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# Recognition of dominant attractants by key chemoreceptors mediates recruitment of plant growth-promoting rhizobacteria

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## Summary

**Chemotaxis to plant root exudates is supposed to be a prerequisite for efficient root colonization by rhizobacteria. This is a highly multifactorial process since root exudates are complex compound mixtures of which components are recognized by different chemoreceptors. Little information is available as to the key components in root exudates and their receptors that drive colonization related chemotaxis. We present here the first global assessment of this issue using the plant growth-promoting rhizobacterium (PGPR) *Bacillus velezensis* SQR9 (formerly *B. amyloliquefaciens*). This strain efficiently colonizes cucumber roots, and here, we show that chemotaxis to cucumber root exudates was essential in this process. We conducted chemotaxis assays using cucumber root exudates at different concentrations, individual exudate components as well as recomposed exudates, taking into account their concentrations detected in root exudates. Results indicated that two key chemoreceptors, McpA and McpC, were essential for root exudate chemotaxis and root colonization. Both receptors possess a broad ligand range and recognize most of the exudate key components identified (malic, fumaric, gluconic and glyceric acids, Lys, Ser, Ala and mannose). The remaining six chemoreceptors did not contribute to exudate chemotaxis. This study provides novel insight into the evolution of the chemotaxis system in rhizobacteria.**

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## Introduction

The rhizosphere, the soil habitat that is influenced by root secretions, can accommodate up to $10^{11}$ cells per gram root (Egamberdieva *et al.,* 2008) that can belong to more than 30 000 bacterial species (Mendes *et al.,* 2011). Whereas some of these microorganisms are plant pathogens, others are plant growth-promoting rhizobacteria (PGPR) with the capacity to enhance plant growth by increasing nutrient acquisition, altering hormone levels or by suppressing plant pathogens (Lugtenberg and Kamilova, 2009). Plants recruit PGPRs to the rhizosphere by the release of specific signal molecules (Berendsen *et al.,* 2012; Bardy *et al.,* 2017). Bacterial chemotaxis is the capacity of directed swimming along a signal gradient (Bi and Sourjik, 2018). It was shown that taxis to root exudates is an essential initial step in the interaction between plants and PGPRs (de Weert *et al.,* 2002; Zhang *et al.,* 2014; Scharf *et al.,* 2016), which promotes root colonization causing in turn mutual benefits for plants and microbes (Szurmant and Ordal, 2004; Bais *et al.,* 2006; Scharf *et al.,* 2016).

The canonical mechanism of bacterial chemotaxis involves chemoreceptor stimulation by either the direct binding of chemoeffectors or periplasmic binding protein-chemoeffector complexes to the chemoreceptor ligand binding domain (Lacal *et al.,* 2010a). The resulting molecular stimulus modulates the activity of the CheA autokinase

and subsequently the transphosphorylation to the CheY response regulator. CheY-P interacts with the flagellar motor causing ultimately chemotaxis.

Next to the study of chemotaxis in established model organisms such as *Escherichia coli*, *Pseudomonas* spp. and *Bacillus subtilis* (Porter *et al.,* 2011; Bi and Lai, 2015; Sampedro *et al.,* 2015), ecophysiological studies of different rhizobacteria have allowed the identification of plant signals that are central to colonization-relevant chemotaxis, as well as the cognate chemoreceptor. In a recent study, Allard-Massicotte *et al*. (2016) reported that chemoreceptors McpB, McpC and TlpC mediated chemotaxis of the plant-associated strain *B. subtilis* 3610 to *Arabidopsis thaliana* root exudates and early root colonization. The oxygen sensing chemoreceptor IcpB of the plant beneficial bacterium *Azorhizobium caulinodans* modulated nodulation and nitrogen fixation on the stems and roots of *Sesbania rostrata* (Jiang *et al.,* 2016). In pathogenic *Ralstonia pseudosolanacearum*, McpM-mediated chemotaxis to L-malate, secreted by tomato roots, was essential for the infection process (Hida *et al.,* 2015). In other plant-associated microorganisms, amino acids responsive chemoreceptors played an important role in chemotaxis to plant exudates and root colonization (Oku *et al.,* 2012; Webb *et al.,* 2014).

Currently, most of these studies describe the contribution of individual chemoreceptors and their cognate signals to root colonization. However, due to the complex composition of root exudates, the resulting chemotactic mechanisms are highly multifactorial and represent the sum of activities of different chemoreceptors, which each may respond to different chemoattractants with potentially different efficiencies. Additionally, the concentration of root exudates in the rhizosphere changes with the distance to the root (Sasse *et al.,* 2018), and this variation in concentration adds to the complexity of chemotaxis and root colonization.

We report here a global analysis of chemoeffectors and chemoreceptors that contribute to the overall chemotaxis to root exudates. In our study, we have used *Bacillus velezensis* SQR9 (formerly known as *B. amyloliquefaciens*) as a model, which is a well-studied and commercially widely used PGPR strain (Cao *et al.,* 2011; Shao *et al.,* 2015). This strain is chemotactically attracted to cucumber root exudates (Weng *et al.,* 2013; Zhang *et al.,* 2015). We have recently determined the chemical composition of cucumber root exudates and demonstrated that 39 compounds (mainly include amino acids, organic acids and sugars) caused chemoattraction whereas five compounds were found to repel the strain (Feng *et al.,* 2018). Of the eight chemoreceptors in SQR9, we have shown that six of them, McpA, McpB, McpC, TlpA, TlpB and McpR, sensed at least one chemoeffector (Feng *et al.,* 2018). However, chemoeffectors in that study were used at the fixed concentration of 1 mM, which is little representative of their concentration *in situ.* To unambiguously define the contribution of individual compounds to root chemotaxis, it is indispensable to use chemoeffector ligand concentrations that correspond to their concentrations detected in root exudates. The main objective of this study is to identify the chemoattractants and their corresponding chemoreceptors that drive rhizospheric chemotaxis and root colonization by SQR9, thus presenting a global model for depicting the rhizospheric chemotaxis of PGPRs. The experimental design enables relationships between compound concentrations in cucumber root exudates and contributions to chemotaxis and root colonization to be identified. Furthermore, information is provided on how this relationship varies with the concentration of root exudates and consequently distance to the root. Based on colonization experiments and chemotaxis assays using natural and recomposed root exudates as well as individual chemoattractants, we were able to identify the central chemoattractants and demonstrate that their responses are mediated primarily by the McpA and McpC chemoreceptors.

## Results

### *Chemotaxis is necessary for efficient root colonization by* B. velezensis *SQR9*

To assess the role of chemotaxis in cucumber root colonization by *B. velezensis* SQR9, we compared the colonization efficiency of its wild-type (wt) with that of the chemoreceptor free mutant SQR9Δ*8mcp*, which had lost the chemotactic response to attractants (Feng *et al.,* 2018). Here cucumber seedlings with fully developed cotyledons (7 days after transplantation) and not at the later six-leaves stage (∼45 days after transplantation) such as in the study by Liu *et al*. (2017) were used in order to follow the initial phase of colonization, which is also relevant for the use of PGPR in agriculture (Cao *et al.,* 2011; Zhang *et al.,* 2015). Data revealed a significant decrease by approximately two orders of magnitude in the population of root attached bacteria for the *mcp* free mutant (Fig. 1). Since the growth kinetics of the wild-type and SQR9Δ*8mcp* strains were similar (Supporting Information Fig. S1), these results indicate that MCP-mediated chemotaxis to exudates is a prerequisite for efficient root colonization by SQR9.

