Identification of Chemotaxis Compounds in Root Exudates and Their Sensing Chemoreceptors in Plant-Growth-Promoting Rhizobacteria *Bacillus amyloliquefaciens* SQR9

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Chemotaxis-mediated response to root exudates, initiated by sensing-specific ligands through methyl-accepting chemotaxis proteins (MCP), is very important for root colonization and beneficial functions of plant-growth-promoting rhizobacteria (PGPR). Systematic identification of chemoattractants in complex root exudates and their sensing chemoreceptors in PGPR is helpful for enhancing their recruitment and colonization. In this study, 39 chemoattractants and 5 chemorepellents, including amino acids, organic acids, and sugars, were identified from 98 tested components of root exudates for the well-studied PGPR strain *Bacillus amyloliquefaciens* SQR9. Interestingly, mutant strain SQR9 Δ mcp, with all eight putative chemoreceptors completely deleted, lost the chemotactic responses to those 44 compounds. Gene complementation, chemotaxis assay, and isothermal titration calorimetry analysis revealed that McpA was mainly responsible for sensing organic acids and amino acids, while McpC was mostly for amino acids. These two chemoreceptors may play important roles in the rhizosphere chemotaxis of SQR9. In contrast, the *B. amyloliquefaciens*-unique chemoreceptor McpR was specifically responsible for arginine, and residues Tyr-78, Thr-131, and Asp-162 were critical for arginine binding. This study not only deepened our

insights into PGPR–root interaction but also provided useful information to enhance the rhizosphere chemotaxis mobility and colonization of PGPR, which will promote their application in agricultural production.

Plant-growth-promoting rhizobacteria (PGPR) are widely used in agricultural production for stimulating plant growth and suppressing soilborne diseases (Lugtenberg and Kamilova 2009), but the performance of their plant-beneficial effects depends on efficient rhizosphere colonization (Compant et al. 2010). Chemotaxis, the ability of motile bacteria to direct their movement in gradients of attractants and repellents, plays an important role during the rhizosphere colonization process of PGPR. In the rhizosphere, a nutrient gradient is formed by plant-released root exudates, which have a complex composition and include some components that may serve as chemoattractants or chemorepellents for plant-beneficial microbes (Badri and Vivanco 2009; Liu et al. 2017). Root exudate-mediated chemotaxis is recognized as the initial step of the recruitment and colonization process (de Weert et al. 2002; Scharf et al. 2016; Zhang et al. 2014), which enhances the ability of bacteria to colonize the roots of diverse plant hosts (Bais et al. 2006; Berendsen et al. 2012; Caetano-Anollés et al. 1988; Scharf et al. 2016).

Bacterial chemotaxis is triggered when a stimulating molecule binds to its cognate chemoreceptor. Typical chemoreceptors are composed of a methyl-accepting (MA) domain, a cytosolic signaling domain, a HAMP (histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein [MCP] and phosphatase) linker (Aravind and Ponting 1999), and a ligand-binding domain (LBD), which is frequently located in the extracytoplasmic space and is responsible for binding extracellular compounds (Baker et al. 2006; Milburn et al. 1991). The binding of the ligand to LBD modulates the autophosphorylation of the histidine kinase CheA, which in turn transfers the phosphoryl groups to the response regulator CheY. Subsequently, the generated CheY-P permits its interaction with the flagellar motor to control cell swimming or tumbling to ultimately mediate chemotaxis (Sourjik and Wingreen 2012).

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A large number of chemoreceptors and their corresponding ligands have been characterized in different microorganisms, including *Escherichia coli*, *Pseudomonas putida*, *Bacillus subtilis*, and *Sinorhizobium meliloti* (Garrity and Ordal 1995; Sampedro et al. 2015; Sourjik 2004; Webb et al. 2017; Yssel et al. 2011). Five chemoreceptors (Tar, Tsr, Tap, Trg, and Aer) have been identified in *E. coli*, which responds to concentration gradients of amino acids, dipeptides, and sugars (Sourjik 2004). Twenty-seven MCP from the free-living environmental bacterium *P. putida* KT2440 have been characterized, while McpS, McpP, and McpQ are found to be chemoreceptors for tri-carboxylic acid cycle intermediates, C₂ and C₃ carboxylic acids, and citrate/metal ion complexes, respectively (Sampedro et al. 2015). The gram-positive model bacterium *B. subtilis* possesses 10 MCP, including McpA, McpB, McpC, TlpA, TlpB, TlpC, HemAT, YfmS, YvaQ, and YoaH (Mowbray and Sandgren 1998; Sourjik 2004). McpB is found to be the sole chemoreceptor for asparagine, and McpC can directly bind 11 amino acids (proline, threonine, glycine, serine, valine, alanine, tyrosine, isoleucine, phenylalanine, leucine, and histidine) and indirectly sense four others (lysine, arginine, methionine, and glutamine). The myoglobin-like transducer HemAT is responsible for aerotaxis (Hou et al. 2000).

Bacterial chemotaxis toward different plant-secreted molecules mediated by specific chemoreceptors in the establishment of plant–microbe interactions has been demonstrated. The chemoreceptor McpU in the alfalfa symbiont *S. meliloti* governed chemotaxis toward host plant root exudates following colonization through direct proline sensing (Webb et al. 2014). Similarly, the chemotaxis receptor IcpB sensing oxygen in *Azorhizobium caulinodans* modulated nodulation and nitrogen fixation on the stems and roots of *Sesbania rostrata* (Jiang et al. 2016). The CtaA, CtaB, and CtaC receptors in *P. fluorescens* Pf0-1 are responsible for sensing amino acids in root exudates and play an important role in root colonization (Oku et al. 2012). Recently, multiple chemotaxis receptors (including McpB, McpC, and TlpC) were found to be involved in the intact

chemotaxis of *B. subtilis* 3610 to *Arabidopsis thaliana* root exudates, and this process was required for bacterial colonization (Allard-Massicotte et al. 2016).

