Plant Soil (2020) 451:357–370
https://doi.org/10.1007/s11104-020-04531-2

REGULAR ARTICLE

L-Glutamic acid induced the colonization of high-efficiency nitrogen-fixing strain Ac63 (*Azotobacter chroococcum*) in roots of *Amaranthus tricolor*

Yi-Fan Wang · Jun-Feng Wang · Zhi-Min Xu · Shao-Hua She · Jun-Qing Yang · Qu-Sheng Li

Received: 6 November 2019 / Accepted: 8 April 2020 / Published online: 27 April 2020

Abstract

Aims The aim of this study was to evaluate its feasibility of a non-symbiotic nitrogen-fixing strain, Ac63 (*Azotobacter chroococcum*), to promote biomass of common vegetable crops and its colonization mechanisms in their roots.

Methods Root exudates of common vegetables, including *Amaranthus tricolor* (AT), *Willow amaranth* (WA), *Chrysanthemum coronarium* (CC), cabbage, and lettuce, were collected, and a chemotaxis assay with Ac63 was performed. Components in exudates for the most differential chemotaxis effect crops (AT and lettuce) were determined with GC-MS. Then, the traditional screening strategies of chemotaxis, swarming, and in vitro were utilized to find the signal molecule for this strain. Finally, the colonization effects of Ac63 in roots of these two crops were verified with a soil pot experiment.

Results Chemotaxis effects of root exudates on Ac63 follow the lists of AT > WA > CC > cabbage ≈ control (water) ≈ lettuce. With a 30-μM concentration of L-glutamic acid, bacterial amount of Ac63 in chemotaxis and in vitro was observed with 2.9- and 7.4-times enhancement compared with that in the control. Laser confocal microscopy indicated that L-glutamic acid induced Ac63 to form a robust biofilm in roots, suggesting that L-glutamic acid is a signal molecule for the colonization of this strain. Soil pot assay showed that the biomass, chlorophyll, and available nitrogen of AT significantly increased than those of lettuce after Ac63 inoculation, resulting from this strain was substantially increased in roots of AT with L-glutamic acid secretion.

Conclusion L-Glutamic acid induced the colonization of high-efficiency nitrogen-fixing strain Ac63 in AT roots, which provides a practical information for its agricultural applications.

Keywords Nitrogen fixation strain · Root exudates · Chemotaxis · Swarming and in vitro · Biofilm formation

Responsible Editor: Luz E. ed-Bashan.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11104-020-04531-2) contains supplementary material, which is available to authorized users.

Y.-F. Wang · J.-F. Wang · Z.-M. Xu · S.-H. She · J.-Q. Yang · Q.-S. Li (✉)
School of Environment, Key Laboratory of Environmental Pollution and Health of Guangdong Province, Jinan University, Guangzhou 510632, China
e-mail: liqusheng@21cn.com

Introduction

Agricultural activities need a large amount of commercial nitrogen fertilizers to effectively promote crop growth, but it can also result in soil compaction and water eutrophication (Carpenter et al. 1998). Development of more environmentally benign nitrogen fertilizers such as biological N_2 fixation would therefore be useful for the decremental application rate of commercial nitrogen fertilizers (Galloway et al. 2004). A biogenous azotobacter and combined nitrogen-fixing bacteria can colonize in rhizosphere of non-legume plants

during the agricultural activities (Carvalho et al. 2016); thus, it is a more effective method to protect environmental pollution caused by nitrogen fertilizers when compared with that of symbiotic nitrogen-fixing bacteria.

It is widely accepted that mass number of non-symbiotic nitrogen-fixing bacteria could be utilized to promote plants' growth after they were effectively colonized in rhizosphere (Fibach-Paldi et al. 2012; Ke et al. 2019; Maheshwari 2011; Puri et al. 2016). The colonization processes of these bacteria were primarily relied on the attraction effects of root exudates (el Zahar Haichar et al. 2014), such as sugars, organic acids, or some secondary metabolites (Huang et al. 2014). The migration of these bacteria to root surface (could be achieved by the bacterial effects of chemotaxis and swarming motility) and the formation of biofilm are two necessary processes for their early and persistent colonization, respectively (Allard-Massicotte et al. 2016; Beauregard et al. 2013; Gao et al. 2016). The chemotaxis responses of bacteria are referred to the flagella rotation to swim in a favorable direction attracted by the chemical compound compositions and concentrations in environment (Eisenbach 1996). Swarming motility is a rapid multicellular movement of bacteria with surviving the semi-solid surface (agar > 0.3%) driven by flagella rotation (Kearns 2010). It has been reported that a larger number of components in root exudates could act as the signaling molecules for the colonization of strains in their rhizosphere. For instance, carbohydrates, dicarboxylates, and L-amino acids in root exudates of corn, *Arabidopsis thaliana*, and tomato can attract the colonization of *Bacillus velezensis* S3-1, *Azospirillum brasilense*, and *Pseudomonas fluorescens* Pf0-1 in their rhizosphere, respectively (Jin et al. 2019; Oku et al. 2012; Singh et al. 2019).

At present, ~92 strains, distributed in bacteria and archaea, are known with a nitrogen fixation capacity (Dos Santos et al. 2012; McRose et al. 2017). In total, ~ 20 high-efficiency non-symbiotic nitrogen-fixing strains are preserved in China. We hypothesized that (i) the nitrogen fixation capacity of 9 representative non-symbiotic nitrogen-fixing bacteria was different even with a similar medium culture; (ii) the distinct attraction ability of crops on strains resulted from different components and concentrations of root exudates; (iii) signal molecules may be found in root exudates of the crop with a high attraction ability for nitrogen-fixing bacteria; and (iv) the efficient colonization of preferred stain will help to crop yield essential for environmental protect by reducing the application rate of commercial nitrogen fertilizers.

Materials and methods

Bacterial strains

Nine non-symbiotic nitrogen-fixing bacterial strains were purchased from China Center of Industrial Culture Collection (CICC), China General Microbiological Culture Collection Center (CGMCC), and Agricultural Culture Collection of China (ACCC), and the detailed information and enrichment medium of these strains are presented in Table S1.

Nitrogen fixation capacity evaluation

Each strain suspension (~ 10^7 colony-forming unit (CFU) mL^{-1} fluid prepared with an enrichment medium, Table S1) was respectively inoculated (1% *v*/*v*) in a conical flask with sterile Ashby nitrogen-free liquid medium (Table S1) and 10% soil solution (pH, 5.52; total nitrogen, 25.38 mg kg^{-1}; ammonia nitrogen, 1.98 mg kg^{-1}; and nitrate nitrogen, 1.26 mg kg^{-1}). Ammonia nitrogen concentrations of the supernatant medium in days 5 and 7 were determined as indicated in the previous study (Bergersen 1980). Then, strain with a higher ammonium excretion (> 1.0 mg L^{-1}) ability was selected to determine its nitrogenase activity in Ashby's nitrogen-free medium (semi-solid medium, 0.5% agar) (Hardy et al. 1973).

Strain Ac63 chemotaxis with root exudates

Sterilized seedlings of five common vegetables, including *Amaranthus tricolor* (AT), *Willow amaranth* (WA), *Chrysanthemum coronarium* (CC), cabbage, and lettuce, were cultured in sterile Hoagland medium (discharged every 2 days) and were illuminated with the cool white fluorescent light with a daylight cycle of 16 h (28 ± 2 °C) for 4 weeks. Crops were transferred in sterile water for 2 days to collect root exudates after their roots were washed with deionized water for three times; then, the solution of root exudates was filtered with a 0.22-μm membrane and then stored at − 20 °C (Haier BCD-625WDGEU1, China). Total organic

carbon (TOC) concentrations of root exudates were measured using a TOC analyzer (Shimadzu TOC-LCPH, Japan). The chemotaxis effects of Ac63 in response to root exudates were carried out according to the previously described method (Ling et al. 2011). Briefly, a 200-μL pipette tip containing 100 μL of Ac63 suspension (~ 10^7 CFU mL^{-1} fluid prepared with an Azotobacter medium, Table S1) was attached to 1-mL tuberculin syringe with a 4-cm 25-gauge needle for 2 h. The syringes contained 200 μL root exudates (TOC concentrations, 0.1, 0.5, 1.0, 3.0, 5.0, and 10.0 mg L^{-1}) or sterile water (as a control groups). Plate counting method was used to evaluate the influences of different concentrations of root exudates of the five crops on their chemotaxis performances of strain Ac63. Components in root exudates of the most differential chemotaxis effects of two crops (AT and lettuce) were identified with a gas chromatography–mass spectrometry (GC-MS) (Shimadzu QP2010 Ultra, Japan) as indicated with the previous derivatization processes (Lisec et al. 2006) and operating conditions (Guo et al. 2018). One-microliter sample was injected into the GC-MS with a splitless mode. Chromatographic separation was set with a DB-5 integrated guard column (30 m × 0.25 mm ID, 0.25-mm film thickness, Agilent, USA). Carrier gas flow rate was 1 mL min^{-1}. Column temperature was initially maintained at 70 °C for 1 min, followed by a 1 °C min^{-1} ramp to 76 °C, then increased to 330 °C with a rate of 5 °C min^{-1}, and a final hold for 6 min at 330 °C. Mass spectra were recorded at 20 scans per second with a scanning range of 50–1000 m/z. The concentration of glutamic acid in root exudates of five crops was measured with the glutamic acid analysis kit (A074 Nanjing Jiancheng, China).

