic responses of seven other virulent *R. pseudosolanacearum* strains to maleate. We confirmed that they harbored functional *mcpP* orthologues, but they showed no chemotactic responses to maleate. Quantitative RT-PCR analysis revealed that these seven *R. pseudosolanacearum* strains did not show negative chemotaxis to maleate because of negligible transcription of the *mcpP* genes. We compared the chemotactic responses of POC14 and POC14[pPS16] toward various chemicals and found that McpP senses inorganic phosphate as a chemoattractant.**

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[**Key words:** Chemotaxis; Chemosensory protein; *Ralstonia pseudosolanacearum*; Plant pathogen; Repellent]

Chemotaxis, the movement of an organism toward or away from a chemical, guides organisms to niches with more optimal environmental conditions (1). Bacterial chemotaxis can be regarded as an important prelude to metabolism, prey–predator relationships, symbiosis, infection, and other ecological interactions (2,3). The molecular mechanisms that underlie bacterial chemotaxis have been studied intensively in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (4,5). Chemoattractants and chemorepellents are detected by cell surface chemoreceptors called methyl-accepting chemotaxis proteins (MCPs). Upon binding a chemotactic ligand, a MCP generates chemotaxis signals that are communicated to the flagellar motor via a series of chemotaxis (Che) proteins. *E. coli* possesses 5 MCPs and 6 Che proteins (CheA, CheB, CheR, CheW, CheY, and CheZ).

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), is a group of gram-negative bacterial plant pathogens that cause bacterial wilt in various economically important crops (6,7). This pathogen generally invades host roots through wounds or natural openings of the roots, from which the pathogen then invades the xylem vessels and spreads up into the stem and leaves (8). The members of the *R. solanacearum* species complex are motile and show positive chemotaxis to various compounds, including amino acids, organic acids, sugars, and inorganic phosphate (9,10). Complete genome sequences have been determined for several strains within the *R. solanacearum* species complex (11). Genomic analyses revealed that each of these strains encodes a set of Che proteins and >20 MCPs. Yao and Allen demonstrated that chemotaxis is required for full virulence in *R. solanacearum* K60 (phylotype II) and that this bacterium depends on chemotaxis to locate and colonize plant roots (10,12). They also reported that aerotaxis (energy taxis) contributes to the ability of *R. solanacearum* K60 to locate and effectively interact with host plants. We identified MCPs for amino acids (McpA), L-malate (McpM), and L-tartrate (McpT) in *R. pseudosolanacearum* Ps29 and MAFF106611 (phylotype I) and found that McpM-mediated chemotaxis is required for full virulence of *R. pseudosolanacearum* MAFF106611 in tomato plants (9,13).

Negative chemotaxis, the movement of an organism away from a chemical, has been reported in several bacteria, including *E. coli*, *S. enterica* serovar Typhimurium, *Bacillus subtilis*, and *Pseudomonas aeruginosa* (5,14–16). However, to our knowledge, no paper has reported negative chemotaxis by members of the *R. solanacearum* species complex. In the course of studying D-malate chemotaxis (13), we found that maleate repelled *R. pseudosolanacearum* Ps29. In the present study, we report negative chemotaxis of

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R. pseudosolanacearum Ps29 to maleate and the identification and characterization of its chemotaxis sensor.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions The bacterial strains and plasmids used in this study are listed in Table 1. *R. pseudosolanacearum* strains Ps29, M4S, and Ps72 were obtained from the Leaf Tobacco Center, Japan Tobacco, Inc. (17), and strains MAFF106603, 106611, 211270, 730103, and 730138 were obtained from the National Institute of Agrobiological Sciences, Japan (17). *R. pseudosolanacearum* strains and their derivatives were cultivated at 28 °C in *R. solanacearum* minimal (RSM) medium for 20 h or in rich CPG medium for 16 h. RSM medium contained 1.75 g/l K_2HPO_4, 0.75 g/l, KH_2PO_4, 0.15 g/l trisodium citrate dihydrate, 1.25 g/l $(NH_4)_2SO_4$, 0.25 g/l $MgSO_4 \cdot 7H_2O$, and 5 g/l glucose. RSM medium was used for growth inhibition tests of maleate. *E. coli* strains were grown at 37 °C in 2X YT medium for 16 h. For plasmid construction and maintenance, kanamycin was provided at 50 μg ml^{-1}.

