Journal of proteome research

Article

pubs.acs.org/jpr

Comparative Proteomics Analysis of *Bacillus amyloliquefaciens* SQR9 Revealed the Key Proteins Involved in in Situ Root Colonization

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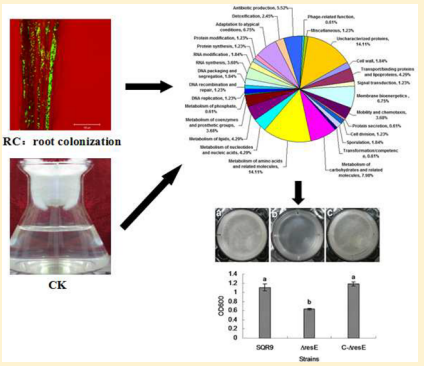
Supporting Information

ABSTRACT: *Bacillus Amyloliquefaciens* SQR9 is a well-investigated plant growth-promoting rhizobacteria with strong root colonization capability. To identify the key proteins involved in in situ root colonization and biofilm formation, the proteomic profiles of planktonic and root colonized SQR9 cells were compared. A total of 755 proteins were identified, of which 78 and 95 proteins were significantly increased and deceased, respectively, when SQR9 was colonized on the root. The proteins that were closely affiliated with the root colonization belonged to the functional categories of biocontrol, detoxification, biofilm formation, cell motility and chemotaxis, transport, and degradation of plant polysaccharides. A two-component system protein ResE was increased 100-fold when compared to the planktonic status; impairment of the *resE* gene postponed the formation of cell biofilm and decreased the root colonization capability, which may be regulated through the *spo0A-sinI-yqxM* pathway. The SQR9 proteomic data provide valuable clues for screening key proteins in the plant−rhizobacteria interaction.



KEYWORDS: *root colonization, proteomics, biofilm formation, resE*

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are bacteria that colonize the plant rhizosphere and stimulate plant growth or suppress soil-borne disease. PGPR colonization of roots is a crucial step for successful control of soil-borne pathogens, which mainly rely on the ability of biofilm formation.[1−3] Bacterial biofilms established on plant root surfaces play an important role in protecting colonization sites and serve as a sink for the nutrients secreted by plants, and thus reduce the availability of nutritional elements in root exudates for pathogens.[4] Biofilm formation for *Bacillus* involves two main operons, *epsA-epsO* and *yqxM-sipW-tasA*,[5−8] as well as transcription factors such as DegU/DegS, Spo0A, SinI/SinR, SlrR/SlrA, and AbrB. A recent study indicated that bacillomycin D acts as a signal for biofilm formation and a weapon for *B. amyloliquefaciens* SQR9 to suppress *Fusarium oxysporum*.[2] Moreover, alteration of the phosphorylation level of DegU in *B. amyloliquefaciens* SQR9 influences the biocontrol activity of soil-borne *Fusarium* wilt disease by coordinating multicellular behavior and regulating the synthesis of bacillomycin D.[9] Disruption of the *abrB* gene in SQR9 can improve biofilm formation and root colonization.[10]

Many studies have illustrated that plant−bacteria communications in the rhizosphere are mainly regulated by root exudates,[11,12] which may act as signals to influence the ability of microbes to colonize the root and survive in the rhizosphere. Chen et al.[13] revealed that tomato root secreted L-malic acid, which strongly promoted root colonization and biofilm formation of *Bacillus subtilis*. In addition, Zhang et al.[14] detected chemotaxis and biofilm formation for two *Bacillus* strains toward citric acid and fumaric acid in cucumber and banana root exudates, respectively. Interestingly, *Fusarium*-infected cucumber seedlings secreted more citric acid and fumaric acid, which stimulated chemotaxis and biofilm formation in *B. amyloliquefaciens* SQR9.[15]

Studies have begun to elucidate the genetic pathways involved in the communication between bacteria and plant roots[16,17] and have elaborated the viewpoint that plant root exudates regulate the expression of bacterial genes involved in establishing microbe−plant interactions. Mark et al.[18] examined the influence of exudates from two varieties of sugar beet on the *Pseudomonas aeruginosa* PA01 transcriptome and found that genes with altered expression included those with functions previously implicated in microbe−plant interactions, such as aspects of metabolism, chemotaxis, and type III secretion, and a subset with putative or unknown function. Alberton et al.[19] used a proteomic approach to study the mechanisms of interaction between *Herbaspirillum seropedicae* SmR1 and rice.

Efficient root colonization is vital for PGPR strains to exert their beneficial plant effects, but most of the *Bacillus* strains' biofilm formation related genes were identified in situ, which is

Received: June 11, 2014

 © XXXX American Chemical Society dx.doi.org/10.1021/pr500565m | *J. Proteome Res.* XXXX, XXX, XXX−XXX

different than in situ root colonization and biofilm formation, because the root surface is a dynamic matrix with continued chemical secretion and root−bacteria interaction.[20] Identification of key proteins/genes of PGPR strains involved in in situ root colonization and biofilm formation can enhance our understanding of the rhizosphere behavior of PGPR and promote their application in agricultural production. *Bacillus amyloliquefaciens* SQR9 (SQR9) is a rhizosphere beneficial strain and can inhibit *Fusarium oxysporum* f. sp. *cucumerinum* J. H. Owen by efficient root colonization and antibiotic production.[21−23,9,10] In this study, we established a system to compare the proteomes of the planktonic and root colonized SQR9 cells using the high-throughput, isobaric tags for relative and absolute quantitation (iTRAQ)-based, quantitative proteomic approach and identified the key proteins involved in in situ root colonization. A novel regulator ResE was found to be involved in root colonization and biofilm formation. Then, its function and regulation mechanism was investigated through site-directed mutagenesis and gene transcription analysis. This work provided new physiological insights into the communication between plants and rhizobacteria and may help to promote the application of SQR9 in agriculture.