Root exudates effected on chemotaxis, swarming, in vitro, and biofilm formation of Ac63

The different components in root exudates of AT and lettuce were respectively selected for a chemotaxis assay with Ac63 as the description in the previous study (Ling et al. 2011). Each component with an excellent chemotactic effect were selected for swarming experiments, and then in vitro assay was performed with the component with an obvious swarming effect on Ac63 as indicated in the previous research (Ling et al. 2011). Component, which shows a significant recruiting ability for Ac63 in chemotaxis, swarming, and in vitro experiments, was considered as the colonization signal molecule of this strain. Sterile water effected on this strain was used as the control treatments.

The qualitative assessment of Ac63 biofilm formation on the roots' surface was visualized with the laser confocal microscopy (Zeiss LSM880, Germany) (Rudrappa et al. 2008). To quantify the biofilm formation as affected by root exudates (AT and lettuce) and single signal molecule in a MSNc liquid medium (potassium phosphate buffer 5.0 mM, Mops (pH 7) 0.1 M, $MgCl_2$ 2.0 mM, $MnCl_2$ 0.05 mM, $ZnCl_2$ 1 μM, thiamine 2.0 μM, $CaCl_2$ 700 μM, NH_4Cl 0.2% (*w*/*v*), cellobiose 0.5% (*w*/*v*)) (Beauregard et al. 2013), the modified crystal violet (CV) assay method was used for evaluation of biofilm formation based on previous study (O'Toole 2011; Wang et al. 2017). MSNc liquid medium (600 μL) and root exudates (20 μL) or signal molecules (20 μL) were added into a 48-well plate, respectively. The final concentrations of root exudates, and signal molecule, were 10 mg L^{-1} and 10, 25, and 50 μM, respectively. Sterile water effected on this strain was used as the control treatments. A 40 μL Ac63 bacterial suspension (~ 10^7 CFU mL^{-1} fluid prepared with an Azotobacter medium, Table S1) was inoculated. The 48-well plate was placed at 28 °C for 12, 24, and 48 h, and biomass of biofilm formation was quantitatively determined.

Soil pot trial with Ac63

Pot experiments were performed in a greenhouse of Jinan University, Guangzhou, in May 2018 (daily temperature, 27 to 33 °C). Vegetables (i.e., AT/lettuce) with/without the secretion of signal molecule (L-glutamic acid) were selected for the comparative tests, and each vegetable was set up with four treatments: without nitrogen fertilizer and uninoculated (C), without nitrogen fertilizer and inoculated with Ac63 (B), nitrogen fertilizer and uninoculated (N), and nitrogen fertilizer and inoculated with Ac63 (NB). Each pot contained with 1.5 kg unsterilized soil mixed with fertilizers: potassium 50 mg kg^{-1} (KCl), phosphate 100 mg kg^{-1} ($Ca(H_2PO_4)_2{\cdot}H_2O$), and nitrogen (N and NB treatments) 20 mg kg^{-1} (NH_4NO_3). We tried to sterilize the seeds of AT and lettuce on 75% ethanol for 30 s; then, we transferred them into 2% sodium hypochlorite for 3 min, and then washed 4 times with sterile water. Then, seeds were transferred into seedling pots consisted of unsterilized soil mixed with potassium 50 mg kg^{-1} (KCl) and phosphate 100 mg kg^{-1} ($Ca(H_2PO_4)_2{\cdot}H_2O$).

The culture conditions were illuminated under cool white fluorescent light with a 16-h daylight cycle (28 ± 2 °C) for 14 days. After seeding (14 days), seedlings were transferred into soil and inoculated with 5 mL Ac63 suspension (CFU $mL^{-1} > 10^9$) at day 5, 10, 15, and 30. Meanwhile, 5 mL sterile water was added into soil as the control treatment. All crops were harvested after a 45-day cultivation. The chlorophyll concentrations in leaf were determined with a spectrophotometry method at 652 nm (Arnon 1949). Approximately 3.0 g rhizosphere soils of each pot were obtained from samples adhering to plant roots. Total nitrogen in roots and leaves and available nitrogen/ammonia nitrogen in rhizosphere soils were determined with the methods of Horneck and Miller (Horneck and Miller 1998) and ISO/TS 14256–1:2003, respectively. Samples for gene quantification was quickly frozen in liquid nitrogen and then stored at − 80 °C (Haier DW-86L728, China).

For root colonization assay, Ac63-gfp was successfully labeled with the plasmid pMP2444 (named as Ac63-gfp) (Stuurman et al. 2000) by previously described electroporation method (Xue et al. 1999). Then, it was tested for the colonization ability in roots after 45-day cultivation with a similar pot condition as mentioned above. The roots were washed with sterile water (three times) to remove root-attached soil and then observed by the laser confocal microscope (Zeiss LSM710, Germany) (Chen et al. 2013).

Root total DNA and rhizosphere soil DNA were extracted using a DNeasy Plant Mini Kit (Qiagen, Germany) and DNeasy PowerSoil Kit (Qiagen, Germany), respectively. The unsterilized soils were intended for pot experiments as a negative control for the DNA extraction. In accordance with the whole genome of *A. chroococcum* (txid1328314), the specific primer (nifH-F 5′ CGAATACGATCCGACCGCCAAG 3′, nifH-R 5′ TTCCATAACGCCGAACTCCATCAG 3′) of nitrogen-fixing gene *nifH* in Ac63 was designed, and real-time quantitative PCR (qPCR) was carried out with a real-time PCR detection system (CFX96 BIORAD, USA). The reaction system includes 1 μL (50 ng) DNA template, 1 μL forward primers (10 μM), 1 μL reverse primers (10 μM), 10 μL 2× QuantiNova SYBR Green PCR Master Mix (Qiagen, Germany) and DNase-free water 7 μL. The reaction conditions follow with the lists: denaturized at 95 °C for 2 min, and 40 cycles include 95 °C for 5 s, then 57 °C for 10 s and 72 °C for 5 s, and the dissolution curve was set to 95 °C for 10 s, and 65 to 95 °C for 0.5 °C plate read once. T plasmid was serially diluted into 10-folds and then subjected to qPCR measurement simultaneously, and the qPCR reactions were carried out separately, in triplicate. The copy number of each sample was calculated in accordance with the previous method (Thonar et al. 2012). Then, CFU was generated with the gene copy number according to referred method (Mosimann et al. 2017).

Data analysis

Relative chemotactic effects of Ac63 in response to root exudates or each component:

$$\mathrm{CFU_{rcr}} = \frac{\mathrm{CFU_{exu}}}{\mathrm{CFU_{ck}}} \quad (1)$$

where $\mathrm{CFU_{rcr}}$, $\mathrm{CFU_{exu}}$, and $\mathrm{CFU_{ck}}$ indicate a value for the evaluation of relative chemotactic response, the bacterial colony number in a syringe needle with root exudates or each component, and the bacterial colony number in a syringe needle with sterile water, respectively.

Relative swarming motility of Ac63 to each component

$$L_{\mathrm{rsr}} = \frac{L_{\mathrm{exu}}}{L_{\mathrm{ck}}} \quad (2)$$

where L_{rsr}, L_{exu}, and L_{ck} represent the relative swarming mobility of bacteria, the diffusion diameter of Ac63 in semi-solid plates with component of root exudates, and the diffusion diameter of Ac63 in semi-solid plates with sterile water, respectively.

For qPCR assay, CFX manager 3.1 (Bio-Rad, USA) was used to calculate the qPCR quantification data. Ensuring the amplification efficiency (E) of qPCR is between 90 and 110%, $R^2 > 0.98$, the dissolution curve is single peak and coincides with the positive control, and no template control (NTC) fluorescence intensity is lower than threshold, and Ct values were between 10 and 35 cycles; the qPCR quantification data (*nifH* abundance) was converted to the equivalent number into copies per gram.