Quantitative chemotaxis assay Computer-assisted capillary assays were carried out as described previously (18). 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 a 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 over 3 min. 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 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). The chemotaxis buffer was 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-ethanesulfonic acid) (pH 7.0).

DNA manipulation Standard techniques were used for plasmid DNA preparation, restriction enzyme digestion, ligation, transformation, and agarose gel electrophoresis (19). PCR was carried out using KOD Plus Neo polymerase (Toyobo, Tokyo, Japan) according to the manufacturer's instructions. Plasmids were introduced into the *R. pseudosolanacearum* strains by transconjugation using *E. coli* S17-1 (20) or by electroporation, as described previously (9).

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

Strain or plasmid	Relevant characteristic(s)	References
Strains		
Ralstonia pseudosolanacearum		
Ps29	Wild-type strain; race 1, biovar 3, phylotype I	17
DPS16	Ps29 derivative; *ΔmcpP* (LC005241)	9
POC14	Ps29 derivative; *ΔmcpA ΔmcpO2 Δmcp05 Δmcp09 Δmcp10 Δmcp11 Δmcp12 ΔmcpM Δmcp15 ΔmcpP Δmcp17 Δmcp18 Δmcp19 Δmcp22*	This study
M4S	Wild-type strain; race 1, phylotype I	17
Ps72	Wild-type strain; race 1, phylotype I	17
MAFF106603	Wild-type strain; race 1, biovar 3, phylotype I	17
MAFF106611	Wild-type strain; race 1, biovar 4, phylotype I	17
MAFF211270	Wild-type strain; race 1, biovar N2, phylotype I	17
MAFF730103	Wild-type strain; race 1, biovar 4, phylotype I	17
MAFF730138	Wild-type strain; race 1, biovar 3, phylotype I	17
Escherichia coli		
JM109	*reaA1 endA1 gyrA96 thi-1 hsdR17*($r_K^- m_K^+$) *e14* negative (*mcrA* negative) *supE44 relA1* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	19
S17-1	MM294 derivative, RP4-2 Tc::Mu-Km::Tn7; chromosomally integrated	20
Plasmids		
pK18mobsacB	Km^r pUC18 derivative; *lacZα mob* site *sacB*	21
pRCII	*E. coli-Ralstonia* shuttle vector derived from pKZ27; IncQ *lac* promoter Km^r	9
pPS16	pRCII with a 1.7-kb PCR fragment including *mcpP* in Ps29	This study

Construction of plasmids for complementation Previously constructed pRCII was used as the plasmid backbone for complementation analysis of *R. pseudosolanacearum* mutants (9). To construct pPS16, primers 5′-ATGAATTCA-GCGGCACTAAAGGTGTGG-3′ and 5′-ATGGATCCAGCGCATTGCCTACGAGTC-3′ were used to amplify the RSp0303 homolog gene of *R. pseudosolanacearum* Ps29. The 1.7-kb PCR product was cloned into *Eco*RI and *Bam*HI sites of pRCII.

Construction of unmarked multiple-deletion mutants Fourteen *mcp* genes in *R. pseudosolanacearum* Ps29 were sequentially deleted using an unmarked gene-deletion technique as described previously (9). Derivatives of the suicide plasmid pK18*sacB* (21), which had been used for construction of a *mcp* single-deletion mutant library of *R. pseudosolanacearum* Ps29 in a previous study (9), were used for unmarked multiple deletion of *mcpA*, *mcpM*, *mcpP* and the RSc1155, RSc1460, RSc3136, RSc3307, RSc3412, RSc0671, RSp0840, RSp1027, RSp1099, RSp1209, and RSp1406 homologs in *R. pseudosolanacearum* Ps29. The resulting deletion mutant was designated *R. pseudosolanacearum* POC14.