However, the chemical composition of root exudates is extremely complex, and the chemotaxis behavior of PGPR to root exudates is an integrative effect of all root-secreted chemoattractants and chemorepellents. Systematic identification of chemoattractants and chemorepellents in root exudates and elucidation of how these various root-secreted compounds are sensed by multiple chemoreceptors of a PGPR strain could give us a comprehensive understanding of PGPR chemotaxis in the rhizosphere. In this study, *B. amyloliquefaciens* SQR9, a well-studied and commercially widely used PGPR strain, which showed chemotaxis toward root exudates and formed obvious biofilms on plant roots (Liu et al. 2017; Weng et al. 2013), was used to explore the chemotaxis compounds in root exudates. The objectives of this study were to (i) detect the chemoattractants (or chemorepellents) in root exudates sensed by SQR9 and (ii) identify the SQR9 chemoreceptors involved that were responsible for these detected compounds. These results will provide guidelines to enhance the rhizosphere colonization and beneficial function of PGPR in agricultural production.

RESULTS

Identification of chemotactic compounds for PGPR strain *B. amyloliquefaciens* SQR9 in root exudates.

B. amyloliquefaciens SQR9 (Table 1) was isolated from the cucumber rhizosphere and showed positive chemotactic responses to root exudates (Liu et al. 2017; Weng et al. 2013). Also, the transcription of its chemotaxis and motility genes was upregulated by root exudates (Zhang et al. 2015). In this study, to have a comprehensive understanding of the chemoattractants in root exudates, 98 commercially available chemical compounds were selected based on results of high-throughput analysis of cucumber root exudates conducted by Liu et al. (2017); this analysis included sugars, sugar alcohols, sugar

Table 1. Bacterial strains and plasmids used in this study

Strains or plasmid	Characteristics ^a	Source or reference
Plasmids		
pTPC	pMD19-T harboring the PC cassette	Zhou et al. 2017
pNW33N	Cm ^R , <i>Escherichia coli</i> – <i>Bacillus</i> shuttle vector	Zhou et al. 2017
p7S6	Spc ^R , pMD18-T ligated with <i>spc</i> gene	Yan et al. 2008
pET-28a	Expression vector	Novagen
pET-28a-McpALBD	pET28a derivative for expression of McpALBD	This work
pET-28a-McpBLBD	pET28a derivative for expression of McpBLBD	This work
pET-28a-McpCLBD	pET28a derivative for expression of McpCLBD	This work
pET-28a-TlpALBD	pET28a derivative for expression of TlpALBD	This work
pET-28a-TlpBLBD	pET28a derivative for expression of TlpBLBD	This work
pET-28a-McpRLBD	pET28a derivative for expression of McpRLBD	This work
Strains		
<i>Bacillus amyloliquefaciens</i>		
SQR9	Wild-type isolate	Cao et al. 2011
SQR9Δ <i>mcp</i>	Deletion in all eight putative <i>mcp</i> genes	This work
SQR9Δ <i>mcp</i> /mcpA	Cm ^R , SQR9Δ <i>mcp</i> with <i>mcpA</i> gene	This work
SQR9Δ <i>mcp</i> /mcpB	Cm ^R , SQR9Δ <i>mcp</i> with <i>mcpB</i> gene	This work
SQR9Δ <i>mcp</i> /mcpC	Spc ^R , SQR9Δ <i>mcp</i> with <i>mcpC</i> gene	This work
SQR9Δ <i>mcp</i> /tlpA	Cm ^R , SQR9Δ <i>mcp</i> with <i>tlpA</i> gene	This work
SQR9Δ <i>mcp</i> /tlpB	Cm ^R , SQR9Δ <i>mcp</i> with <i>tlpB</i> gene	This work
SQR9Δ <i>mcp</i> /mcpR	Spc ^R , SQR9Δ <i>mcp</i> with <i>mcpR</i> gene	This work
SQR9Δ <i>mcp</i> /hemAT	Cm ^R , SQR9Δ <i>mcp</i> with <i>hemAT</i> gene	This work
SQR9Δ <i>mcp</i> /yfmS	Cm ^R , SQR9Δ <i>mcp</i> with <i>yfmS</i> gene	This work
<i>E. coli</i>		
BL21 (DE3)	F [−] <i>ompT</i> <i>hsdSB</i> (rB [−] mB [−]) <i>gal</i> <i>dcm</i> λDE3 (harboring gene 1 of the RNA polymerase from the phage T7 under the <i>PlacUV5</i> promoter)	Invitrogen
Top 10	F [−] <i>mcrA</i> Δ(<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) ψ80 <i>lacZ</i> ΔM15 Δ <i>lacX74</i> <i>nupG</i> <i>recA1</i> <i>araD139</i> Δ(<i>ara-leu</i>) 7697 <i>galE15</i> <i>galK</i> 16 <i>rpsL</i> (Str ^R) <i>end</i> A1λ [−]	Invitrogen

^a Cm^R = chloramphenicol resistance, Spc^R = spectinomycin resistance, and Str^R = streptomycin resistance.

acids, amines, amino acids, fatty acids, organic acids, and others (Table 2). These chemicals were tested individually for the chemotactic response of SQR9 using a simple and reusable microfluidic SlipChip device, as described by Shen et al. (2014). Results showed that SQR9 has a chemotactic response to 44 of these 98 tested compounds (Table 2; Supplementary Table S1). Among them, 23 compounds were strong chemoattractants, with a chemotaxis index (I_{30}) of more than 0.8 (Table 2). Sixteen compounds were demonstrated as medium chemoattractants, with an I_{30} between 0.6 and 0.8 (Table 2). Additionally, few root exudate compounds (DL-dithiothreitol, hydroxycarbamate, pentadecanoic acid, salicylic acid, and sodium decanoate) were characterized as repellents for SQR9, with an I_{30} less than 0.4. These results indicated that most of these responsive components served as chemoattractants, which may explain the general positive chemotactic response of SQR9 to total root exudates, as reported previously (Liu et al. 2017; Weng et al. 2013). However, more than half (54 of 98) of the tested components did not have any chemotaxis effect on SQR9.

Detection of MCP in *B. amyloliquefaciens* SQR9.