Three parallels for each treatment were set up separately for all treatments. One-way ANOVA was performed in SPSS 22.0 software (IBM, USA), and

Duncan's multiple range tests with $P < 0.05$ or $P < 0.01$ were considered the significant levels.

Results

Nitrogen fixation capacity of strains

Strain number 6 (*Azotobacter chroococcum* CICC22663, Ac63) showed a highest ammonium excretion ability (Fig. S1), and its nitrogenase activity reached to 77.1 nmol mg^{-1} h^{-1}. Compared with the previously described non-symbiotic nitrogen-fixing bacteria (Chen et al. 2018; Goswami et al. 2016), Ac63 has a stronger ability in N_2 fixation. However, there is no report about the stable colonization performance of this strain on its potential host plant.

Strain Ac63 chemotaxis with root exudates

Chemotactic response of strain Ac63 to root exudates of crops, except for lettuce, increased with the TOC concentrations of root secretions (Fig. 1). When these concentrations reached to 10 mg L^{-1}, the relative chemotactic response (CFU_{rcr}) value of AT, WA, CC, cabbage, and lettuce was 3.0, 1.7, 1.4, 1.3, and 0.9, respectively. Compared with the control treatment (water), a strongest chemotactic response of Ac63 was observed in AT treatment ($P < 0.05$), but no chemotactic effect was observed in root exudates of lettuce treatment ($P > 0.05$) (Fig. 1).

Components of root exudates and their effects on strain Ac63

As shown in Fig. 2, in total, root exudates of AT (highest CFU_{rcr}) consisted of 9 amino acids, 14 organic acids, 3 sugars, 3 alcohols, and 1 amine, and for lettuce (lowest CFU_{rcr}), root exudates consisted of 7 amino acids, 14 organic acids, 1 alcohol, and 1 amine (Table S2). Chemotactic effects of strain Ac63 to each component in root exudates (AT and lettuce) are presented with Fig. 3. The colony number of Ac63 attracted by L-glutamic acid, acetic acid, and malic acid was significantly higher (i.e., by 289%, 13%, and 7%, respectively) than that of the control treatment (water) ($P < 0.05$). However, the average number of Ac63 colonies attracted by the remaining substances was lower than that of the control treatment (water), and 15 compounds showed an inhibitory effect ($P < 0.05$) (Fig. 3). The application of L-glutamic, acetic, and malic acids for bacteria swarming experiments showed that the diffusion diameter of acetic and malic acids on the surface of Ac63 semi-solid medium did not result in a significant diffusion promotion ($P > 0.05$), whereas the diameter of Ac63 diffusion of L-glutamic acid increased by 20% ($P < 0.05$) when compared with that of control treatment (water). Furthermore, the results of in vitro experiments suggested that after adding L-glutamic acid into crop's root, the average number of Ac63 colonies attracted by root was 1.51×10^7 CFU $groot^{-1}$, which was 738.0% higher than that of the control treatment (water) ($P < 0.01$) (Fig. 4a). Laser confocal microscopy indicated that the crop's roots with L-glutamic acid secretion function formed a dense biofilm-like structure with a strong green fluorescence (Fig. 4b), whereas control treatment (water) presented with an individual and relatively weak fluorescence (Fig. 4c). Chemotaxis, swarming, and in vitro experiments demonstrated that L-glutamic acid was the signal molecule for Ac63 for its colonization in crops' roots (Figs. 3a, d and 4a).

L-Glutamic acid concentration in root exudates of these five crops followed the lists of AT > WA > cabbage > CC > lettuce. When TOC concentrations of root exudates were 10 mg L^{-1}, the corresponding glutamic acid concentrations were 38.0 ± 3.2, 22.4 ± 2.1, 14.0 ± 3.2, 11.9 ± 2.6, and 0.4 ± 0.3 μM (Fig. 5), respectively. In summary, L-glutamic acid concentration in root exudates of these five crops was significantly positively correlated with the chemotactic effects of root exudates on Ac63 ($P < 0.01$) (Fig. 5).

Effects of root exudates or L-glutamic acid on biofilm formation of strain Ac63

Figure 6 shows the effects of root exudates collected from crops of AT and lettuce on biofilm formation of strain Ac63. Biomass of Ac63 biofilm formed in MSNc medium with root exudates of AT was ~ 8 times higher than that of control treatment (water) ($P < 0.05$), but its formation in lettuce treatment was similar with that of control treatment (water) ($P > 0.05$). In addition, the biofilm formation biomass of Ac63 in MSNc medium with different L-glutamic acid concentrations was 3–34 times higher than that of control treatment (water).

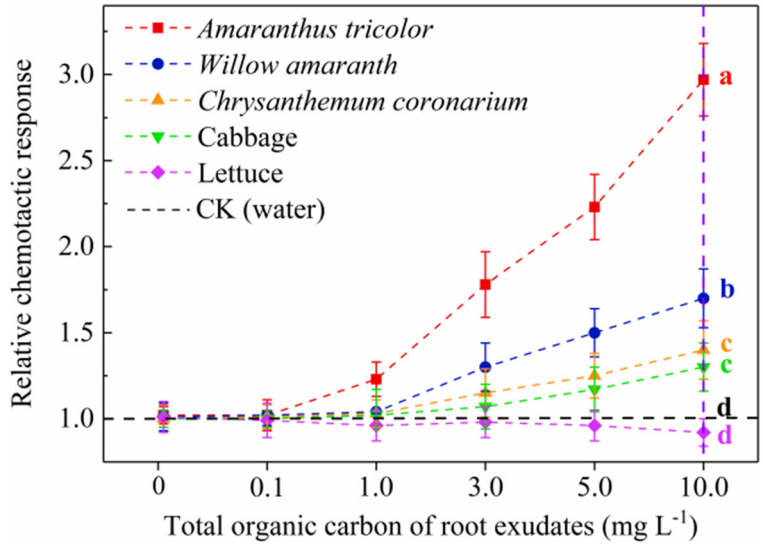

Fig. 1 Chemotactic responses of Ac63 to root exudates of five crops. Relative chemotactic response is the value of bacterial number in root exudates/control (water) treatments. Lowercase letters represent the significant differences in 6 treatments according to Duncan's test ($P < 0.05$)

Effects of Ac63 on *Amaranthus tricolor* and lettuce in soil pot

As shown in Table 1, without nitrogen fertilizer, the physiological indicators, including plant heights, chlorophyll concentrations, and total nitrogen concentrations in roots and shoots, and soil bioavailable nitrogen concentrations of AT and lettuce treatments inoculated with Ac63 were promoted significantly when compared with that of the corresponding non-inoculated treatments ($P < 0.05$). Except for total nitrogen concentrations in roots, the enhancements of other indicator rates of AT were higher than those of lettuce. With nitrogen fertilizer, the above physiological indicators and soil bioavailable nitrogen concentrations in AT treatment inoculated with Ac63 were significantly higher than that of non-inoculated treatments ($P < 0.05$). Except for total nitrogen in roots and ammonia nitrogen in rhizosphere soil, the enhancements of other parameter rates of AT were greater than those of lettuce (Table 1).

Ac63 showed a relatively excellent colonizing ability in roots of AT, rather than that of lettuce (Fig. 7(I)); the

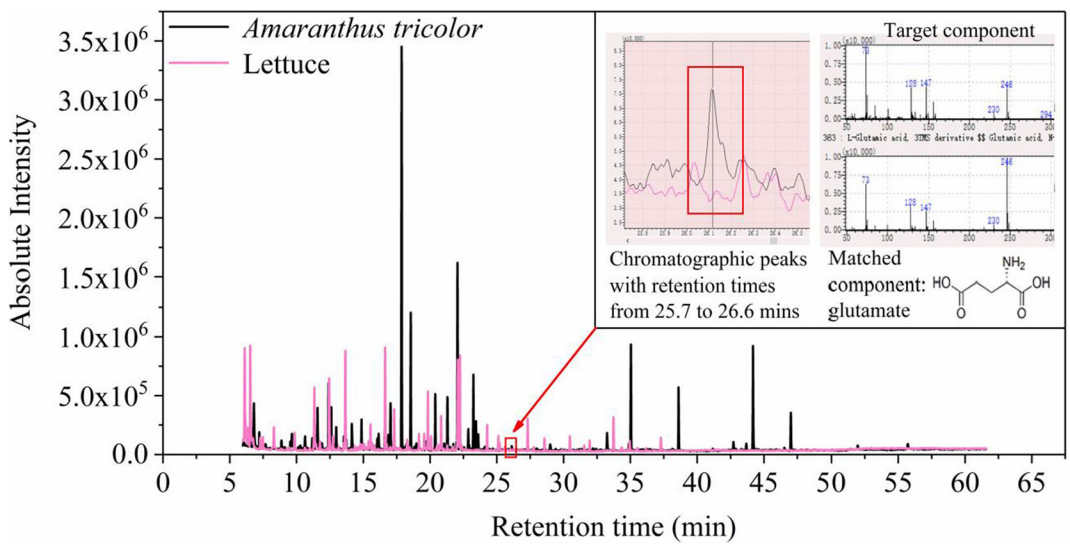

Fig. 2 The components chromatogram of root exudates collected from *Amaranthus tricolor* and lettuce