### *Essential roles for the McpA and McpC chemoreceptors in root colonization*

Subsequently, we aimed at assessing the contribution of each of the individual receptors in this process. To this end, the MCP-free mutant was complemented with each of the eight individual receptors and the colonization of

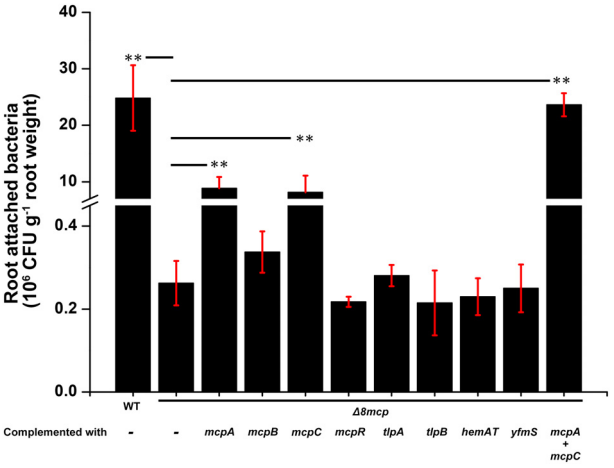


**Fig. 1.** Comparison of root colonization capacity of wild-type SQR9, the MCP-free mutant SQR9Δ*8mcp*, as well as this mutant complemented with different chemoreceptor. ** indicates $P < 0.01$. Data are means and standard deviations from six independent experiments. [Color figure can be viewed at wileyonlinelibrary.com]

cucumber seedlings was monitored. Complementation of the mutant with *mcpA* or *mcpC*, separately restored in a similar way the capacity to colonize roots, and the amount of root attached bacteria amounted to approximately a third as compared to the wild-type strain (Fig. 1). In marked contrast, the complementation with each of the remaining six chemoreceptors resulted in colonization efficiencies that were statistically not different to that of the SQR9Δ*8mcp* strain (Fig. 1). When this mutant strain was complemented with the *mcpA* and *mcpC* genes, the root colonization was not different to that of the wt (Fig. 1). These results demonstrate that both, McpA and McpC, play key roles in root colonization, but also indicate that the effects caused by both receptors are additive.

### *McpA and McpC mediate chemotaxis to root exudates*

Not all chemoreceptors mediate chemotaxis (Wuichet and Zhulin, 2010) and there is a significant body of data showing that chemoreceptor-based mechanisms are responsible for twitching motility (Whitchurch *et al.,* 2004) or are associated with alternative cellular functions such as the control of second messenger levels (Hickman *et al.,* 2005; Fulcher *et al.,* 2010). To determine the function of both receptors, we initially carried out chemotaxis assays measuring wt responses to different concentrations of cucumber root exudates, since exudates form gradients depending on the distance from root surface. The chemotaxis index $I_{30} > 0.6$ or $< 0.4$ indicates attractant and repellence responses, respectively, whereas $0.4 \leq I_{30} \leq 0.6$ means no taxis. It was found that root exudates at 1× (concentration in the cucumber seedling containing recipient used to generate root exudates, ~0.02 mg ml$^{-1}$) or 10× concentrations hardly attracted the wt strain ($0.4 \leq I_{30} \leq 0.6$) (Supporting Information Fig. S2). Since chemoattraction was observed at a 100× or 1000× root exudates ($I_{30}$ = 0.90 for 100× and 0.95 for 1000×; Fig. 2), these two conditions were used to study the tactic behavior of the different strains mentioned above. Mutant SQR9Δ*8mcp* was devoid of chemotaxis to both concentrations (0.51 and 0.50 for 100× and 1000×, respectively; Fig. 2) whereas this mutant complemented with *mcpA* (0.68 and 0.91 for 100× and 1000×, respectively) or *mcpC* (0.65 and 0.84) showed partially restored taxis (Fig. 2). No chemotaxis was observed for the mutant complemented with any of the remaining six chemoreceptors (Fig. 2). As expected, complementation of SQR9Δ*8mcp* with *mcpA* and *mcpC* resulted also in wt-like taxis (0.75 and 0.91 for

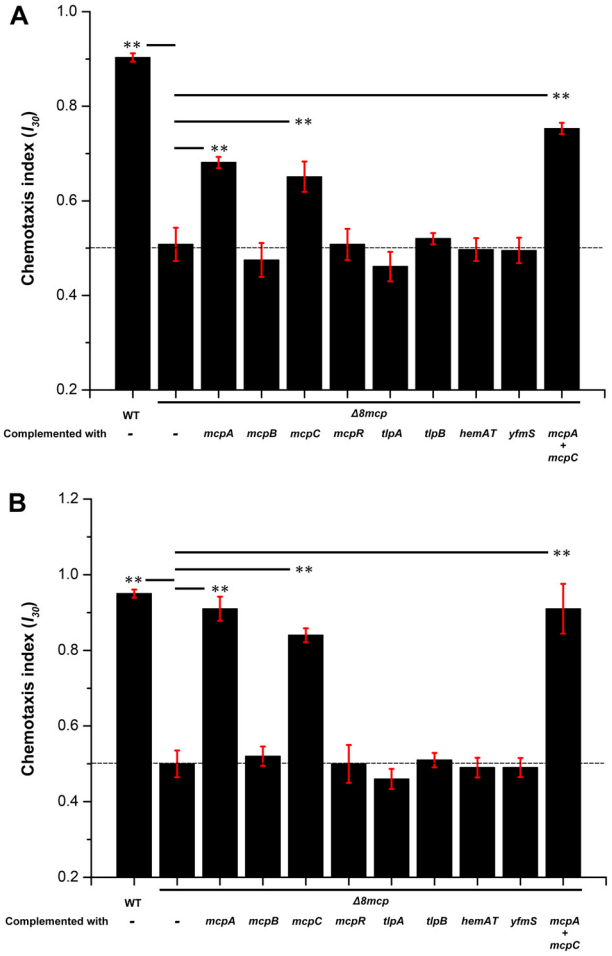


**Fig. 2.** Chemotaxis of wild-type SQR9, the MCP-free mutant SQR9Δ*8mcp* as well as this mutant complemented with different chemoreceptors, towards a 100× (A) and 1000× (B) cucumber root exudates solution. Shown is the chemotaxis index as determined by the SlipChip assay as described in the Materials and Methods. $I_{30}$ is chemotaxis index, $I_{30} > 0.6$ or $< 0.4$ indicates attractant or repellent responses, respectively; while $0.4 \leq I_{30} \leq 0.6$ designates an absence of taxis. ** $P < 0.01$. Data are means and standard deviations from 13 independent measurements. [Color figure can be viewed at wileyonlinelibrary.com]

100× and 1000×, respectively; Fig. 2). Overall, the chemotaxis measurements correlated well with the root colonization data shown in Fig. 1, indicating that chemotaxis mediated by McpA and McpC drives the colonization of cucumber roots.

### *Quantification of McpA and McpC sensed attractants present in root exudates*

The cotyledon stage of the cucumber seedlings used in our experiments differed from a previous study where exudate composition was determined from roots of the six-leaves stage (Liu *et al.,* 2017). Therefore, the composition of cucumber root exudates from seedlings with fully developed cotyledons was determined by gas chromatography–mass spectrometry (GC–MS) in this study. The data were generally similar to that of the previous study but has permitted to identify several additional compounds (Table 1). In total, 188 compounds were detected of which 116 could be identified, which were then classified into 11 categories based on their structure. Organic acids, amino acids and sugars were identified as the predominant compound families (Table 1).