Chemoattractants or chemorepellents are sensed by bacterial chemoreceptors. To elucidate how these identified root-secreted compounds were recognized by SQR9, the potential MCP genes were predicted according to the existing of MA domain (Alexander and Zhulin 2007) by using the whole-genome sequence of SQR9 (National Center for Biotechnology Information [NCBI] accession number CP006890) (Zhang et al. 2015). Eight potential MCP-encoding genes were detected, which included *mcpA* (V529_30840), *mcpB* (V529_30860), *mcpC* (V529_13280), *tlpA* (V529_30850), *tlpB* (V529_30830), V529_10510, *hemAT* (V529_09980), and *yfmS* (V529_06960) (Fig. 1A). Compared with the well-studied *B. subtilis* strains 168 and OI1085, seven predicated MCP in SQR9 were conserved, and a unique chemoreceptor gene, V529_10510 (designated as *mcpR*), was found to only exist in SQR9 (Fig. 1B). *B. amyloliquefaciens* SQR9 appeared to have fewer MCP, because three chemoreceptors (TlpC, YoaH, and YvaQ) of strain 168 were found to be absent in SQR9. According to the report of Yssel et al. (2011), the similarity of homologous chemotaxis proteins between *B. subtilis* and *B. amyloliquefaciens* was only about 70%. These findings suggested that, though they are close-related organisms, the specific functions of MCP in *B. amyloliquefaciens* are quite different from those in *B. subtilis*.

The domain architecture analysis of the eight predicated MCP based on the SMART database (Schultz et al. 1998) indicated that, except for HemAT and YfmS, the predicted extracellular domain existed in all six other MCP, and five of them (excluding *McpR*) had a conserved LBD for sensing environmental signals (Fig. 1A). The LBD of *McpA*, *McpB*, *McpC*, *TlpA*, and *TlpB* in *B. amyloliquefaciens* SQR9 contain dCache domains and belong to cluster II based on their molecular sizes (239, 246, 242, 239, and 245 amino acids, respectively). However, the LBD of *McpR* contains CHASE3 and belongs to cluster I (138 amino acids).

BLAST analysis based on the *McpR* amino acid sequence retrieved 67 alignments that shared high similarity (identity >51%, including *McpR* itself) in NCBI (Supplementary Table S2), most of which originated from *B. amyloliquefaciens* and *B. velezensis* (30 alignments for each). Additionally, sequences from *B. nakamurai* (two alignments), *B. subtilis* (one alignment), and *Bacillus* sp. (three alignments) were also obtained. *B. amyloliquefaciens* and *B. velezensis* are considered to be a taxonomic unit above the species level and form “operational group *B. amyloliquefaciens*” within the *B. subtilis* species complex (Fan et al. 2017). More than 50% of *B. amyloliquefaciens*

(55.4%) and *B. velezensis* (53.6%) strains contain *McpR*, whereas only 12.5% *B. nakamurai* strains and 1.8% *B. subtilis* strains contain *McpR*. These analyses indicate that *McpR* may be a unique chemoreceptor of *B. amyloliquefaciens*.

SQR9 Δ *mcp*, deficient in all eight putative *mcp* genes, completely lost chemotactic reaction to 44 compounds.

To elucidate the roles of these detected MCP in SQR9's chemotactic response to these root-secreted chemoattractants

Table 2. Ranking and summary of the chemotaxis index (I_{30}) of SQR9 in response to the 98 targeted compounds

Ranking (n), substances ^a	SQR9		SQR9 Δ <i>mcp</i>	
	I_{30} ^{b,c}	Standard error ^c	I_{30} ^{b,c}	Standard error ^c
Approximately 0.8–1.0 (23)				
Valine ^d	0.99	0.0026	0.48	0.052
Glutamine	0.99	0.0017	0.51	0.047
Serine	0.99	0.0018	0.53	0.049
Threonine	0.98	0.0024	0.49	0.036
Succinic acid	0.98	0.0030	0.43	0.049
Cystine	0.98	0.0034	0.47	0.029
DL-malic acid	0.98	0.0038	0.53	0.057
Gluconic acid	0.97	0.0035	0.49	0.039
Asparagine	0.97	0.0071	0.47	0.039
Citric acid	0.95	0.016	0.47	0.019
D-maltose	0.94	0.0048	0.54	0.030
Methionine	0.93	0.016	0.47	0.032
Alanine	0.93	0.014	0.55	0.040
Leucine	0.93	0.036	0.45	0.043
Arginine	0.91	0.027	0.55	0.029
Histidine	0.90	0.022	0.47	0.064
Isoleucine	0.90	0.0089	0.53	0.029
Glycine	0.88	0.031	0.58	0.061
Fumaric acid	0.86	0.019	0.57	0.027
Proline	0.81	0.035	0.57	0.062
Dulcitol	0.81	0.028	0.48	0.075
Lysine	0.81	0.015	0.51	0.038
D-mannose	0.81	0.026	0.50	0.062
Approximately 0.6–0.8 (16)				
Adipic acid	0.79	0.022	0.52	0.026
Glutamic acid	0.78	0.019	0.52	0.046
Phthalic acid	0.77	0.019	0.48	0.033
Aspartic acid	0.76	0.078	0.51	0.058
Fructose	0.74	0.033	0.52	0.039
Tryptophan	0.73	0.025	0.50	0.021
Phenylalanine	0.73	0.038	0.50	0.029
DL-glyceric acid	0.72	0.025	0.50	0.037
Dehydroascorbic acid	0.71	0.048	0.50	0.037
Oxalic acid	0.70	0.030	0.45	0.054
Ribitol	0.69	0.038	0.50	0.055
Tyrosine	0.67	0.025	0.49	0.023
D(+)-Ribose	0.63	0.033	0.45	0.028
Inosine	0.63	0.050	0.50	0.058
3-Hydroxypropionic acid	0.62	0.042	0.49	0.038
D-(+)-fucose	0.61	0.029	0.48	0.049
Approximately 0.4–0.6 (54) ^e				
...	...	...	...	...
Approximately 0.0–0.4 (5)				
DL-dithiothreitol	0.31	0.080	0.49	0.041
Salicylic acid	0.29	0.023	0.51	0.034
Hydroxycarbamate	0.29	0.041	0.51	0.045
Pentadecanoic acid	0.20	0.016	0.49	0.043
Sodium decanoate	0.16	0.028	0.50	0.061

^a Compounds were used at a concentration of 1 mM.