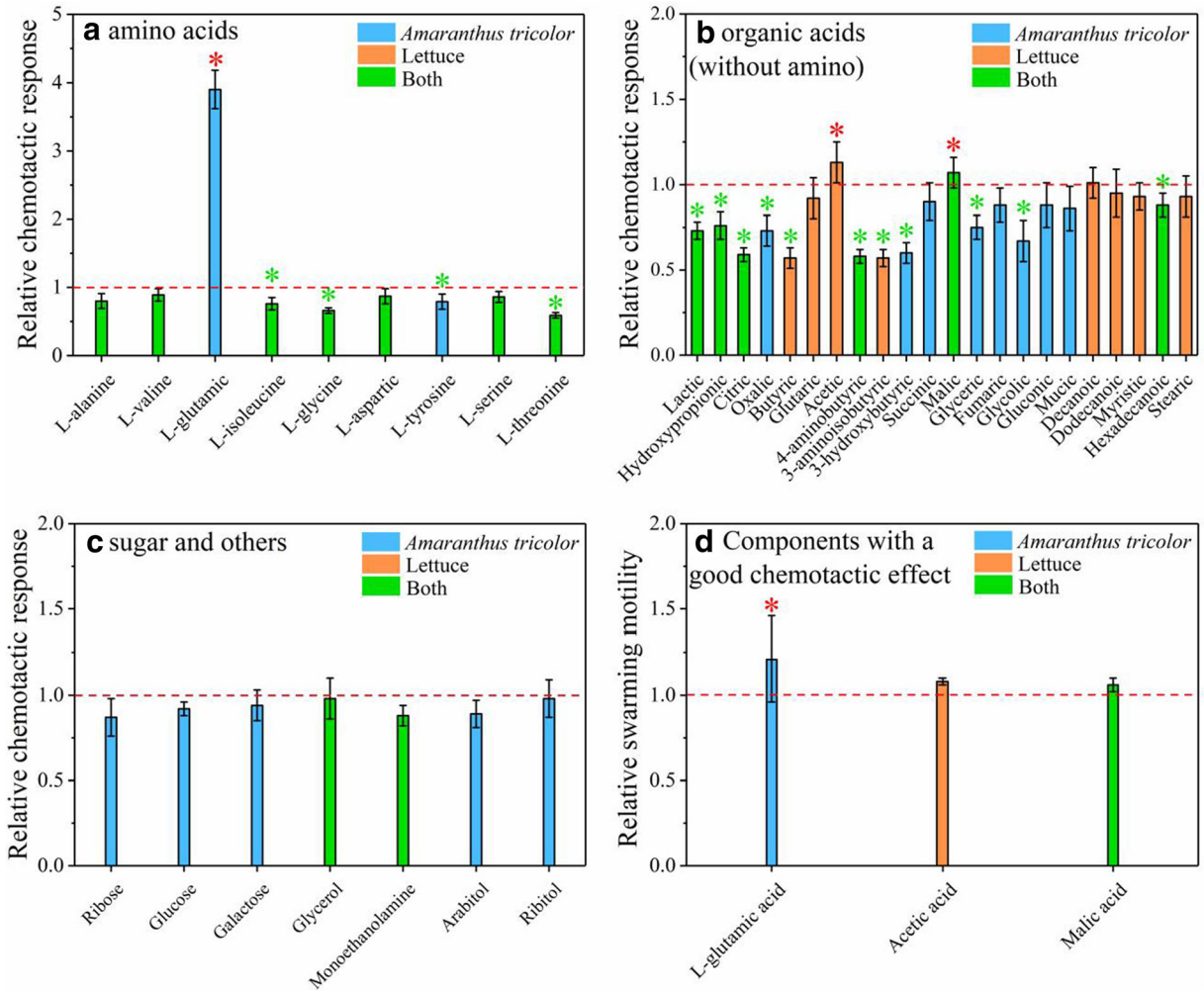

Fig. 3 Relative chemotactic and swarming responses of Ac63 towards carbon source components (30 μM) in root exudates of *Amaranthus tricolor* and lettuce. The asterisk indicates a significant difference from the control condition (water, 1.00 ± 0.15) according to Duncan's test ($P < 0.05$), and red asterisk and green asterisk indicate a relative chemotaxis response index > 1 or < 1, respectively

abundances of nitrogen-fixing gene *nifH* in roots of AT with and without nitrogen fertilizer treatments after strain Ac63 inoculation were 5.0×10^7 and 3.5×10^7, which represents the corresponding colony number of Ac63 in these roots were 8.5×10^7 and 6.0×10^7 CFU g^{-1} root, respectively. However, the abundance of nitrogen-fixing gene *nifH* and the colony number of Ac63 in the roots of lettuce were lower than detection limit after Ac63 inoculation (Fig. 7(II)). Meanwhile, the abundance of nitrogen-fixing gene *nifH* of the unsterilized soils intended for pot experiments (negative control for the DNA extraction) was also lower than that of the detection limit; the number of Ac63 colonies in the rhizosphere soil of AT was significantly higher than that of lettuce ($P < 0.05$) (Fig. 7(III)).

Discussion

The results demonstrated L-glutamic acid is a signal molecule for attractions of high-efficiency nitrogen-fixing strain Ac63. *Pseudomonas sojae* zoospores (Suo et al. 2016), *Pseudomonas chlororaphis* PCL1606 (Polonio et al. 2017), nitrogen-fixing bacteria *A. chroococcum* X-50 (Gupta Sood 2003), and rhizosphere-promoting bacteria *Bacillus*

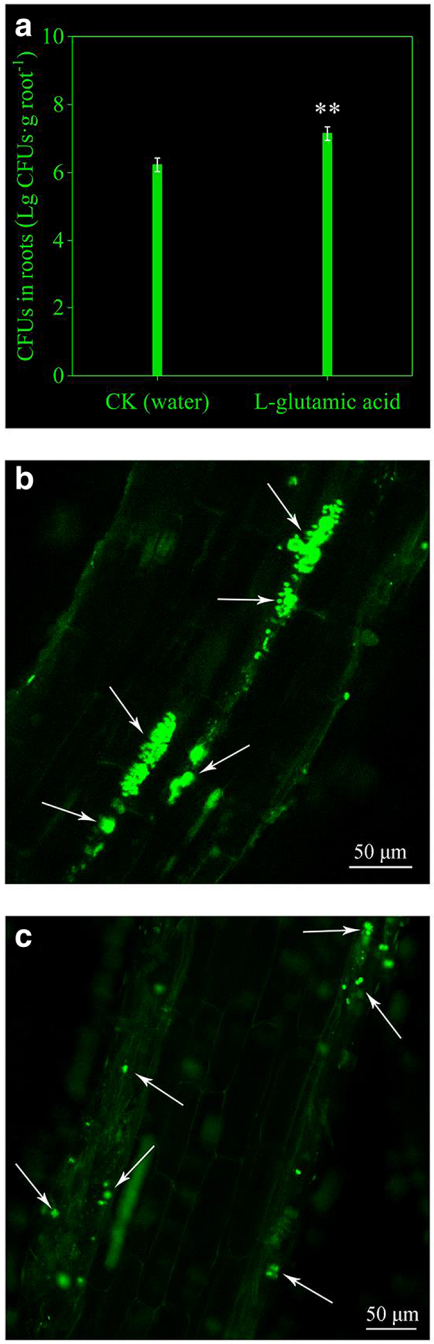

Fig. 4 Root colonization and biofilm formation of Ac63 with L-glutamic acid and water treatments: the population levels of Ac63 recruited by roots of *Amaranthus tricolor* after the addition of L-glutamic acid (**a**) (Duncan's test, $^{**}P < 0.01$); biofilm formed by the addition of L-glutamic acid (30 μM, 10 μL) (**b**), or water (**c**). Green indicates individual Ac63 visualized by staining with SYTO9; white arrows indicate biofilm-like structure formed by Ac63

amyloliquefaciens SQR9 (Feng et al. 2018) also could be attracted by L-glutamic acid in a chemotactic experiment as indicated in the previous studies.