From the chemotaxis and colonization data it is clear that McpA and McpC are central receptors. A previous study has identified the ligands for both receptors (Feng *et al.,* 2018). From the 188 compounds detected in root exudates at the cotyledon stage, McpA and McpC were found to respond to 29 compounds of which 28 served as chemoattractants while capric acid was a repellent (Feng *et al.,* 2018). In detail, McpA responded to 20 attractants including four amino acids (Tyr, Ser, Ile and Asp), 11 organic acids (succinic, phthalic, oxalic, malic, glyceric, fumaric, dehydroascorbic, citric, adipic, 3-hydroxypropionic and gluconic acids) and five sugars/sugar alcohols (ribose, mannose, fucose, fructose and ribitol); while McpC mediated taxis to eight amino acids (Val, Thr, Ser, Pro, Ala, Leu, Met and His) as well as succinic acid, gluconic acid and maltose (Feng *et al.,* 2018). These compound-receptor relationships are indicated in Table 1.

However, analysis of the root exudate composition is of qualitative nature. To understand the contribution of these individual compounds to root colonization related chemotaxis, it is indispensable to obtain quantitative measurements of the abundance of compounds in exudates. Using an ultra-high performance liquid chromatography- tandem mass-spectrometry (UHPLC–MS/MS) approach, attempts have been made to quantify the 28 attractants for McpA and McpC in exudates. A number of compounds were below the detection limit of this technique, but reliable quantitative measurements could be obtained for 20 of them (Table 2). Citric acid was by far the most abundant compound in the exudate sample, followed by malic acid and lysine. In addition,

**Table 1.** Qualitative assessment of root exudate composition as determined by GC–MS.

| Category | Compounds |
|---|---|
| Sugars (16) | xylose, tagatose, sucrose, **ribose**[a], **mannose**[a], **maltose**[c], lactose, glucose-6-phosphate, glucose, **fucose**[a], fructose-6-phosphate, **fructose**[a], cellobiose, glucose-1-phosphate, isomaltose, sedoheptulose |
| Sugar alcohols (6) | xylitol, **threitol**, **ribitol**[a], erythritol, sorbitol, mannitol |
| Sugar amines (2) | *N*-acetylmannosamine, galactosamine |
| Amino acids (19)[c] | **Val**[c], **Tyr**[a]**, Trp, Thr**[c], **Ser**[ab], **Pro**[c], **Phe**, **Ile**[a], **Gly**, **Asp**[a], **Ala**[c], **Asn**, **Leu**[c], **Met**[c], **His**[c], **Gln**, *N*-acetyl-tryptophan, ornithine, canavanine |
| Organic acids (51) | **succinic acid**[ab], stearic acid, shikimic acid, **phthalic acid**[a], **pentadecanoic acid**, pelargonic acid, palmitoleic acid, palmitic acid, **oxalic acid**[a], nicotinic acid, myristic acid, **malic acid**[a], lauric acid, **glyceric acid**[a], glutaric acid, **fumaric acid**[a], **dehydroascorbic acid**[a], **citric acid**[a], **capric acid**[a] **(r)**, benzoic acid, azelaic acid, **adipic acid**[a], 4-aminobutyric acid, **3-hydroxypropionic acid**[a], 3-aminoisobutyric acid, glycolic acid, vanillic acid, **salicylic acid**, hydroxybenzoic acid, threonic acid, **gluconic acid**[a], chlorogenic acid, 3-phosphoglycerate, α-ketoglutaric acid, aconitic acid, isocitric acid, 2,4-diaminobutyric acid, 2-hydroxybutanoic acid, 3-hydroxybutyric acid, D-galacturonic acid, citramalic acid, linoleic acid, heptadecanoic acid, stearic acid, cinnamic acid, tartaric acid, malonic acid, ferulic acid, 6-phosphogluconate, glucosaminic acid, fructose 2,6-biphosphate |
| Alcohols (2) | myo-inositol, β-glycerolphosphate |
| Ketones (2) | dihydroxyacetone, acetophenone |
| Amines (5) | tyramine, putrescine, hydroxylamine, cyclohexylamine, spermidine |
| Amides (3) | urea, biuret, indole-3-acetamide |
| Esters (3) | 1-monostearin, 1-monopalmitin, methyl palmitoleate |
| Others (7) | galactinol, 4-hydroxybenzoate, nicotinamide, **inosine**, uracil, guanosine, glutathione |

**Note:** Apart from the 116 compounds listed in this Table; there is evidence for the presence of at least 72 other compounds that, however, could not be identified. Compounds identified as attractants or repellents (r) in a previous study (Feng *et al.,* 2018) are shown in bold. Chemicals detected in this study but absent in the previous study are underlined.
**a**. and **b**. represents SQR9 McpA and McpC sensed ligands respectively.
**c**. All amino acids were L-isomers.

**Table 2.** Quantification of the compounds in 1× cucumber root exudates that sensed by McpA and McpC in SQR9 by UHPLC–MS/MS.

| Compound | Concentration (μmol $g^{-1}$ DW[a] root exudates) | Concentration in 1× root exudates solution (nM)[b] | Sensed by chemoreceptor (s) (Feng *et al.,* 2018) |
|---|---|---|---|
| Citric acid | 15.66 ± 4.95 | 313.19 | McpA |
| Malic acid | 3.46 ± 0.43 | 69.13 | McpA, TlpB |
| Lys | 1.16 ± 0.21 | 23.29 | McpA |
| Succinic acid | 0.81 ± 0.14 | 16.22 | McpA, McpC, McpR, TlpB |
| Glyceric acid | 0.76 ± 0.06 | 15.17 | McpA, McpB |
| Glu | 0.31 ± 0.01 | 6.10 | McpA |
| Gluconic acid | 0.29 ± 0.06 | 5.77 | McpC, McpA, McpB, TlpA |
| Val | 0.19 ± 0.02 | 3.73 | McpC |
| Fumaric acid | 0.17 ± 0.04 | 3.35 | McpA, TlpB |
| Mannose | 0.12 ± 0.01 | 2.35 | McpA |
| Asp | 0.12 ± 0.02 | 2.34 | McpA |
| Ile | 0.11 ± 0.03 | 2.17 | McpA |
| Ser | 0.10 ± 0.04 | 2.05 | McpC, McpA, McpB |
| Leu | 0.10 ± 0.01 | 1.94 | McpC |
| Pro | 0.09 ± 0.01 | 1.80 | McpC |
| Thr | 0.09 ± 0.03 | 1.71 | McpC, TlpB |
| Adipic acid | 0.07 ± 0.02 | 1.41 | McpA, McpB |
| Tyr | 0.07 ± 0.01 | 1.37 | McpA |
| Ala | 0.04 ± 0.01 | 0.83 | McpC |
| Met | 0.02 ± 0.00 | 0.37 | McpC, McpB |

**a.** DW means dry weight.
**b.** Concentration in 1× cucumber root exudates (0.02 mg $ml^{-1}$ of total compounds). Data are means and standard errors of three biological replicates of cucumber root exudates.

significant levels of several organic acids (succinic, glyceric, gluconic and fumaric acids), other amino acids (Glu, Val, Asp, Ile, Ser and Leu) and mannose were also detected, while the remaining six compounds were present at a concentration inferior to 0.10 μmol $g^{-1}$ DW root exudate (Table 2).