^b When the I_{30} was between 0.4 and 0.6, this was considered to be no chemotaxis response. When the I_{30} was less than 0.4, the compound was considered to be repellent. When the I_{30} was more than 0.6, the compound was considered to be an attractant.

^c Data for each compound are the means for the 13 biological replicates. Error bars represent the standard error from the mean.

^d All 20 amino acids are L-amino acids.

^e Data provided in Supplementary Table S1.

and chemorepellents, all eight MCP-encoding genes were deleted from the SQR9 genome to obtain the strain SQR9 Δ 8mcp. Chemotaxis experiments of SQR9 Δ 8mcp with the 44 identified chemoattractants and chemorepellents showed that SQR9 Δ 8mcp completely lost the chemotaxis ability to the 44 compounds (Table 2). These results suggest that these eight predicted genes encode the major functional MCP, which are critical for SQR9's chemotactic response to root-secreted chemoattractants and chemorepellents.

McpA is a major chemoreceptor of SQR9 for a broad range of chemoattractants.

To determine the chemoreceptor responsible for these root-secreted chemoattractants and chemorepellents, each of the eight MCP-encoding genes was individually complemented in SQR9 Δ 8mcp, and the chemotaxis of the complementary strains to these 44 compounds were tested. Expression of McpA (SQR9 Δ 8mcp/mcpA) significantly restored chemotaxis to 20 compounds (Fig. 2A), including five amino acids (aspartic acid, glutamic acid, isoleucine, lysine, and tyrosine), 10 organic acids (citric acid, malic acid, oxalic acid, fumaric acid, succinic acid, phthalic acid, adipic acid, dehydroascorbic acid, glyceric acid, and 3-hydroxypropionic acid), and five other compounds (hydroxycarbamate, mannose, ribose, fucose, and ribitol). SQR9 Δ 8mcp/mcpA also partially restored the chemotactic reaction to sodium decanoate, serine, gluconic acid, and fructose, indicating that McpA probably participated in but did not dominate the chemotaxis to these compounds (Table 3; Supplementary Table S3). These results suggest that McpA senses a broad range of root-secreted compounds and is a major chemoreceptor of SQR9.

To further verify the above chemotaxis assay, the amino-terminal sensing domain of McpA (Ala33-Pro278) was expressed and purified (Supplementary Fig. S1), and its binding of citric acid and aspartic acid (representing the McpA sensed organic acids and amino acids, respectively) was investigated using isothermal titration calorimetry (ITC) analysis. The ITC

results indicated that the LBD of McpA directly bound the two compounds in vitro and that the binding reactions were exothermic, with an apparent dissociation constant (K_D) value of 0.39 ± 0.15 and 0.24 ± 0.16 μ M for citric acid and aspartic acid, respectively. The enthalpy change (ΔH) values for the two ligands were calculated to be -14.34 ± 0.47 and -10.37 ± 0.48 kcal mol $^{-1}$, respectively (Fig. 2B).

Other chemoreceptors of SQR9 were responsible for fewer compounds.

Compared with McpA, other chemoreceptors of SQR9 showed a relatively narrow binding ligand range (Fig. 3A). Complementation of *hemAT* and *yfmS* (SQR9 Δ 8mcp/*hemAT* and SQR9 Δ 8mcp/*yfmS*) did not have any chemotactic effect regarding these tested compounds (Supplementary Table S4). Thus, these two genes were not involved in the chemotaxis of SQR9 to the root-secreted compounds, which may be due to their lack of extracellular region. For the 44 tested compounds, McpB was only responsible for four amino acid attractants (glycine, tryptophan, asparagine, and glutamine), as well as two chemorepellents (salicylic acid and sodium decanoate) (Fig. 3A). McpC was mostly responsible for amino acids, because nine of the SQR9 Δ 8mcp/mcpC-restored chemoattractants were amino acids (valine, alanine, threonine, proline, serine, leucine, cystine, methionine, and histidine) (Fig. 3A). TlpA only showed a response to a chemorepellent, DL-dithiothreitol, while TlpB responded to five chemoattractants (phenylalanine, maltose, fructose, dulcitol, and inosine), mostly sugars, and a potential chemorepellent of pentadecanoic acid verified by ITC subsequently (Fig. 3A).

ITC analysis of the purified LBD of McpB (Glu33-Pro278), McpC (Lys33-Met274), TlpA (Ala33-Pro278), and TlpB (Ala33-Met282) with certain compounds was conducted to verify the results of the chemotaxis experiments of complementary strains (Fig. 3B). The K_D and ΔH values for McpBLBD and sodium decanoate binding were 3.44 ± 2.17 μ M and -1.30 ± 0.18 kcal mol $^{-1}$, respectively; the K_D values for binding of

Fig. 1. Eight potential methyl-accepting chemotaxis proteins (MCP) in SQR9. **A**, Domain architectures of the eight MCP in the SQR9 were analyzed according to the SMART database (Schultz et al. 1998). **B**, Comparison of the MCP in *Bacillus amyloliquefaciens* SQR9 and *B. subtilis* 168. There were 8 MCP genes in the *B. amyloliquefaciens* SQR9 genome and 10 MCP genes in *B. subtilis* 168. The double-slash stands for a substantial distance between the adjacent genes.

McpCLBD with leucine and proline were 3.64 ± 0.78 and 3.62 ± 0.78 μM , respectively; and the ΔH values were calculated to be -81.05 ± 7.01 and -85.63 ± 7.48 kcal mol^{-1} , respectively. The K_D and ΔH values for the binding of TlpBLBD with phenylalanine and pentadecanoic acid were 3.17 ± 0.89 and 3.03 ± 0.66 μM , and -32.24 ± 3.39 and -25.68 ± 1.86 kcal mol^{-1} , respectively.

The unique chemoreceptor McpR in *B. amyloliquefaciens* specifically mediates chemotaxis to arginine.