Chemotactic response of bacteria to a specific component includes two steps: (i) the signaling molecule binds to its corresponding methyl-accepting chemotaxis proteins (MCPs) on the surface of bacteria, and it will promote the phosphorylation of *CheA* kinase to transfer phosphate group to *CheY* protein; (ii) *CheY* protein acts as motor proteins to induce flagellum move and turn, resulting in the bacteria tumbling and producing chemotaxis (Hazelbauer 2012). In general, ~ 14 MCP genes are found in bacterial genome (Lacal et al. 2010), and different MCPs are the receptor proteins for their specific signal molecules, respectively. For instance, *CheA1* and *CheA2*, *McpC*, and *Tip12* are the receptors of *Pseudomonas putida* KT2440, *B. subtilis*, and *Campylobacter jejuni* for the recognition of maize's root exudates, glutamate and glutamate, respectively (Glekas et al. 2012; López-Farfán et al. 2019; Lübke et al. 2018). Unfortunately, there are few reports about the information of MCPs in *A. chroococcum* and their functional verification. We performed a sequence comparison analysis based on the protein annotation results in the NCBI database. The query cover of amino acid sequences between the MCP of *A. chroococcum* (WP_089168023.1) and *McpC* and *Tip12* are 64% and 29%, respectively. Therefore, the homolog of *McpC* may mediate the chemotactic response of Ac63 to L-glutamic acid. In addition, bacterial chemotaxis is also related to the concentrations of signal molecules in their growth environment (Martín-Mora et al. 2016). Although the in situ concentration of root exudates in soil environment is hard to determine (Feng et al. 2018), quantitative analysis of root exudate components based on the GC-MS and then using of each component with different concentration rates could efficiently simulate the chemotactic effect of a specific component on bacteria than that of a certain concentration (30 μM).

The results of chemotactic responses to root exudates were based on the activities of all chemoreceptors (Feng et al. 2019). Six MCPs of *Ralstonia pseudosolanacearum* Ps29, including *McpA*, *McpT*, *Mcp*09, *McpM*, *Mcp*15, and *Mcp*19, presented with a negative chemotaxis response to ethanol (Oku et al. 2017). The negative chemotactic effects of strain Ac63 were found with the components of isoleucine, glycine, aspartic, tyrosine, threonine, lactic, hydroxypropionic, citric, oxalic, 4-aminobutyric, 3-hydroxybutyric,

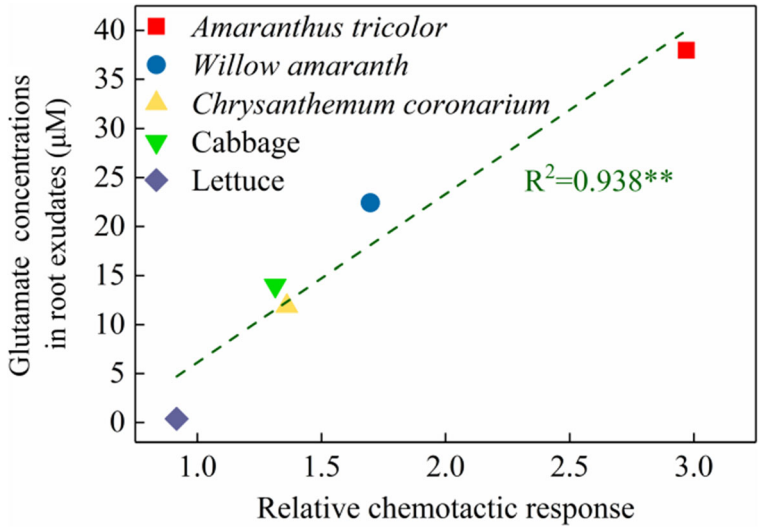

Fig. 5 Correlation analysis of glutamate concentrations in root exudates (TOC, 10 mg L^{-1}) with chemotactic responses of Ac63. Two asterisks indicate the significant correlation at $P = 0.01$ level (both sides, Pearson correlation coefficient)

glyceric, glycolic, and hexadecenoic acids in root exudates of AT, whereas a positive chemotactic effect was observed with mixture compounds of root exudates. These results demonstrated that L-glutamic acid could act as a really strong attractant for the attraction of Ac63 in rhizosphere. Feng reported that malic acid could be used as an attractant for *B. velezensis* SQR9 (Feng et al. 2019), inducing a significantly positive chemotaxis for this strain even at a relatively low concentration (10 μM). Acetic and malic acids in root exudates of lettuce presented a weak positive chemotaxis effect, but the lack of strong attractant L-glutamic acid and the existence of many negative chemotactic components resulted in no significant Ac63 chemotaxis in its root.

Swarming is also an important factor for the colonization ability of bacteria in rhizosphere (Gao et al. 2016). Nutrient-rich and soft substrates in rhizosphere soils may also promote the swarming motility and bacterial colonization. However, swarming is determined by the collision between bacterial cells instead of tumbling and running; thus, swarming effects of bacteria are unlikely to be driven by chemotaxis (Darnton et al. 2010). During the colonization of *B. subtilis* SWR01 onto tomato roots, the swarming motility rather than

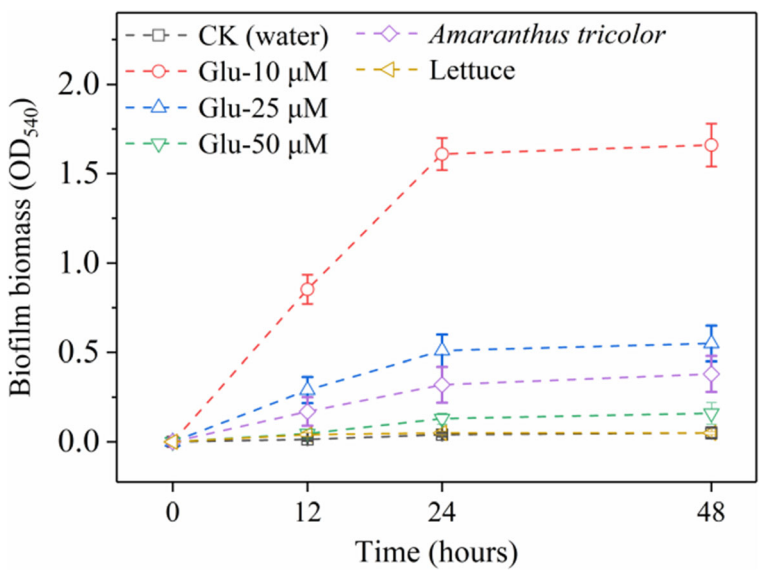

Fig. 6 Ac63 biofilm formed in MSNc medium with water (CK), 10, 25, and 50 μM L-glutamic acid (Glu), *Amaranthus tricolor* and lettuce root exudates (TOC = 10 mg L^{-1}), respectively

Table 1 Effects of inoculation with Ac63 on bio-characteristic parameters and soil nitrogen of *Amaranthus tricolor* (AT) and lettuce

Vegetable	Nitrogen application	Inoculated with Ac63	Treatment	Height (cm)	Root weight (g)	TN in root (mg g^{-1})	TN in shoot (mg g^{-1})	Chlorophyll in leaf (mg g^{-1})	$NH4^+$–N in soil (mg kg^{-1})	Available N in soil (mg kg^{-1})
AT	−	−	C	5.73 ± 0.25 c	0.25 ± 0.04 d	6.18 ± 0.06 c	4.92 ± 0.03 d	0.06 ± 0.01 d	0.64 ± 0 d	1.57 ± 0.01 d
	−	+	B	9.57 ± 0.12 b	0.66 ± 0.22 c	7.53 ± 0.06 a	12.21 ± 0.11 b	0.32 ± 0 b	1.27 ± 0.05 c	3.75 ± 0.07 c
	+	−	N	10.1 ± 0.96 b	1.87 ± 0.14 b	6.01 ± 0.08 d	7.1 ± 0.08 c	0.18 ± 0.01 c	3.91 ± 0.07 b	5.52 ± 0.04 b
	+	+	NB	13.33 ± 0.58 a	2.28 ± 0.22 a	6.37 ± 0.11 b	13.14 ± 0.09 a	0.49 ± 0.08 a	4.26 ± 0.08 a	5.93 ± 0.01 a
Lettuce	−	−	C	8.6 ± 0.36 C	1.06 ± 0.07 D	4.4 ± 0.06 D	2.75 ± 0.01 D	0.08 ± 0.01 C	0.5 ± 0 C	2.67 ± 0 B
	−	+	B	12.33 ± 0.58 B	2 ± 0.11 C	8.71 ± 0.41 A	5.87 ± 0.02 C	0.27 ± 0.04 A	0.73 ± 0.01 A	2.63 ± 0.06 AB
	+	−	N	20.73 ± 2.05 A	3.53 ± 0.3 B	4.8 ± 0.06 C	7.33 ± 0.35 B	0.15 ± 0.03 B	0.45 ± 0.01 D	2.68 ± 0 AB
	+	+	NB	18.57 ± 1.52 A	4.02 ± 0.18 A	5.21 ± 0.03 B	10.31 ± 0.1 A	0.16 ± 0.05 B	0.64 ± 0.01 B	2.7 ± 0.03 A
AT	−		B/C	1.67	2.64	1.22	2.48	5.33	1.98	2.39
	+		NB/N	1.32	1.22	1.06	1.85	2.72	1.09	1.07
Lettuce	−		B/C	1.43	1.89	1.98	2.13	3.38	1.46	0.99
	+		NB/N	0.90	1.14	1.09	1.41	1.07	1.42	1.01