### *A few key attractants in root exudates contribute to overall recruitment of SQR9*

Having determined the concentration of the individual chemoattractants in root exudates now permitted to decipher their individual contribution to root-exudate chemotaxis and colonization. In initial experiments, we have generated a mixture of these 20 attractants listed in Table 2 (referred to as Mix-20) at a concentration that corresponded to that in 1×, 10×, 100× and 1000× concentrated root exudates samples. We then conducted chemotaxis assays of the wt and SQR9Δ*8mcp* mutant as well as this mutant complemented with either *mcpA* or/and *mcpC.* Like observed in the root exudates assay, the wt strain failed to show chemotaxis to 1× or 10× Mix-20 (Supporting Information Fig. S3), but showed a significant response to 100× and 1000× Mix-20 ($I_{30}$ = 0.90 and 0.96, respectively; Fig. 3). As expected, SQR9Δ*8mcp* was devoid of chemotaxis to the 100× and 1000× Mix-20 solutions (Fig. 3), and a partial or complete restoration of the chemotaxis phenotype was noted upon complementation with *mcpA* or/and *mcpC*, respectively ($I_{30}$ ranging from 0.64 to 0.93; Fig. 3). Results obtained with Mix-20 (Fig. 3) are thus very similar to those obtained with root exudates sample, which validates the Mix-20-based experimental approach and the quantification of the individual root exudates compounds.

Next we conducted chemotaxis assays of wt SQR9 towards the 20 individual components of Mix-20 at their concentration in the 100× and 1000× cucumber root exudates, respectively (Table 2; since 1× and 10× Mix-20 did not induce taxis, individual compounds were not tested at these concentrations). At the 100× concentration, malic acid was the strongest attractant ($I_{30}$ = 0.85, Fig. 4A) that showed taxis comparable to that observed with Mix-20 and 100× root exudates, followed by Lys, Ser and fumaric acid that triggering moderate attraction of SQR9 ($I_{30}$ ranging from 0.70 to 0.74; Fig. 4A); whereas mannose, glyceric acid, Ile, gluconic acid and Glu induced only weak chemotaxis of SQR9 ($I_{30}$ ranging from 0.61 to 0.69; Fig. 4A); the remaining 11 individual components caused only very minor responses with an $I_{30}$ ranging from 0.50 to 0.59 (Fig. 4A). The general pattern at the 1000× concentration was similar to that at the 100× sample, but additional compounds caused attraction of SQR9 (Fig. 4B). In detail, malic and gluconic acid triggered strongest chemotaxis of SQR9, of which the $I_{30}$ (0.91 and 0.85, respectively) were significantly higher than all the other compounds (Fig. 4B); fumaric acid, Ala, mannose and glyceric acid were found to be moderate attractants ($I_{30}$ ranging from 0.71 to 0.73; Fig. 4B); and Lys, Thr, Val, succinic acid and Glu induced weak responses of the bacteria ($I_{30}$ ranging from 0.62 to 0.65; Fig. 4B); finally,

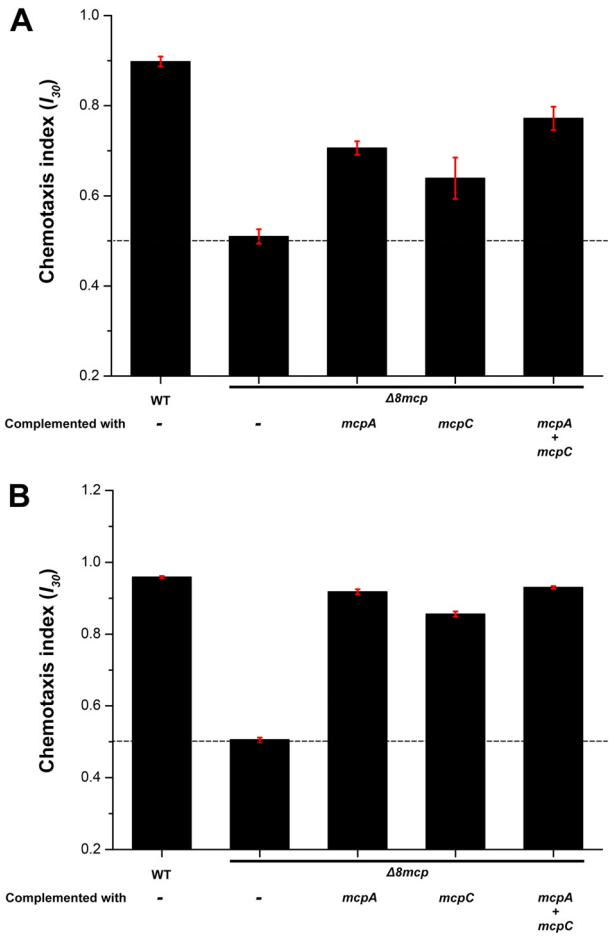


**Fig. 3.** Chemotaxis of wild-type SQR9, SQR9Δ8*mcp*, SQR9Δ8*mcp*/*mcpA*, SQR9Δ8*mcp*/*mcpC* and SQR9Δ8*mcp*/*mcpAmcpC* towards the 100× (A) and 1000× (B) compound mixture Mix-20. Shown are the chemotaxis indices from SlipChip assays. Data are means and standard errors from 13 replicates. The compounds that form part of Mix-20 are shown in Table 2 (Mix-20). [Color figure can be viewed at wileyonlinelibrary.com]

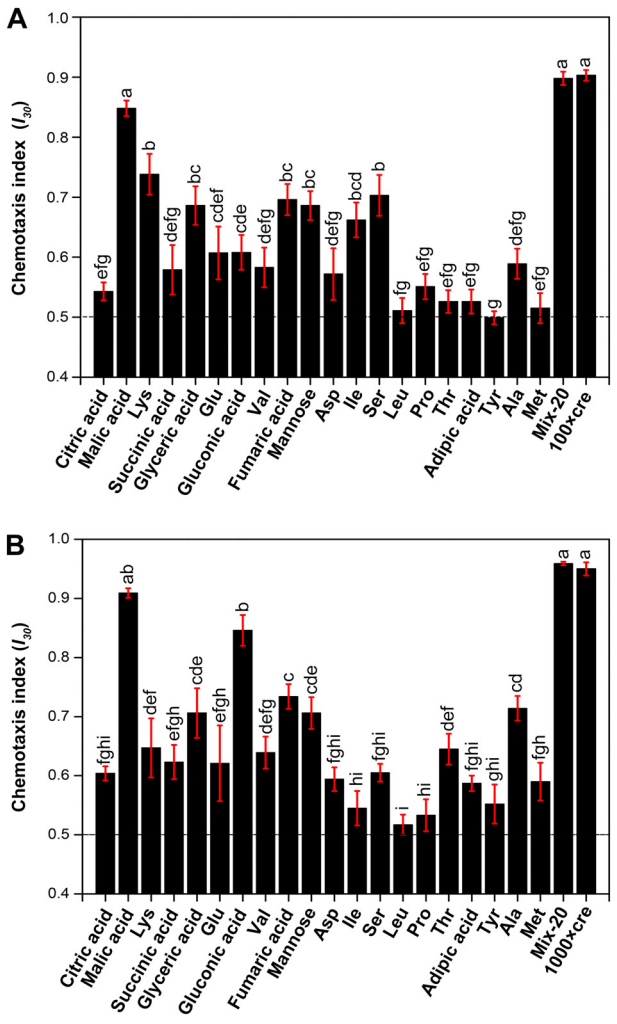


**Fig. 4.** Chemotaxis of SQR9 towards the 20 individual compounds at their concentrations in the 100× (A) and 1000× (B) cucumber root exudates (cre for short in the Figure). All amino acids were L-isomers; the D-isomer of mannose was used; and other seven compounds (citric, malic, succinic, glyceric, gluconic, fumaric and adipic acids) were racemic mixtures. Shown are chemotaxis indices from SlipChip assays. Data are means and standard errors from 13 replicates. Columns with different letters are statistically different according to the Duncan test ($P < 0.05$). [Color figure can be viewed at wileyonlinelibrary.com]

the other nine chemicals did not attract SQR9 cells (Fig. 4B).