RNA extraction and qrt-RT-PCR Cells grown in RSM medium for 20 h without any supplementation were harvested, and total RNA was extracted using a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Two-stage quantitative real-time (qrt) reverse transcriptase PCR (RT-PCR) was performed. First, cDNA was generated from total RNA using ReverTraAce qPCR RT Master Mix with gDNA remover (Toyobo). The cDNA was later applied in the qrt-PCR stage using KOD SYBR qPCR Mix (Toyobo) on a LightCycler (version 1.5) thermocycler (Roche Diagnostics, Basel, Switzerland). Thermal cycling conditions for qrt-PCR were as follows: 98 °C (2 min), followed by 40 cycles of 98 °C (10 s), 60 °C (10 s), and 68 °C (30 s) using primers for *mcpP* or *gyrB*. The gene expression data were normalized to the level of expression of the endogenous reference *gyrB* gene and are reported as the relative value. The *mcpP* primers were 5′-TGCTCTTCGTTCTGGCTGTC-3′/5′-GCCAGGTTGTTGGAATAGGTTTC-3′, designed in the ligand binding domain (LBD) region to give an expected product size of 106 bp. The primers for *gyrB* were 5′-TTGTCGGCGATGTACTTGTTG-3′/5′-GAGCAGGTGCTCTGCTTCAC-3′, giving an expected product size of 110 bp.

Nucleotide sequence accession numbers The nucleotide sequences of the *mcpP* genes and its upstream in *R. pseudosolanacearum* strains have been deposited in the DDBJ, EMBL-Bank, and GenBank nucleotide sequence databases under accession numbers MF138064 (Ps29), MF138065 (Ps72), MF138066 (M4S), MF138067 (MAFF106603), MF138068 (MAFF106611), MF138069 (MAFF211270), MF138070 (MAFF730103) and MF138071 (MAFF730138).

RESULTS

Negative chemotaxis to maleate The chemotactic responses to maleate of *R. pseudosolanacearum* Ps29 were assayed using a computer-assisted capillary method (18) that generates reproducible quantitative data regarding bacterial behavioral responses to both attractants and repellents. The number of bacteria near the mouth of a capillary containing HEPES buffer (negative control) increased slightly, which is a normal phenotypic response of this strain. The movement of bacteria away from maleate was so rapid that the number of cells near the mouth of the capillary containing 5 mM maleate decreased by approximately 60% within the first 30 s (Fig. 1A). Fig. 1B shows that the negative chemotactic response to maleate increased in a concentration-dependent manner.

Most reported repellents are indeed harmful to bacteria (14). We therefore examined whether maleate is toxic to *R. pseudosolanacearum* Ps29 by monitoring bacterial growth. Fig. 2 shows that maleate only moderately inhibited the growth of strain Ps29, with inhibition of 30 and 45% after 24 h of cultivation in RSM medium containing maleate at 5 and 10 mM, respectively.

Identification of the maleate MCP Analysis of the genome sequence of *R. pseudosolanacearum* GMI1000 revealed that it harbors 22 putative *mcp* genes. In a previous study, we demonstrated that *R. pseudosolanacearum* Ps29 harbors homologs of 22 GMI1000 *mcp* genes (9). To identify the gene encoding the maleate MCP, we examined a library of 22 *R. pseudosolanacearum* Ps29 *mcp* single-deletion mutants (9) for their ability to respond to maleate. Strain DSP16, in which the RSp0303 ortholog was deleted, was not repelled by maleate (Fig. 3). The remaining 21 mutants exhibited negative chemotaxis to maleate comparable to that of wild-type Ps29 (data not shown). Introduction of a plasmid

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