McpR is a unique chemoreceptor of *B. amyloliquefaciens*. Complementation of McpR (SQR9 Δ mcp/mcpR) fully restored the chemotactic response to arginine but not to the other 19 amino acids (Supplementary Table S5). Furthermore, the sensing of arginine by McpR was specific in SQR9. The other seven chemoreceptor gene complementary strains did not show chemotactic response to arginine (Fig. 4A), and SQR9 Δ mcp/mcpR hardly showed any chemotactic response to

the other tested compounds, except a slight positive chemotaxis toward succinic acid and fucose with very low I_{30} values compared with the other seven chemoreceptor gene complementary strains, which suggested that McpR may partially participate in the response to succinic acid and fucose (data not shown). The expressed and purified amino-terminal sensing domain of McpR (Ser35-Gln165) directly bound to arginine in vitro in the ITC analysis, with K_D and ΔH values of 8.38 ± 1.41 μM and 40.36 ± 1.91 kcal mol^{-1} , respectively (Fig. 4B).

To further uncover the potential arginine-binding residues in the McpRLBD the sequences of the reported chemoreceptors for arginine were obtained. The McpC in *B. subtilis* OI1085 was reported to bind L-Arg with significantly higher affinity (K_D of approximately 100 μM) (Glekas et al. 2012), while McpB was also involved in chemotaxis to L-Arg (Yssel et al. 2011). Both PctALBD and PctBLBD of *P. aeruginosa* PAO1 could bind L-Arg with a K_D of 1 and 60 μM , respectively, and PctA is the major receptor for L-Arg (Rico-Jiménez et al.

Fig. 2. Chemotaxis activity mediated by the McpA chemoreceptor. **A**, Chemotactic responses of SQR9, SQR9 Δ mcp, and SQR9 Δ mcp/mcpA to 20 compounds. The black dotted line represents a chemotaxis index of 0.5. The concentration of all the compounds was 1 mM. Data for each strain are the means of 13 biological replicates. Error bars represent the standard error of these 13 biological replicates. Different letters above the bars indicate significant differences between the three strains for each compound ($P \leq 0.05$). **B**, Titration of 18 μM McpALBD with 200 μM citric acid, aspartic acid, and arginine (as a negative control). Upper panels contain the titration raw data and the lower panels show the integrated and dilution-corrected peak areas of the raw data. Data were fitted with the "one binding site model" of the MicroCal version of ORIGIN. Abbreviations: Mali = malic acid, Citr = citric acid, Oxal = oxalic acid, Succ = Succinic acid, Fuma = Fumaric acid, Phth = phthalic acid, Adip = adipic acid, Asp = aspartic acid, Glu = glutamic acid, Ile = isoleucine, Lys = lysine, Tyr = tyrosine, Hyca = hydroxycarbamate, Mano = mannose, Ribo = ribose, Fuco = fucose, Ribi = ribitol, DeAs = dehydroascorbic acid, Glyc = glyceric acid, and 3HP = 3-hydroxypropionic acid.

2013). Amino acid sequence alignment (using ClustalW and MAFFT) of the five arginine-receptor LBD revealed that residues Tyr-78, Asp-162, and Thr-131 may be crucial for arginine binding in McpR of SQR9 (Supplementary Fig. S2). For confirmation, these three residues were individually substituted by glycine, which cannot form hydrogen bonds with small ligands and is the smallest amino acid, to avoid causing possible structure disruption in the putative binding pockets. Chemotaxis analysis demonstrates that the three SQR9 Δ 8mcp/mcpR strains with single amino-acid mutations of mcpR showed significantly decreased chemotaxis to arginine compared with wild-type mcpR (Fig. 5). Taken together, these results showed that the residues Tyr-78, Thr-131, and Asp-162 are the amino acid residues in McpR that are involved in arginine binding.

DISCUSSION

B. amyloliquefaciens SQR9 is a plant-beneficial rhizobacterium with a strong root colonization capability and positive chemotaxis toward cucumber root exudates (Weng et al. 2013), whose composition has been identified through gas chromatography–mass spectrometry (Liu et al. 2017). In this study, we show that PGPR strain *B. amyloliquefaciens* SQR9 chemotactically responded to nearly half of the tested root exudate components (44 of the 98 compounds); 39 of them were chemoattractants, while only 5 compounds served as chemorepellents. Amino acids, organic acids, and sugars were the major chemoattractant compounds and accounted for 45, 32, and 11% of the identified chemoattractants, respectively. The chemoreceptors McpA and McpC, which are mainly responsible for sensing amino acids and organic acid chemoattractants, may play critical roles in the chemotaxis mobility of SQR9 to the plant root (Fig. 6).

Generally, root exudates were divided into two different classes of compounds: the low-molecular-weight compounds, including a variety of amino acids, organic acids, sugars, and secondary metabolites, and high-molecular-weight compounds, including proteins and polysaccharides (Badri and Vivanco 2009; Liu et al. 2017). Thus far, most of the identified chemoattractants of root exudates for rhizobacteria were low-molecular-weight compounds, which are carbon and energy resources in the environment. Additionally, chemotaxis is believed to assist rhizobacteria in moving to areas suitable for growth (Jiang et al. 2016; Lacal et al. 2010; Porter et al. 2011; Szurmant and Ordal 2004).

As an important microorganism that is closely related to *B. amyloliquefaciens*, *B. subtilis* OI1085 showed a chemotaxis response to all 20 L-amino acids, which were predominantly mediated by McpC (supports chemotaxis to 17 and directly binds to 11 amino acids) and McpB (supports chemotaxis to 4 amino acids) (Hanlon and Ordal 1994). Here, we found that the sensing of amino acids in *B. amyloliquefaciens* SQR9 was dispersed among different chemoreceptors, which is significantly different from *B. subtilis* OI1085. In SQR9, McpA, McpB, McpC, TlpB, and McpR responded to 6, 7, 9, 2, and 1 amino acids, respectively (Table 3). Interestingly, the extracellular regions of the chemoreceptors McpA, McpB, and McpC of SQR9 were similar to the chemoreceptors McpB and McpC of *B. subtilis*, which were predicted to possess double PDC sensor domains (Glekas et al. 2010, 2012; Rico-Jiménez et al. 2013; Sampedro et al. 2015). McpC was the dominant amino acid sensor in SQR9, because 9 of 10 of its ligands were amino acids (Table 3). Amino acid sequence alignments showed that the identities of McpCLBD between SQR9 and OI1085 were 61.63%.