Four treatments: without nitrogen fertilizer and uninoculated (C), without nitrogen fertilizer and inoculated with Ac63 (B), nitrogen fertilizer and uninoculated (N), nitrogen fertilizer and inoculated with Ac63 (NB). Different lowercase letters represent the significant differences among treatments of AT. Different uppercase letters represent the significant differences among treatments of lettuce (Duncan's test, $P < 0.05$)

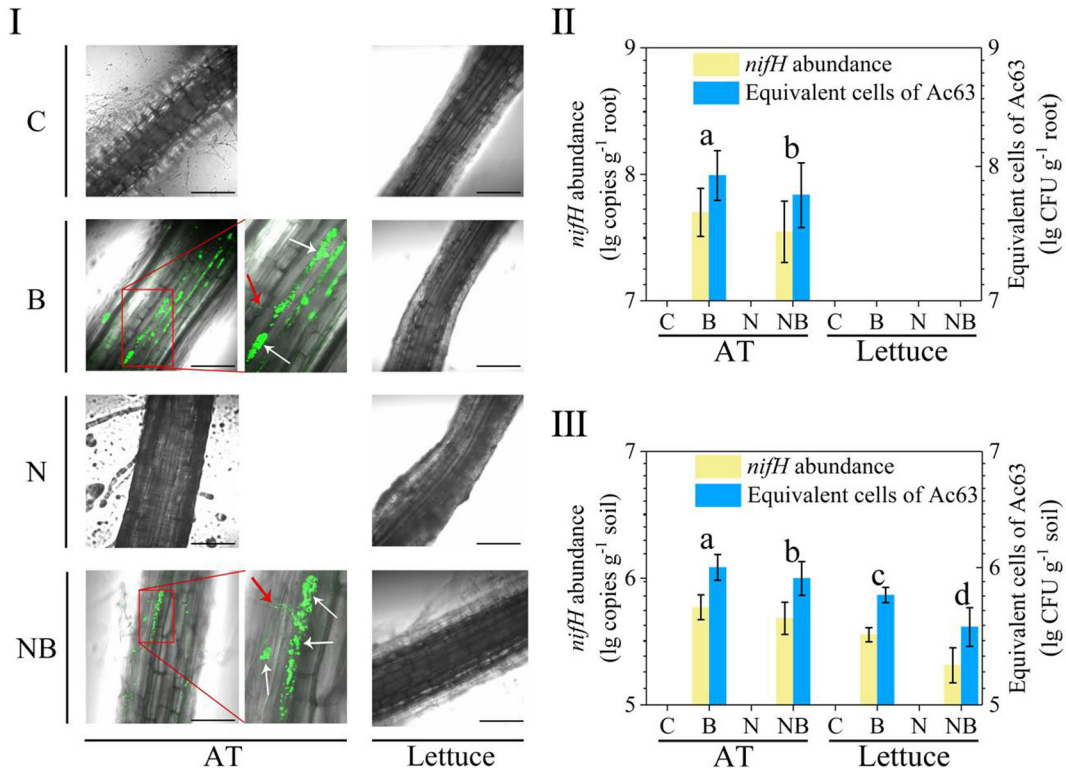

Fig. 7 Colonization of Ac63 in roots and rhizosphere soil of *Amaranthus tricolor* (AT) and lettuce. Visualization of root colonization by strain Ac63-gfp under a laser confocal microscope (scale bar = 200 μm) (I); *nifH* abundance and colonization number of Ac63 in roots (II) and rhizosphere soil (III) of AT and lettuce. Four treatments: without nitrogen fertilizer and uninoculated (C), without nitrogen fertilizer and inoculated with Ac63 (B), nitrogen fertilizer and uninoculated (N), nitrogen fertilizer and inoculated with Ac63 (NB). Different lowercase letters represent significant differences among the treatments (including AT and lettuce) (Duncan's test, $P < 0.05$), no letter above indicates the *nifH* abundance is below the detection limit. Red/white arrows indicate individual Ac63 and biofilm-like structure formed by Ac63, respectively

chemotactic response plays a major role (Gao et al. 2016). Results indicated that L-glutamic acid could also significantly increase the swarming motility of Ac63, which is consistent with glutamate inducing the swarming motility of *P. aeruginosa* (Köhler et al. 2000). Glutamate affects the activity of protein *DipA* and then regulates c-di-GMP amount in intracellular (Mattingly et al. 2018). Different concentrations of c-di-GMP can induce or inhibit the swarming motility of *P. aeruginosa* PA14 by regulating on the flagellar motor stator proteins *MotCD* and *MotAB*, respectively (Kuchma et al. 2015).

Biofilm formation is one of the marks of bacterial colonization in niche (Ramey et al. 2004). Bacteria form a biofilm matrix through extracellular proteins, EPS, and eDNA, thereby joining free cells into a membrane (Chen et al. 2015). The present study showed that L-glutamic acid can effectively stimulate Ac63 to form biofilm in roots and medium culture. Glutamate also promotes *B. subtilis* (Yu et al. 2016) and *Pseudomonas stutzeri* (Wang et al. 2017) to form a biofilm as indicated in the previous researches. Related studies showed that acetic acid affected on *Ywbl* protein of *B. subtilis* and promoted the biofilm formation of *B. subtilis* through holin-antiholin-like protein (*YwbHG*, *YsbAB*, and *YxaKC*) and eDNA efflux (Chen et al. 2015). Citric acid and malic acid affected on *B. amyloliquefaciens* SQR9 genes *epsD* and *tapA*, resulting in the secretion of EPS/extracellular protein and the formation of bacterial biofilm (Zhang et al. 2015). Recent study indicated that gene *gltA* encoded glutamate synthetase (GOGAT) with a function to convert glutamine into glutamate, and the increasing expression of this gene could significantly promote the biofilm formation of *Enterococcus faecalis* (Yu 2019). Thus, this gene possibly promoted biofilm formation by synthesizing glutamic acid. However, the specific mechanism on L-glutamic acid to promote the bacterial biofilm formation is unclear. Interestingly, we

found that the biofilm formation of Ac63 was decreased with the concentration of L-glutamic acid in the medium from 10 to 25 μM or 50 μM. Similar studies have showed that the low concentrations (0.01 mM, 0.1 mM) of threonine could significantly increase the biofilm formation of *Bacillus cereus* AR156 than that of a high concentration (10 mM) (Wang et al. 2019).

Furthermore, results of soil pot experiment indicated that L-glutamic acid can strongly attract strain Ac63 to colonize in the unsterilized soil. The colonization number of Ac63 in roots (8.5×10^7 and 6.0×10^7 CFU g^{-1} root) is close to the previous reports (Ansari and Ahmad 2018; Ke et al. 2019; Puri et al. 2016). This study also found that the amount of Ac63 in rhizosphere soils of two crops after Ac63 inoculation could reach up to ~ 10^6 CFU g^{-1} soil, which might result from the short interval, mass inoculation, and well survivability of Ac63 in this study. In general, only a few of the rhizosphere inoculum infiltrates the micropores of soil matrix which could directly attach to the root surface of crops. Therefore, with the attraction of signal molecule (L-glutamic acid), mass inoculation of strains Ac63 attracted and colonized the roots of AT; however, lettuce was not. Rhizosphere soils have a high content of these bacteria, which slightly promotes the growth of lettuce. The amount of Ac63 in roots of AT decreased significantly after the application of nitrogen fertilizer, and the growth indicators of plant also decreased apparently. These results indicated that the application of nitrogen fertilizer affected the colonization number of Ac63 in AT root and its plant-promoting effects.