To get a comprehensive understanding of the dose–response relationships of these compounds, we summarize in Fig. 5 the tactic responses of wild-type SQR9 to the 20 attractants as determined here and in our previous study (Feng *et al.,* 2018). It can be observed that most chemotactic response was dependent on ligand dose concentration, but the $EC_{50}$ value (the concentration at which the compound can induce half of the maximal response) for the different compounds was quite variable. In particular, the $EC_{50}$ value of many attractants were in the micromolar range, such as those for malic acid, Lys, Glu, gluconic acid, Val, Leu, adipic acid and Met (Fig. 5). Some of the chemoeffectors had an $EC_{50}$ value at lower concentrations, including fumaric acid, mannose and Ala, which are present in root exudates at lower concentrations, but that induce significant SQR9 taxis (Table 2 and Fig. 4). On the other side, although citric acid at 1 mM was previously reported as a strong attractant of SQR9 previously (Feng *et al.,* 2018), it can only mediate weak chemotaxis even at 0.3 mM (Fig. 5). This could be related to the fact that citrate was the most abundant chemoeffector in cucumber root exudates, but has had barely any effect on inducing taxis of SQR9 in rhizospheric condition.

Finally, to assess the additive effects of these compounds, we ordered these 20 attractants according to their magnitude of chemotaxis as determined in Fig. 4 for 100× and 1000× root exudates. Starting with the compound that caused least chemotaxis, that is, Tyr in

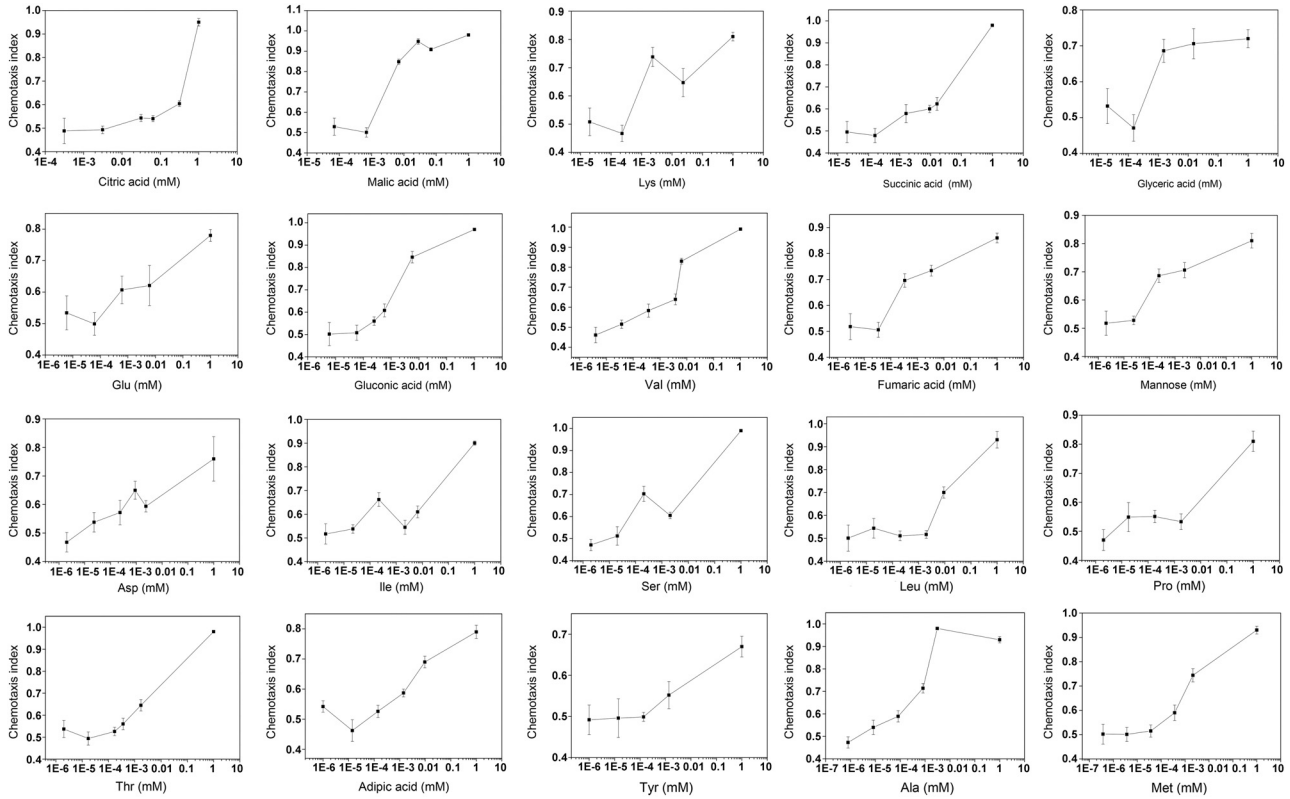


**Fig. 5.** Chemotaxis of wild-type SQR9 towards the 20 attractants at different concentrations. Shown are the $I_{30}$ values at concentrations corresponding to that in 1×, 10×, 100× and 1000× cucumber root exudates (obtained in this study), and those obtained using 1 mM chemoeffector solutions, as reported previously (Feng *et al.,* 2018). Taxis was measured to an additional concentration of citric acid, malic acid, succinic acid, gluconic acid, Val, Asp, Ile, Leu, Thr, adipic acid, Ala and Met. The chemotaxis assays were performed by the SlipChip assay as described in the Methods. Error bars represent the standard error from the mean of 13 replicates.

100× and Leu in 1000× concentrations, respectively, we then generated two sets of 19 different compound mixtures (corresponding to 100× and 1000× Mix-20), by adding consecutively individual compounds according to the sequence of increasing chemotaxis. The composition of these different compound mixtures is provided in Supporting Information Fig. S4, and the respective concentration of the individual compounds corresponds to that in a 100× or 1000× root exudates solution. Chemotaxis assays indicated that at 100× root exudates, a significant increase was noted following the addition of Leu to Tyr (Mix-2), while the subsequent addition of a series of chemoattractants did not significantly alter the chemotactic response (Supporting Information Fig. S4). Importantly, supplement of the strongest attractant, malic acid, led to a second significant increase in the $I_{30}$ value (Supporting Information Fig. S4). With regard to the 1000× conditions, a significant increase was only observed following the inclusion of Pro (Mix-2), whereas the addition of Glu (Mix-10), Thr (Mix-13), gluconic acid (Mix-19) and malic acid (Mix-20) caused minor increases in response (Supporting Information Fig. S4). Supplying most of other chemicals did not significantly alter the chemotaxis index; and addition of succinic acid even significantly decreased the $I_{30}$ value from 0.85 to 0.74 (Supporting Information Fig. S4).

Taken together, initial mix of two weak attractants lead to a significant increase of chemotaxis, whereas further inclusion of most compounds induced only limited additive effects, indicative of a saturation of the system with ligands. The chemotaxis to cucumber root exudates by SQR9 is driven by a handful of strong attractants ($I_{30} \geq 0.7$), such as malic acid (both concentrations), Lys (100×), Ser (100×), fumaric acid (both), gluconic acid (1000×), Ala (1000×), mannose (1000×) and glyceric acid (1000×). Malic acid, Lys, fumaric acid, mannose and glyceric acid stimulate the McpA receptor; Ala, another strong attractant, is sensed by McpC, whereas Ser and gluconic acid are sensed by both receptors (Table 2).