More than 140 compounds have been found to induce chemotaxis in *Pseudomonas* strains (Sampedro et al. 2015), which made these strains models for establishing chemoreceptor

Table 3. Summary of the corresponding ligands of the six chemoreceptors with an extracellular ligand-binding domain region in *Bacillus amyloliquefaciens* SQR9 and comparison with *B. subtilis* OI1085^a

MCP	Ligands ^b	Category	Compounds ^c	In <i>B. subtilis</i> ^d
McpA	Dominant	Amino acids	Aspartic acid*, glutamic acid*, isoleucine*, lysine*, tyrosine*	Glucose (r), α -methylglucoside (r)
		Acids	Phthalic acid*, citric acid*, oxalic acid*, malic acid, succinic acid, fumaric acid, adipic acid, dehydroascorbic acid*, glyceric acid, 3-hydroxypropionic acid	...
McpB	Involved	Others	Ribitol*, mannose*, ribose, fucose, hydroxycarbamate (r)	...
			Sodium decanoate (r), serine, gluconic acid, fructose	...
			Glycine*, tryptophan*, asparagine*, glutamine*	Asparagine, aspartate, glutamine, glutamate, and histidine
McpC	Dominant	Acids	Salicylic acid (r), sodium decanoate (r)	...
			Adipic acid, ribose, glyceric acid, 3-hydroxypropionic acid, serine, cystine, methionine, gluconic acid, fructose	...
			Valine*, alanine*, proline*, leucine*, histidine*, serine, threonine, cystine, methionine	All amino acids except asparagine, tryptophan, and histidine
TlpA	Involved	Acids	Gluconic acid	...
			Succinic acid, maltose	...
TlpB	Dominant		DL-dithiothreitol* (r)	Unknown
			Hydroxycarbamate (r), sodium decanoate (r), gluconic acid, maltose	...
McpR	Involved	Amino acids	Phenylalanine*	Unknown
		Acids	Pentadecanoic acid* (r)	...
		Others	Dulcitol*, inosine*, maltose, fructose	...
McpR	Dominant		Malic acid, succinic acid, fumaric acid, ribose, fucose, salicylic acid (r), sodium decanoate (r), threonine, gluconic acid	...
			Arginine*	ND
			Succinic acid, fucose	...

^a (r) indicates that this compound is chemorepellent and ND means that this methyl-accepting chemotaxis protein (MCP) is absent in *B. subtilis* OI1085.

^b Ligands information. “Dominant” indicates that this chemoreceptor predominantly mediates the chemotaxis of SQR9 to these compounds, while “involved” means that the MCP is partially involved in chemotaxis to these chemicals.

^c Asterisk (*) indicates that these compounds were only sensed by the single chemoreceptor.

^d Ligands identified for those orthologous proteins in *B. subtilis* OI1085 according to the previous literature.

structure–function relationships (Kato et al. 2008). García et al. (2015) reported that McpP of *P. putida* KT2440 had a CACHE-type LBD and mediated taxis to some C₂ and C₃ carboxylic acids. In this study, the large-scale identification of chemoattractants in root exudates and their responsible chemoreceptors in SQR9 facilitated revealing the chemoreceptor structure–function relationship. Among the 20 ligands of McpA, nine compounds had carboxyl groups at both ends of the chains, which included two amino acids (aspartic acid and glutamic acid) and seven acids (citric acid, malic acid, oxalic acid, fumaric acid, succinic acid, phthalic acid, and adipic acid) (Supplementary Fig. S3). McpA had a dCache domain, which was different from the CACHE domain of McpP in *P. putida* KT2440. In SQR9, McpC predominantly mediated the chemotaxis to aliphatic amino acids (leucine, alanine, and valine), sulfur-bearing amino acids (cystine and methionine), and hydroxy amino acids (threonine and serine), and McpB seemed to mediate the chemotaxis to the only two amide amino acids, asparagine and glutamine. These results suggest that compounds with similar structure may be sensed by the same chemoreceptor.

Compounds that are attractants for some bacteria may be repellents for others. For example, acetate is an attractant for

Rhodobacter sphaeroides but a repellent for *E. coli* (Packer and Armitage 2000; Tso and Adler 1974). Interestingly, a compound can switch from being an attractant at a low oxygen concentration to a repellent at atmospheric oxygen concentrations, depending on the growth conditions and the needs of the *R. sphaeroides* cell (Romagnoli et al. 2002). In our work, though 1 mM concentration of the tested ligands in the chemotaxis assay was higher than their actual concentration in the rhizosphere, the SlipChip device allowed the formation of diffusion concentration gradients of the chemoeffector, which mimics the natural condition that plant-released root exudates form a nutrient gradient with distance (Shen et al. 2014). Under this condition, a substantial amount of the tested root exudate components were sensed by SQR9 as chemoattractants, while only a few compounds were chemorepellents. These results reinforce the idea that many PGPR strains show strong chemotactic responses to root exudates, which are very important for the beneficial functions of PGPR.

ITC analysis in vitro is an important approach for verifying whether the binding between chemoreceptor and ligand is direct or not. In this study, the dominant objectives were focused on identification of the MCP mediating chemotactic response of SQR9 to the 39 chemoattractants and 5 chemorepellents by