Conclusion

L-Glutamic acid is a signal molecule for the colonization of highly efficient nitrogen-fixing strain Ac63. The root system of *Amaranthus tricolor* can secrete a high concentration of L-glutamic acid, inducing Ac63 colonization in its roots and promoting its growth, whereas lettuce cannot secrete L-glutamic acid, and no corresponding effects were observed in this study. This means that crops which can secrete L-glutamic acid may promote the colonization of strain Ac63, exert a good growth-promoting effect, and decrease the using rate of commercial nitrogen fertilizers. These findings can enhance the understanding of mutually beneficial symbiotic relationships between non-symbiotic nitrogen-fixing bacteria and crops, which provides an important information for the application of high-efficiency nitrogen-fixing strains during the agricultural activities.

Author contributions Q.S.L. conceived the project; Q.S.L. and Y.F.W. designed the experiments. Y.F.W., S.H.S., and J.Q.Y. performed the experiments. Q.S.L. supervised the project. Y.F.W. analyzed the data and wrote the manuscript. Q.S.L., J.F.W., and Z.M.X. complemented the writing.

Funding information This works was supported by the National Natural Science Foundation of China (No. 41673094) and the Key Project of National Natural Science Foundation (U1901212).

References

Allard-Massicotte R, Tessier L, Lécuyer F, Lakshmanan V, Lucier J-F, Garneau D, Caudwell L, Vlamakis H, Bais HP, Beauregard PB (2016) *Bacillus subtilis* early colonization of *Arabidopsis thaliana* roots involves multiple chemotaxis receptors. MBio 7(6): e01664-01616. doi. https://doi.org/10.1128/mBio.01664-16

Ansari FA, Ahmad I (2018) Biofilm development, plant growth promoting traits and rhizosphere colonization by *Pseudomonas entomophila* FAP1: a promising PGPR. Adv Microbiol 8(3):235. https://doi.org/10.4236/aim.2018.83016

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris*. Plant Physiol 24(1):1–15. https://doi.org/10.1104/pp.24.1.1

Beauregard PB, Chai Y, Vlamakis H, Losick R, Kolter R (2013) Bacillus subtilis biofilm induction by plant polysaccharides. Proc Natl Acad Sci U S A 110(17):E1621–E1630. https://doi.org/10.1073/pnas.1218984110

Bergersen FJ (1980) Methods for evaluating biological nitrogen fixation. Willey and Sons, Chichester pp 702

Carpenter SR, Caraco NF, Correll DL, Howarth RW, Sharpley AN, Smith VH (1998) Nonpoint pollution of surface waters with phosphorus and nitrogen. Ecol Appl 8(3):559–568. https://doi.org/10.1890/1051-0761(1998)008[0559:NPOSWW]2.0.CO;2

Carvalho T, Ballesteros H, Thiebaut F, Ferreira P, Hemerly A (2016) Nice to meet you: genetic, epigenetic and metabolic controls of plant perception of beneficial associative and endophytic diazotrophic bacteria in non-leguminous plants. Plant Mol Biol 90(6):561–574. https://doi.org/10.1007/s11103-016-0435-1

Chen S-L, Tsai M-K, Huang Y-M, Huang C-H (2018) Diversity and characterization of *Azotobacter* isolates obtained from rice rhizosphere soils in Taiwan. Ann Microbiol 68(1):17–26. https://doi.org/10.1007/s13213-017-1312-0

Chen Y, Gozzi K, Yan F, Chai Y (2015) Acetic acid acts as a volatile signal to stimulate bacterial biofilm formation. MBio 6(3):e00392–e00315. https://doi.org/10.1128/mBio.00392-15

Chen Y, Yan F, Chai Y, Liu H, Kolter R, Losick R, Guo J (2013) Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. Environ Microbiol 15(3):848–864. https://doi.org/10.1111/j.1462-2920.2012.02860.x

Darnton NC, Turner L, Rojevsky S, Berg HC (2010) Dynamics of bacterial swarming. Biophys J 98(10):2082–2090. https://doi.org/10.1016/j.bpj.2010.01.053

Dos Santos PC, Fang Z, Mason SW, Setubal JC, Dixon R (2012) Distribution of nitrogen fixation and nitrogenase-like sequences amongst microbial genomes. BMC Genomics 13(1):162. https://doi.org/10.1186/1471-2164-13-162

Eisenbach M (1996) Control of bacterial chemotaxis. Mol Microbiol 20(5):903–910. https://doi.org/10.1111/j.1365-2958.1996.tb02531.x

el Zahar HF, Santaella C, Heulin T, Achouak W (2014) Root exudates mediated interactions belowground. Soil Biol Biochem 77:69–80. https://doi.org/10.1016/j.soilbio.2014.06.017

Feng H, Zhang N, Du W, Zhang H, Liu Y, Fu R, Shao J, Zhang G, Shen Q, Zhang R (2018) Identification of chemotaxis compounds in root exudates and their sensing chemoreceptors in plant-growth-promoting rhizobacteria *Bacillus amyloliquefaciens* SQR9. Mol Plant Microbe In 31(10): 995–1005. https://doi.org/10.1094/MPMI-01-18-0003-R

Feng H, Zhang N, Fu R, Liu Y, Krell T, Du W, Shao J, Shen Q, Zhang R (2019) Recognition of dominant attractants by key chemoreceptors mediates recruitment of plant growth-promoting rhizobacteria. Environ Microbiol 21(1):402–415. https://doi.org/10.1111/1462-2920.14472

Fibach-Paldi S, Burdman S, Okon Y (2012) Key physiological properties contributing to rhizosphere adaptation and plant growth promotion abilities of *Azospirillum brasilense*. FEMS Microbiol Lett 326(2):99–108. https://doi.org/10.1111/j.1574-6968.2011.02407.x

Galloway JN, Dentener FJ, Capone DG, Boyer EW, Howarth RW, Seitzinger SP, Asner GP, Cleveland CC, Green P, Holland EA (2004) Nitrogen cycles: past, present, and future. Biogeochemistry 70(2):153–226. https://doi.org/10.1007/s10533-004-0370-0

Gao S, Wu H, Yu X, Qian L, Gao X (2016) Swarming motility plays the major role in migration during tomato root colonization by *Bacillus subtilis* SWR01. Biol Control 98:11–17. https://doi.org/10.1016/j.biocontrol.2016.03.011

Glekas GD, Mulhern BJ, Kroc A, Duelfer KA, Lei V, Rao CV, Ordal GW (2012) The *Bacillus subtilis* chemoreceptor McpC senses multiple ligands using two discrete mechanisms. J Biol Chem 287(47):39412–39418. https://doi.org/10.1074/jbc.M112.413518

Goswami D, Thakker JN, Dhandhukia PC (2016) Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. Cogent Food Agric 2(1):1127500. https://doi.org/10.1080/23311932.2015.1127500

Guo S-H, Hu N, Li Q-S, Yang P, Wang L-L, Xu Z-M, Chen H-J, He B-Y, Zeng EY (2018) Response of edible amaranth cultivar to salt stress led to Cd mobilization in rhizosphere soil: a metabolomic analysis. Environ Pollut 241:422–431. https://doi.org/10.1016/j.envpol.2018.05.018

Gupta Sood S (2003) Chemotactic response of plant-growth-promoting bacteria towards roots of vesicular-arbuscular mycorrhizal tomato plants. FEMS Microbiol Ecol 45(3): 219-227. doi: https://doi.org/10.1016/S0168-6496(03)00155-7

Hardy R, Burns RC, Holsten RD (1973) Applications of the acetylene-ethylene assay for measurement of nitrogen fixation. Soil Biol Biochem 5(1): 47-81. doi: https://doi.org/10.1016/0038-0717(73)90093-X

Hazelbauer GL (2012) Bacterial chemotaxis: the early years of molecular studies. Annu Rev Microbiol 66:285–303. https://doi.org/10.1146/annurev-micro-092611-150120

Horneck DA, Miller RO (1998) Determination of total nitrogen in plant tissue. Handbook of reference methods for plant analysis 2:75–83

Huang X-F, Chaparro JM, Reardon KF, Zhang R, Shen Q, Vivanco JM (2014) Rhizosphere interactions: root exudates, microbes, and microbial communities. Botany 92(4):267–275. https://doi.org/10.1139/cjb-2013-0225

Jin Y, Zhu H, Luo S, Yang W, Zhang L, Li S, Jin Q, Cao Q, Sun S, Xiao M (2019) Role of maize root exudates in promotion of colonization of *Bacillus velezensis* strain S3-1 in rhizosphere soil and root tissue. Curr Microbiol 76(7):855–862. https://doi.org/10.1007/s00284-019-01699-4