## Discussion

Chemoreceptor-mediated taxis to root exudates is frequently essential to initiate the interaction between plants and rhizobacteria. This is the case for symbiotic and non-symbiotic beneficial bacteria as well as for pathogenic

species (Hazelbauer and Lai, 2010; Hida *et al.,* 2015; Allard-Massicotte *et al.,* 2016; Scharf *et al.,* 2016; Matilla and Krell, 2018). Studies of bacterial chemotaxis to plant root exudates, as well as its role in rhizosphere colonization, have received increasing attention in recent years (Scharf *et al.,* 2016; Massalha *et al.,* 2017). Amino acids such as Arg, Ala and Ile, as well as organic acids like malic, citric or fumaric acid have been shown to serve as chemoattractants for different rhizobacteria (de Weert *et al.,* 2002; Gupta Sood, 2003; Rudrappa *et al.,* 2008; Tan *et al.,* 2013; Liu *et al.,* 2014; Zhang *et al.,* 2014; Webb *et al.,* 2017b). In addition, inactivation of chemoreceptors responsible for sensing amino acids, malic acid and oxygen significantly impaired chemotaxis to root exudates or/and root colonization by *Sinorhizobium meliloti*, *R. pseudosolanacearum* and *A. caulinodans*, respectively (Webb *et al.,* 2014; Hida *et al.,* 2015; Jiang *et al.,* 2016). These studies focus on one or several stimuli and their cognate MCPs. Here, we present the first global analysis of the contribution of individual chemoreceptors and chemoeffectors to chemotaxis to root exudates. Whereas in the previous study we assessed chemotaxis to chemoeffectors at 1 mM (Feng *et al.,* 2018), the experimental design of the present study was based on the compound concentration detected in cucumber root exudates. The main result of this work resides in the demonstration that a handful of chemoattractants play key roles, and the nature of the dominant attractants partially depends on the exudate concentration (Fig. 6).

The physiological relevance of bacterial chemotaxis depends largely on the ligand concentration in the corresponding physiological habitat (Glekas *et al.,* 2010; Martin-Mora *et al.,* 2016a). Therefore, the identification of SQR9 chemoeffectors based on experimentation with 1 mM solution did not permit to derive conclusions as to the contribution of each root exudate component to overall taxis in the rhizosphere (Feng *et al.,* 2018). Here, the quantification of chemotaxis in response to single attractants at their detected concentration in root exudates, as well as the different mixtures, suggested that a significant number of weak-attractants had only a limited contribution to root exudate taxis, whereas strong attractants were found to drive the overall taxis to cucumber root exudates (Figs 4 and 5; Supporting Information Fig. S4). Additionally, several issues related to our experimental design have to be mentioned: (i) Since it is very difficult to determine the compound concentrations *in situ*, we have determined the concentrations of selected attractants in root exudates. (ii) The composition of root exudates and the relative abundance of the individual compounds vary with cucumber growth stages and the root sites (Chaparro *et al.,* 2014). Here, we have chosen seedlings with fully developed cotyledons for exudate collection because a potential microbial fertilizer would be applied at this stage. Future studies will be aimed at determining the effects of growth stages and root site on the composition and function of the root exudates. (iii) The GC–MS analysis is limited in providing information on the stereochemistry of root exudate components. Therefore, we have selected the isomer for further studies that naturally occurs in the rhizosphere. (iv) This work was performed in liquid medium and not in verified in soil system. Several previous studies have shown that the colonization pattern of rhizobacteria in hydroponic/agar medium system agreed with that observed under soil/culture medium system (de Weert *et al.,* 2002; Cai *et al.,*

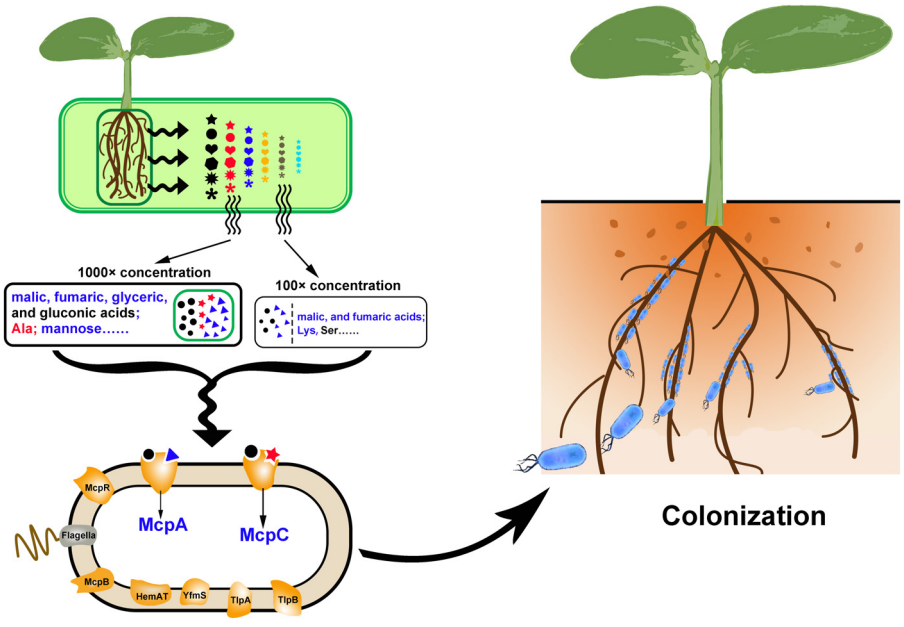


**Fig. 6.** Model of *B. velezensis* SQR9 recruitment to cucumber roots. McpA and McpC mediate chemotaxis to several dominant attractants (malic, fumaric, gluconic and glyceric acids, Lys, Ser, Ala and mannose). Stimuli sensed by McpA are shown in blue, those sensed by McpC in red, whereas ligands sensed by both receptors in black. [Color figure can be viewed at wileyonlinelibrary.com]

2009; Chen *et al.,* 2013). Therefore, and considering the better controllability of hydroponic system, the colonization assays in this study were performed in liquid MS medium, which is also used as a planting medium. Future studies will be carried outin a soil/medium system.

The data presented in this article permit for the first time to determine whether there is a relationship between the abundance of a given compound in root exudates and its contribution to overall taxis to root exudates. Using the compound concentration in cucumber root exudates (Table 2) and their capacity to induce taxis (Fig. 4), we have performed a Spearman's correlation test, which showed that compound abundance positively correlated with their capacity to induce chemotaxis; both for 100× (coefficients = 0.549, $P$ = 0.012) and 1000× (coefficients = 0.455, $P$ = 0.044) root exudates, suggesting that more abundant compounds in general exert key roles in the overall root exudate taxis. However, why are the key chemoattractants not the same for different root exudates concentrations? For example, Lys was the second strongest attractant at 100× but induced only an average response at the 1000× root exudates (Fig. 4). One possible explanation may be related to the fact that chemotaxis dose–response relationships are frequently biphasic (Martin-Mora *et al.,* 2016b) and in some cases even polyphasic (Lacal *et al.,* 2010b). This implies that the maximum of a given chemotactic response is frequently not observed for the highest concentration. Since each chemoattractant has a different dose–response behavior, their relative contribution to overall root exudates taxis may vary with the exudation concentration (Fig. 5).