Fig. 3. Chemotaxis activity mediated by the McpB, McpC, TlpA, and TlpB chemoreceptors. **A**, Chemotactic responses of SQR9, SQR9 Δ mcp, SQR9 Δ mcp/mcpB, SQR9 Δ mcp/mcpC, SQR9 Δ mcp/tlpA, and SQR9 Δ mcp/tlpB to 23 compounds. The black dotted line represents a chemotaxis index of 0.5. The concentration of all the compounds was 1 mM. Data for each strain are the means of 13 biological replicates. Error bars represent the standard error of 13 biological replicates. Different letters above the bars indicate significant differences between the three strains for each compound ($P \leq 0.05$). **B**, Titration of 20 μ M McpBLBD, 20.5 μ M McpCLBD, and 18 μ M TlpBLBD with 200 μ M sodium decanoate, leucine, proline, phenylalanine, pentadecanoic acid, and arginine (as a negative control). Upper panels contain the raw titration data and the lower panels show the integrated and dilution-corrected peak areas of the raw data. Data were fitted with the “one binding site model” of the MicroCal version of ORIGIN. Abbreviations: Asn = asparagine, Gln = glutamine, Gly = glycine, Sali = salicylic acid, Trp = tryptophan, DeNa = sodium decanoate, Leu = leucine, Ala = alanine, Val = valine, Cys = cysteine, Met = methionine, Thr = threonine, Ser = serine, Pro = proline, His = histidine, Gluc = gluconic acid, DTT = dl-dithiothreitol, Phe = phenylalanine, Malt = maltose, Fruc = fructose, Inos = inosine, PeDe = pentadecanoic acid, and Dulc = dulcitol.

direct binding or other ways. To explore the correspondence between these ligands and their potential MCP, we randomly picked eight representative compounds to perform the ITC analysis, and excitedly found that all of them can directly bind to the identified chemoreceptors in SQR9.

In conclusion, we have identified 44 compounds in root exudates that the plant-beneficial rhizobacterium *B. amyloliquefaciens* SQR9 chemotactically responded to, 39 of which

served as attractants to recruit SQR9 to colonize in the rhizosphere. Among the multiple chemoreceptors of the PGPR strains, the receptors mainly responsible for the root-secreted organic acids and amino acids may play important roles in the rhizosphere chemotaxis mobility of PGPR. These results not only deepened our insights of PGPR–root interaction but also provided useful information to enhance the rhizosphere chemotaxis mobility and colonization of PGPR with

the in vitro addition of a suitable chemoattractant. This approach will increase the efficiency of PGPR in agricultural production and reduce the input of chemical fertilizers and pesticides.

Fig. 5. Chemotactic responses of SQR9 Δ 8mcp complemented the wild-type or mutated *mcpR* to arginine. The concentration of arginine was 1 mM. Data for each strain are the means of 13 biological replicates. Error bars represent the standard error of 13 biological replicates. Different letters above the bars indicate significant differences ($P \leq 0.05$).

MATERIALS AND METHODS

Bacterial strains, media and growth conditions.

The strains and plasmids used in this study are described in Table 1. *B. amyloliquefaciens* SQR9 (China General Microbiology Culture Collection Center, CGMCC accession number 5808) was isolated from the cucumber rhizosphere. SQR9 was grown at 37°C in low-salt Luria-Bertani (LB) medium (peptone at 10 g liter⁻¹, yeast extract at 5 g liter⁻¹, and NaCl at 3 g liter⁻¹) that was solidified with 15 g liter⁻¹ agar. *E. coli* BL21 (DE3) cells were grown at 37°C in Luria-Bertani medium (peptone at 10 g liter⁻¹, yeast extract at 5 g liter⁻¹, and NaCl at 5 g liter⁻¹) solidified with agar at 15 g liter⁻¹. When necessary, the final concentrations of antibiotics were added as follows: chloramphenicol (Cm) at 5 mg liter⁻¹, zeocin at 20 mg liter⁻¹, spectinomycin (Spc) at 100 mg liter⁻¹, and kanamycin (Kan) at 30 mg liter⁻¹.

MCP gene prediction and deletion in *B. amyloliquefaciens* SQR9.

Based on the whole genome sequence of *B. amyloliquefaciens* SQR9 (NCBI accession number CP006890) (Zhang et al. 2015), the potential genes encoding for MCP were predicated according to the standard existence of a MA domain (Alexander and Zhulin 2007).

Considering the functional redundancy of MCP for sensing chemoeffectors as reported in many organisms (Glekas et al. 2012, Ni et al. 2013, Oku et al. 2012, Webb et al. 2014), the *B. amyloliquefaciens* SQR9 mutant deficient in all eight

Fig. 6. Proposed model of the chemotaxis mobility of *Bacillus amyloliquefaciens* SQR9 toward cucumber root-secreted compounds mediated by different chemoreceptors.

predicated *mcp* genes was constructed using an unmarked genetic manipulation based on multiple gene deletions that rely on a counter-selectable marker, *pheS* (Zhou et al. 2017). First, four adjacent genes (*tlpB-mcpA-tlpA-mcpB*, 8,393 bp) were deleted in the first-round knockout to generate SQR9 Δ 4*mcp*. Then, *mcpC* (1,964 bp), *mcpR* (V529_10510; 1,647 bp), *hemAT* (1,290 bp), and *yfmS* (852 bp) were further deleted one by one in the next rounds of knockout to finally acquire SQR9 Δ 8*mcp* (through SQR9 Δ 5*mcp*, SQR9 Δ 6*mcp*, and SQR9 Δ 7*mcp*, respectively). All of these mutants were confirmed by polymerase chain reaction (PCR) and sequencing with the primer sets listed in Supplementary Table S6.

Complementation of the disrupted genes.

The eight *mcp* genes were separately integrated into the chromosomal *amyE* locus of the chemoreceptor-deficient SQR9 Δ 8*mcp* to obtain single-*mcp*-gene complementary strains (SQR9 Δ 8*mcp/mcpA*, SQR9 Δ 8*mcp/mcpB*, SQR9 Δ 8*mcp/mcpC*, SQR9 Δ 8*mcp/tlpA*, SQR9 Δ 8*mcp/tlpB*, SQR9 Δ 8*mcp/mcpR*, SQR9 Δ 8*mcp/hemAT*, and SQR9 Δ 8*mcp/yfmS*). First, the full sequence of each of the eight *mcp* genes (*mcpA*, *mcpB*, *mcpC*, *tlpA*, *tlpB*, *mcpR*, *hemAT*, and *yfmS*) with its native promoter was amplified from SQR9 genomic DNA, which partially overlapped with *Cm* or *Spc* resistance genes (*Cm^R* or *Spc^R*) and the downstream fragments. After that, an upstream 0.70-kb fragment and a downstream 0.74-kb fragment of the 1.97-kb *amyE* gene were amplified with the primer set *amyE*-UF/*amyE*-UR. Subsequently, the 1.0-kb *Cm^R* and *Spc^R* genes were amplified from the plasmid pNW33n and p7S6 (Yan et al. 2008) with primer sets P1/P2 and P3/P4, respectively, which overlapped with the upstream fragments and the complemented genes. Finally, the obtained four fragments were fused by overlap PCR, and the fused fragments for each of the eight target genes were individually transformed into Δ 8*mcp*-competent cells. The obtained transformants were verified using primer sets (Zhou et al. 2017).