Ke X, Feng S, Wang J, Lu W, Zhang W, Chen M, Lin M (2019) Effect of inoculation with nitrogen-fixing bacterium *Pseudomonas stutzeri* A1501 on maize plant growth and the microbiome indigenous to the rhizosphere. Syst Appl Microbiol 42(2):248–260. https://doi.org/10.1016/j.syapm.2018.10.010

Kearns DB (2010) A field guide to bacterial swarming motility. Nat Rev Microbiol 8(9):634–644. https://doi.org/10.1038/nrmicro2405

Köhler T, Curty LK, Barja F, Van Delden C, Pechère J-C (2000) Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. J Bacteriol 182(21):5990–5996. https://doi.org/10.1128/JB.182.21.5990-5996.2000

Kuchma S, Delalez N, Filkins L, Snavely E, Armitage J, O'Toole G (2015) Cyclic di-GMP-mediated repression of swarming motility by *Pseudomonas aeruginosa* PA14 requires the MotAB stator. J Bacteriol 197(3):420–430. https://doi.org/10.1128/JB.02130-14

Lacal J, García-Fontana C, Muñoz-Martínez F, Ramos JL, Krell T (2010) Sensing of environmental signals: classification of chemoreceptors according to the size of their ligand binding regions. Environ Microbiol 12(11):2873–2884. https://doi.org/10.1111/j.1462-2920.2010.02325.x

Ling N, Raza W, Ma J, Huang Q, Shen Q (2011) Identification and role of organic acids in watermelon root exudates for recruiting *Paenibacillus polymyxa* SQR-21 in the rhizosphere. Eur J Soil Biol 47(6):374–379. https://doi.org/10.1016/j.ejsobi.2011.08.009

López-Farfán D, Reyes-Darias JA, Matilla MA, Krell T (2019) Concentration dependent effect of plant root exudates on the chemosensory systems of *Pseudomonas putida* KT2440. Front Mcrobiol 10(78). https://doi.org/10.3389/fmicb.2019.00078

Lübke A-L, Minatelli S, Riedel T, Lugert R, Schober I, Spröer C, Overmann J, Groß U, Zautner AE, Bohne W (2018) The transducer-like protein Tlp12 of *Campylobacter jejuni* is involved in glutamate and pyruvate chemotaxis. BMC

Microbiol 18(1):111. https://doi.org/10.1186/s12866-018-1254-0

Maheshwari DK (2011) Bacteria in agrobiology: plant growth responses. Springer Science & Business Media

Martín-Mora D, Reyes-Darias JA, Ortega Á, Corral-Lugo A, Matilla MA, Krell T (2016) McpQ is a specific citrate chemoreceptor that responds preferentially to citrate/metal ion complexes. Environ Microbiol 18(10):3284–3295. https://doi.org/10.1111/1462-2920.13030

Mattingly AE, Kamatkar NG, Morales-Soto N, Borlee BR, Shrout JD (2018) Multiple environmental factors influence the importance of the phosphodiesterase DipA upon *Pseudomonas aeruginosa* swarming. Appl Environ Microbiol 84(7): e02847–e02817. https://doi.org/10.1128/AEM.02847-17

McRose DL, Zhang X, Kraepiel AM, Morel FM (2017) Diversity and activity of alternative nitrogenases in sequenced genomes and coastal environments. Front Microbiol 8:67. https://doi.org/10.3389/fmicb.2017.00267

Mosimann C, Oberhänsli T, Ziegler D, Nassal D, Kandeler E, Boller T, Mäder P, Thonar C (2017) Tracing of two *Pseudomonas* strains in the root and rhizoplane of maize, as related to their plant growth-promoting effect in contrasting soils. Front Microbiol 7: 2150. doi: https://doi.org/10.3389/fmicb.2016.02150

O'Toole GA (2011) Microtiter dish biofilm formation assay. Jove-J Vis Exp 47:e2437. https://doi.org/10.3791/2437

Oku S, Hida A, Mattana T, Tajima T, Nakashimada Y, Kato J (2017) Involvement of many chemotaxis sensors in negative chemotaxis to ethanol in *Ralstonia pseudosolanacearum* Ps29. Microbiology+ 163(12):1880–1889. https://doi.org/10.1099/mic.0.000574

Oku S, Komatsu A, Tajima T, Nakashimada Y, Kato J (2012) Identification of chemotaxis sensory proteins for amino acids in *Pseudomonas fluorescens* Pf0-1 and their involvement in chemotaxis to tomato root exudate and root colonization. Microbes Environ ME12005. https://doi.org/10.1264/jsme2.ME12005

Polonio Á, Vida C, de Vicente A, Cazorla FM (2017) Impact of motility and chemotaxis features of the rhizobacterium *Pseudomonas chlororaphis* PCL1606 on its biocontrol of avocado white root rot. Int Microbiol 20(2):95–104. https://doi.org/10.2436/20.1501.01.289

Puri A, Padda KP, Chanway CP (2016) Evidence of nitrogen fixation and growth promotion in canola (*Brassica napus* L.) by an endophytic diazotroph *Paenibacillus polymyxa* P2b-2R. Biol Fertil Soils 52(1):119–125. https://doi.org/10.1007/s00374-015-1051-y

Ramey BE, Koutsoudis M, von Bodman SB, Fuqua C (2004) Biofilm formation in plant–microbe associations. Curr Opin Microbiol 7(6):602–609. https://doi.org/10.1016/j.mib.2004.10.014

Rudrappa T, Czymmek KJ, Paré PW, Bais HP (2008) Root-secreted malic acid recruits beneficial soil bacteria. Plant Physiol 148(3):1547–1556. https://doi.org/10.1104/pp.108.127613

Singh VS, Tripathi P, Pandey P, Singh DN, Dubey BK, Singh C, Singh SP, Pandey R, Tripathi AK (2019) Dicarboxylate transporters of *Azospirillum brasilense* Sp7 play an important role in the colonization of finger millet (*Eleusine coracana*) roots. Mol Plant Microbe In 32(7):828–840. https://doi.org/10.1094/MPMI-12-18-0344-R

Stuurman N, Bras CP, Schlaman HR, Wijfjes AH, Bloemberg G, Spaink HP (2000) Use of green fluorescent protein color variants expressed on stable broad-host-range vectors to visualize rhizobia interacting with plants. Mol Plant Microbe In 13(11):1163–1169. https://doi.org/10.1094/MPMI.2000.13.11.1163

Suo B, Chen Q, Wu W, Wu D, Tian M, Jie Y, Zhang B, Wen J (2016) Chemotactic responses of *Phytophthora sojae* zoospores to amino acids and sugars in root exudates. J Gen Plant Pathol 82(3):142–148. https://doi.org/10.1007/s10327-016-0651-1

Thonar C, Erb A, Jansa J (2012) Real-time PCR to quantify composition of arbuscular mycorrhizal fungal communities—marker design, verification, calibration and field validation. Mol Ecol Resour 12(2):219–232. https://doi.org/10.1111/j.1755-0998.2011.03086.x

Wang D, Xu A, Elmerich C, Ma LZ (2017) Biofilm formation enables free-living nitrogen-fixing rhizobacteria to fix nitrogen under aerobic conditions. ISME J 11(7):1602. https://doi.org/10.1038/ismej.2017.30

Wang N, Wang L, Zhu K, Hou S, Chen L, Mi D, Gui Y, Qi Y, Jiang C, Guo J-H (2019) Plant root exudates are involved in *Bacillus cereus* AR156 mediated biocontrol against *Ralstonia solanacearum*. Front Microbiol 10:98. https://doi.org/10.3389/fmicb.2019.00098

Xue G-P, Johnson JS, Dalrymple BP (1999) High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. J Microbiol Methods 34(3):183–191. https://doi.org/10.1016/S0167-7012(98)00087-6

Yu X (2019) A preliminary study of the effects of *gltA* gene on biofilm formation of *Enterococcus*. Probe-Dentistry 1(1). doi: https://doi.org/10.18686/den.v1i1.1188

Yu Y, Yan F, Chen Y, Jin C, Guo J-H, Chai Y (2016) Poly-γ-glutamic acids contribute to biofilm formation and plant root colonization in selected environmental isolates of *Bacillus subtilis*. Front Microbiol 7:1811. https://doi.org/10.3389/fmicb.2016.01811

Zhang N, Yang D, Wang D, Miao Y, Shao J, Zhou X, Xu Z, Li Q, Feng H, Li S (2015) Whole transcriptomic analysis of the plant-beneficial rhizobacterium *Bacillus amyloliquefaciens* SQR9 during enhanced biofilm formation regulated by maize root exudates. BMC Genomics 16(1):685. https://doi.org/10.1186/s12864-015-1825-5