Some discrepancies were observed between the chemotaxis of the individual compounds (Fig. 4) and when present in the different reconstituted root exudates (Supporting Information Fig. S4). For example, inclusion of succinic acid, a weak attractant under 1000× condition, even decreased the $I_{30}$ value or the addition of moderate stimuli did not cause a significant increase in chemotaxis (Supporting Information Fig. S4). However, McpA and McpC respond to multiple ligands. When the taxis to individual compounds is monitored, receptors respond to a single compound. In the response to mixtures multiple ligands will interact with both receptors and the addition of further ligands, depending on their affinity, may not result in additional receptor stimulation. We also noticed that no chemotaxis was observed to 1× and 10× root exudates by SQR9 cells. A 10× concentration translates to a concentration of 3 μM for citric acid, whereas the remaining compounds are present at nanomolar concentrations. Typically, the dissociation constants of chemoeffectors by chemoreceptors are in the μM range (Matilla and Krell, 2017); therefore, the effector concentrations in 10× root exudates are too low for chemoreceptor binding. Taken together the facts that chemotaxis is essential for root colonization by SQR9 and that the strain is unable to respond chemotactically to 1× and 10× root exudates indicates that the strain senses local compound concentrations that are well above those in exudate samples. The chemotaxis assays were conducted in liquid medium, and the concentration used here (1×, 10×, 100× and 1000×) are artificial recomposed root exudates; thus it is difficult to directly correlate these concentrations to those in natural soil. In a more diffusion limited soil environment, the tested strain may sense and respond to these local concentrations that are well above those measured in the liquid exudate samples. Similarly, it has been shown that lower acyl-homoserine lactones (AHLs) concentrations are required for gene activation *in situ* (soil) than in liquid culture (Schuster *et al.,* 2013).

The dominant attractants identified for SQR9 have also been reported to be chemoeffectors for other rhizobacteria or to carry out other functions in plant-microbe interactions. For example, malic acid that is present both in the root exudates of *Arabidopsis thaliana* and tomato, is a chemoattractant for *B. subtilis* (Rudrappa *et al.,* 2008), *R. pseudosolanacearum* (Hida *et al.,* 2015) and *P. fluorescens* (de Weert *et al.,* 2002; Gupta Sood, 2003) and contributes to root colonization. Further examples illustrate that several of the other key attractants (fumaric and gluconic acids, Lys, Ser and Ala) that were identified in exudates of *Cicer arietinum* L., tomato and alfalfa, were also important attractants for rhizospheric microorganisms (Gitte *et al.,* 1978; de Weert *et al.,* 2002; Gupta Sood, 2003; Glekas *et al.,* 2012; Webb *et al.,* 2017a). It appears that organic acids (especially the tricarboxylic acid cycle intermediates) and amino acids serve frequently as chemoattractants for various rhizobacteria (Bardy *et al.,* 2017). Interestingly, citric acid, the most abundant compound in cucumber root exudates as well as in root exudates of tomato and sweet pepper (Kamilova *et al.,* 2006), failed to induce chemotaxis of SQR9 even at 1000× concentrations (Fig. 4). It has also been reported that PGPR *P. putida* KT2440 harboured a specific citrate receptor; which however mediated low sensitivity responses since first significant chemotaxis was observed at 5 mM (Martin-Mora *et al.,* 2016a). It seems that although citric acid is an abundant root exudate component, some rhizobacteria show only moderate taxis to this tricarboxylic acid cycle intermediate.

A parallel study using the plant-associated *B. subtilis* NCIB3610 strain indicated that chemoreceptors McpB, McpC and TlpC dominate the bacterial chemotaxis to *A. thaliana* root exudates, while the early colonization of plant roots by *B. subtilis* was dependent on additional chemoreceptors (Allard-Massicotte *et al.,* 2016). In contrast, only McpA and McpC were found to be essential for root colonization by *B. velezensis* SQR9. This can at least partially be attributed to the fact that the ligands of

both receptors are different to those in their *B. subtilis* homologous (Garrity *et al.,* 1998; Glekas *et al.,* 2012). This discrepancy suggests that although *B. velezensis* (formerly known as *B. amyloliquefaciens* subsp. *plantarum*) and *B. subtilis* are closely related at the sequence level including the conservation of most *mcp* genes, the roles of these chemoreceptors are quite different, both in terms of ligand profile and ecological functions, which is in accordance with the bioinformatics-based predication by Yssel *et al*. (2011).

McpA and McpC are the two receptors with the broadest ligand range in SQR9 (Feng *et al.,* 2018) and both contain a dCACHE_1 type of ligand binding domain (LBD) (Upadhyay *et al.,* 2016; Feng *et al.,* 2018). Interestingly, a dCACHE_1 domain containing chemoreceptor, CcmL, with an equally broad ligand range has been identified in the human pathogen *Campylobacter jejuni* and its activity was essential for the bacterial survival in the host (Rahman *et al.,* 2014). In general, chemoreceptor activation can be achieved by either the direct binding of chemoeffectors or in complex with ligand binding proteins, and potentially these different binding modes may account for this broad ligand range (Matilla and Krell, 2017; Ortega *et al.,* 2017).

In conclusion, we show here that McpA and McpC work in an additive manner to drive the chemotaxis of *B. velezensis* SQR9 to cucumber root exudates and rhizospheric colonization, by responding to a handful of key chemoattractants. This work also forms the scientific basis for biotechnological applications to improve plant colonization by PGPR using genetic engineering approaches to increase, for example, the cellular abundance of both key chemoreceptors or, alternatively, by enhancing and the exudation of certain attractants by host plants.

## Experimental procedures

### *Bacterial strains, media and growth conditions*

The strains and plasmids used in this study are listed in Supporting Information Table S1. *B. velezensis* SQR9 (China General Microbiology Culture Collection Center, CGMCC accession no.5808; formerly known as *B. amyloliquefaciens*) was isolated from the cucumber rhizosphere; the mutant strain deficient in all eight *mcp* genes (SQR9Δ*8mcp*) and various complementary strains were constructed in a previous study (Feng *et al.,* 2018). All strains were grown at 37°C in low-salt Luria-Bertani (LLB) medium (peptone, 10 g $l^{-1}$; yeast extract, 5 g $l^{-1}$; NaCl, 3 g $l^{-1}$) solidified with 15 g $l^{-1}$ agar; when necessary, the final concentrations of antibiotics were added as follows: 5 mg $l^{-1}$ chloramphenicol (Cm); 20 mg $l^{-1}$ zeocin (Zeo); 100 mg $l^{-1}$ spectinomycin (Spc); 30 mg $l^{-1}$ kanamycin (Kan).

### *Plant material and growth condition*

Cucumber seeds of cultivar 'Jinchun 4' were surface-sterilized with 2% (w/v) NaClO for 15 min followed by three rinses in sterile distilled water, and were then placed into sterile tissue culture bottles containing vermiculite (Xianlin Garden Center, Nanjing) for germination. After incubation for four days in a growth chamber at 22°C with a photoperiod of 16 h light/8 h dark, the germinated seedlings were then transplanted into 50-ml conical flasks containing 40 ml sterile liquid 1/4 sucrose-free Murashige Skoog (MS) medium (Murashige and Skoog, 1962), with one seeding in each flask. The MS medium was changed every two days during the growth period. Subsequently, the seedling containing flasks were cultured on a shaker at 50 r.p.m. and illuminated under cool white fluorescent light with a photoperiod of 16-h light/8-h dark at 25 ± 5°C. The cucumber plants with fully developed cotyledons were used for further studies.

### *Root colonization assay*

Suspension of SQR9 and derived strains (SQR9Δ*8mcp* and complemented strains) were prepared in LLB medium (with suitable antibiotics if necessary) and grown for 12 h at 37°C with 200 r.p.m. shaking. The cells were pelleted by centrifugation at 10 000 *g* for 10 min and resuspended in 1/4 sucrose-free Murashige Skoog medium at an $OD_{600}$ of approximately 1.0. The resulting cell suspension was used to inoculate sterile conical flasks containing 40 ml MS medium and the germinated seedlings at ratio of 1%, leading to a final cell concentration of approximately $5 \times 10^7$ CFUs $ml^{-1}$. After incubation at the conditions described above for three days, the cucumber roots were cut and briefly washed with sterile distilled water. Subsequently, the collected root samples were weighed and ground with a mortar in sterile distilled water; the obtained suspensions were diluted and plated onto LLB agar medium (with suitable antibiotics if necessary). Cell quantification was achieved by counting the bacterial colonies after incubation at 37°C for 12 h. The data were normalized to root wet weight as described by Liu *et al*. (2017).