Chemotaxis assay.

The chemotaxis assay was performed as described by Shen et al. (2014) by using a simple and reusable microfluidic SlipChip device. By a simple slipping operation, three microwells were disconnected from other units and interconnected by the ducts, which allowed the formation of diffusion concentration gradients of the chemoeffector for inducing cell migration from the cell microcells toward the other two microwells. As a result, the concentration of the chemoeffector sensed by bacteria was lower than its initial concentration. Briefly, a 10-mg mL⁻¹ bovine serum albumin (BSA) solution was injected into all channels and used to wash the microwells for 5 min. After removing the BSA solution using a vacuum across the access holes, the solutions containing different chemoeffectors, bacterial cells suspended in phosphate-buffered saline (PBS) buffer (NaCl at 8 g liter⁻¹, KCl at 0.2 g liter⁻¹, Na₂HPO₄ at 1.44 g liter⁻¹, and KH₂PO₄ at 0.24 g liter⁻¹; pH 7.4), and PBS buffer alone (negative control) were loaded to the top, middle, and bottom microwells, respectively. Subsequently, the SlipChip device was slipped to ensure that the cells could migrate freely from the middle microwells to the ducts and the microwells were loaded with the chemoeffector or PBS. The device was placed on an inverted fluorescence microscope (Ti-Eclipse; Nikon) and kept for 30 min in the dark at room temperature. Then, the cells in the top microwells and bottom microwells were monitored. The chemotaxis index (*I_t*) was used to indicate the chemotactic ability of the bacterial cells with a chemoeffector at a certain concentration. *I_t* was defined as $N_e/(N_e + N_c)$, where *N_e* is the number of cells that migrated into the chemoeffector-containing microwells and *N_c* is the number of cells that migrated into the control microwells in a certain time period.

In this study, *I₃₀* was used to characterize chemotaxis; cells were allowed to migrate freely in the gradient for 30 min. In theory, an *I₃₀* value approximately equal to 0.5 means that the cells do not respond to the chemoeffector, while an *I₃₀* value of more than 0.5 indicates that the cells are attracted by the chemoeffector and an *I₃₀* value lower than 0.5 suggests the cells are repelled by the chemoeffector. Here, to ensure the stability and reliability of the assessment, we redefined the nonresponse interval of *I₃₀* to the range between 0.4 and 0.6, while >0.6 and <0.4 were defined as positive and negative responses to the chemoeffector, respectively.

Protein expression and purification.

Expression and purification of the LBD of McpA, McpB, McpC, TlpA, TlpB, and McpR in *B. amyloliquefaciens* SQR9 were performed. The DNA fragments encoding the LBD of McpA (Ala33-Pro278), McpB (Glu33-Pro278), McpC (Lys33-Met274), TlpA (Ala33-Pro278), TlpB (Ala33-Met282), and McpR (Ser35-Gln165) were amplified with primer sets that contained restriction sites for *NdeI* and *HindIII* using SQR9 genomic DNA as a template. The PCR products were digested and cloned into the expression plasmid pET28a(+), which was linearized with the *NdeI* and *HindIII* enzymes. The insert and flanking regions of the six reconstructed plasmids (pET28a-McpALBD, pET28a-McpBLBD, pET28a-McpCLBD, pET28a-TlpALBD, pET28a-TlpBLBD, and pET28a-McpRLBD) were verified by DNA sequencing. The expressed proteins contained an N-terminal His tag.

The six constructed expression vectors were individually introduced into *E. coli* BL21 (DE3). The obtained strains were grown in 1-liter Erlenmeyer flasks containing 200 ml of LB medium supplemented with Kan at 30 mg liter⁻¹ at 37°C, with shaking at 220 rpm. When the optical density at 600 nm of the culture reached 0.6, the growth temperature was lowered to 16°C. After another 30-min incubation, the protein expressions were induced by adding 0.05 mM isopropyl- β -D-thiogalactopyranoside. The growth was continued at 16°C, with shaking at 220 rpm overnight, prior to cell harvest by centrifugation at 8,000 $\times$ g for 10 min. Then, the cells were washed at least twice with 0.01 M PBS. The cell pellets were resuspended in 50 ml of 0.01 M PBS and disrupted by sonication (2-s pulse on, 3-s pulse off). The cellular lysates were centrifuged at 20,000 $\times$ g for 50 min. Finally, for further clarification, the lysates were passed through a 0.22- μ m filter (Millipore) to remove any other aggregates or insoluble particles, followed by purification with His-affinity resin chromatography according to the manufacturer's (Hua Chun) instructions. All the purified proteins were collected and stored in TKMDmod buffer (50 mM Tris, pH 8.0; 50 mM KCl; and 5 mM MgCl₂) at -80°C.

Isothermal titration calorimetry.

All ITC measurements were performed using an iTC200 titration calorimeter (MicroCal; GE) at 25°C, and the data were extracted and processed using the Origin software package. All of the ligands were dissolved in TKMDmod buffer. For ligand binding analysis, the protein was placed into the sample cell and was titrated with aliquots of ligand solution in the injector syringe. The 400- μ l reaction cell was constantly stirred at 750 rpm. The mean enthalpies measured from the injection of the ligands into the buffer were subtracted from raw titration data prior to data analysis using the "one binding site model" of the MicroCal version of ORIGIN. The two important constants, *K_D* and ΔH , were gained. The smaller the *K_D* value, the stronger the binding of the protein and ligand. Moreover, the binding is endothermic when the ΔH is greater than zero. When the ΔH is less than zero, the binding is exothermic.

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