### *Generation of cucumber root exudates*

Before collecting the root exudates from cucumber seedlings with fully developed cotyledons, each conical flask was checked for sterility by plating 100-μl aliquots onto LLB agar medium. Plants were extracted from their recipients and cucumber roots were washed using sterile distilled water. In total 90 plants were placed into a 50-ml conical flask and the roots were submerged into 40 ml sterile distilled water. All plants were placed in a plant

growth chamber for 24 h (16-h light/8-h dark) at 25 ± 5°C with gentle shaking (50 r.p.m.). Plants were then removed from their flasks and the exudate solution was filtered using a 0.45 μm cut-off (Millipore), and sterility was verified by plating 100 μl exudate samples onto LLB agar medium. Samples were then lyophilized and the powder stored at −80°C for further study (Liu *et al.,* 2014). In total, 72 mg of exudates from three biological replicates of each 30 plants were collected that amount to a total volume of 3.6 l (~0.02 mg $ml^{-1}$ in the flasks).

### *Identification of root exudate components*

The collected root exudates from three individual biological replicates were analysed by gas chromatography–mass spectrometry (GC–MS) at Shanghai Biotree biotechnology limited company, China, for qualitative analysis of their components. Based on the GC–MS data, the ligands sensed by the SQR9 chemoreceptors were further analysed by the same company using ultra-high performance liquid chromatography- tandem mass-spectrometry (UHPLC–MS–MS) for quantification (Supporting Information Methods). Absolute quantification of each target compound in cucumber root exudates was calculated based on their detected peak areas and the calibration curves of relevant standard.

### *Preparation of recomposed root exudates*

Different recomposed root exudates of the cucumber exudation components were prepared by dissolving compounds in phosphate buffer saline (PBS), taking into account their concentration in root exudates as determined by LC–MS/MS.

### *Chemotaxis assay*

The chemotaxis assays were based on a simple and reusable microfluidic SlipChip device fabricated by State Key Laboratory of Microbial Resources, Institute of Microbiology, Chinese Academy of Sciences, Beijing, China (Shen *et al.,* 2014). Prior to the assay, a 10 mg $ml^{-1}$ bovine serum albumin (BSA) solution was injected into all channels of this device and left for 5 min to reduce cell adherence. After removing the BSA solution by vacuum sucking, the chemoattractant solution (either root exudates or recomposed root exudates) dissolved in PBS (cucumber root exudates with a concentration of 0.02 mg $ml^{-1}$ was defined as the 1 × was, and so on for 10×, 100× and 1000× root exudates) and the buffer control were placed into the top and bottom microwells, respectively; the prepared bacterial suspension in PBS was loaded to the middle microwells (Supporting Information Fig. S5). Subsequently, the SlipChip device was slipped to ensure that the cells can migrate freely from the middle microwells to the ducts and microwells were loaded with the chemoeffector or PBS (Supporting Information Fig. S5). The device was placed on an inverted fluorescence microscope (Ti-Eclipse, Nikon, Japan) and kept for 30 min in the dark. Then, the migration of the cells in the presence of the top microwells and bottom microwells were monitored by counting the number of cells in the chemoeffector microwells and PBS microwells according to the pictures obtained at the 30 min point. The chemotaxis index ($I_{30}$) was used to indicate the chemotactic ability of the bacterial cells with a chemoeffector at a certain concentration. $I_{30}$ is defined as $N_e/(N_e + N_c)$, where $N_e$ is the number of cells that have migrated to the chemoeffector, and $N_c$ is number of cells that have migrated to the control microwells in a certain time period.

An $I_{30}$ value between 0.4 and 0.6 ($0.4 \leq I_{30} \leq 0.6$) indicates an absence of taxis; an $I_{30}$ value more than 0.6 ($I_{30} > 0.6$) indicates that the cells are attracted by the chemoeffector; while $I_{30}$ value lower than 0.4 ($I_{30} < 0.4$) indicates repellent responses.

### *Construction of the SQR9Δ8*mcp *mutant complemented with the* mcpA *and* mcpC *genes*

The double-complementary strain expressing *mcpA* and *mcpC* was constructed by integrating the two genes into the *amyE* locus of SQR9Δ*8mcp*, following the experimental protocol reported by Zhou *et al*. (2017). The primers used are listed in Supporting Information Table S2.

### *Statistical analysis*

The Duncan's multiple rang tests ($P < 0.05$) of the SPSS version 22.0 (IBM, Chicago, IL, version 22.0) was used for statistical analysis.

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## Conflict of interest

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Table S1.** Bacterial strains used in this study.

**Table S2.** Primers used in this study.

**Fig. S1.** Growth of *B. velezensis* SQR9, SQR9*Δ8mcp*, and different complemented strains. Overnight cultures of wt SQR9 in LLB were adjusted to an $OD_{600}$ = 1.0, and the resulting suspension was used to inoculate fresh LLB broth at ratio of 1%.

**Fig. S2.** Chemotaxis of wild-type SQR9 towards cucumber root exudates at 1× and 10× concentration. The chemotaxis assay was performed by the SlipChip assay as described in the Methods. Error bars represent the standard error from the mean of 13 replicates.

**Fig. S3.** Chemotaxis of wild-type SQR9 towards the 1× and 10× compound mixture Mix-20. The chemotaxis assay was performed by the SlipChip assay as described in the Methods. Error bars represent the standard error from the mean of 13 replicates. The compounds that form part of Mix-20 are shown in Table (Mix-20).

**Fig. S4.** Chemotaxis of SQR9 towards recomposed root exudates. The chemotaxis to 100× and 1000× cucumber root exudates (cre for short in the Figure) is also shown for comparison. In these mixtures, compounds are present at the equivalent concentration in 100× (A) and 1000× (B) cucumber root exudates. Compounds are ordered according to the magnitude of chemotaxis the individual compounds induce at the 100× and 1000× concentration. The added compound in each Mix is shown in the X-axis legends. Abbreviations: Tyr = tyrosine, Leu = leucine, Met = methionine, Thr = threonine, Adip = adipic acid, Citr = citric acid, Pro = proline, Asp = aspartic acid, Succ = succinic acid, Val = valine, Ala = alanine, Glu = glutamic acid, Gluc = gluconic acid, Ile = isoleucine, Glyc = glyceric acid, Mann = mannose, Fuma = fumaric acid, Ser = serine, Lys = lysine, Mali = malic acid. The Error bars represent the standard error from the mean of 13 replicates. Columns with different letters are statistically different according to the Duncan test ($P < 0.05$).

**Fig. S5.** Scheme of SlipChip device used in this study.

A. Schematic view of the SlipChip device.

B. Feature view of the assembly of the top and the bottom plates.

C–E. The individual steps in a chemotaxis assay are illustrated using dyes. The device was loaded with chemoeffector, bacterial cells and buffer solution at state I. After slipping, the loading ducts were disconnected from the microwells, which created a diffusion concentration gradient for bacterial chemotaxis at state II. Finally, the device was slipped again to terminate the experiment and collect the migrated cells at state III.

**Appendix S1:** Supporting Information Methods Identification of root exudate components.