MATERIALS AND METHODS

Strains and Growth Conditions

A 1 mL sample of overnight cultures of *Bacillus amyloliquefaciens* SQR9 (SQR9) and the *gfp*-tagged strain *Bacillus amyloliquefaciens* SQR9-*gfp* (SQR9-*gfp*)[21] were incubated in 100 mL of liquid Luria−Bertani medium on a shaker at 170 rpm and 37 °C for 4 h. The cultures were centrifuged at 12 000 × *g* at 4 °C for 10 min, and the cell pellets were washed three times in $^1/_4$ sterile Murashige and Skoog (MS) medium and resuspended in the same medium. The concentration of the suspensions was determined by the serial dilution plate counting method.

Planting System Description

Cucumber seeds (Jinchun No. 4) were surface sterilized for 3 min using 2% sodium hypochlorite, rinsed three times in sterile distilled water, and then planted in sterile vermiculite in a tissue-culture container (Figure S1A, Supporting Information). The sealing film on the top of the tissue-culture container can help the seedlings ventilate and avoid contamination. When two cotyledons appeared, the cucumber seedlings were removed from the tissue-culture container using sterile tweezers, washed carefully in sterile water to remove the vermiculite on the cucumber root surface, and then transferred to an aseptic conical flask containing 50 mL of $^1/_4$ sterile MS medium in a biological hood. The function of the axenic cotton plug in the cultivating device was to avoid microbial contamination of cucumber root as described for the sealing film above. The plants were cultured in a growth chamber at 30 °C on a 16-h light/8-h dark cycle. The cultivating bottle was shaken slightly three times every day, and the medium was changed every 3 days, which provided fresh, adequate nutrients for plant growth. When the seedlings had 3−4 true leaves (Figure S1B, Supporting Information), they were checked for contamination by the Petri dish plating method. The contaminated seedlings were discarded, and the completely sterile ones were used for SQR9 inoculation.

Experimental Design

To investigate the key SQR9 proteins involved in root colonization, the two treatments described were set up as follows. An aseptic flask without cucumber roots was inoculated with SQR9 as a control (CK) (Figure S1D, Supporting Information), and another treatment used an aseptic flask with cucumber that was inoculated with SQR9 for root colonization (RC) (Figure S1E, Supporting Information). Each treatment was replicated 30 times. After the defined number of days, SQR9 cells with CK treatment were collected by centrifugation at 12 000 × *g* for 10 min; SQR9 cells with RC treatment were first detached from the cucumber root before collection using the method from Oosthuizen et al.[24] with some modification. SQR9 colonized cucumber roots were aseptically removed from the container, washed three times in sterile water, and placed on axenic filter paper to remove the planktonic cells. Then, the roots were placed into a 250 mL sterile flask containing 45 g of glass beads (6 mm in diameter) and 100 mL of sterile water, which was shaken vigorously by hand for 20 min to separate the attached cells from the roots. The detached cells were harvested by centrifugation at 12 000 × *g* for 10 min. There were three biological repeats for this experiment.

Determination of SQR9 Colonization Dynamics on Cucumber Roots under the Study Condition

To determine the optimum collection time for SQR9 colonized on the cucumber rhizoplane, SQR9-*gfp* colonized roots were collected at different time points (1, 2, 3, 4, 5, 6, and 7 days after inoculation) and cut into 1−2 cm long pieces, washed with sterile distilled water, placed on microslides, and directly observed by a confocal laser scanning microscope (CLSM) (Leica Model TCS SP2, Heidelberg, Germany). Images were formed using Leica confocal software, version 2.61. On the basis of the above observations, the optimum time for root colonized SQR9 collection was determined. The roots colonized by SQR9 were also visualized under a scanning electron microscope (XL-30 ESEM, Philips, The Netherlands).

Proteins Extraction Analysis

The proteins from the SQR9 cells obtained from the two treatments were extracted using a protein isolation kit (KeyGEN Biotech, Nanjing, China), according to the manufacturer's instruction, and quantified using a BCA protein quantification kit (Dingguo Changsheng Biotech Co., Ltd., Beijing, China). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method described by Laemmli[25] with some modification. Protein samples mixed with the same volume of 1 × Laemmli buffer [62 mM Tris-HCl, pH 6.8; 2% SDS (w/v); 10% glycerin (v/v); 5% 2-mercaptoethanol (v/v); 0.02% bromophenol blue (w/v)] were boiled for 5 min. SDS-PAGE was performed using a 10% (w/v) polyacrylamide gel at 120 V for 90 min. The gel was stained in staining solution [10% Coomassie Brilliant Blue R-250 (w/v); 50% methanol (v/v); 7% glacial acetic acid (v/v)] for 1 h and subsequently destained with destaining solution [2.5% methanol; 10% acetic acid].

iTRAQ Labeling and Automated 2D LC−MS/MS Protein Identification

A 1 g sample of solid intracellular protein powder of SQR9 from each treatment was dissolved in 3 mL of ddH_2O and then concentrated by running through an Amicon YM-3 centrifugal filter unit with a 3-kDa molecular mass cutoff membrane filter (Millipore, Bedford, MA). Protein quantification was measured

dx.doi.org/10.1021/pr500565m | J. Proteome Res. XXXX, XXX, XXX−XXX

using the BCA protein assay kit (Sangon Biotech, SK3061). Samples of different treatment were performed using SDS-PAGE again to determine whether they were accurate or equivalent. Then, 50 μg of each protein was used for enzymolysis and iTRAQ labeling. iTRAQ labeling was carried out by the method of Liu et al.,[26] and the peptide samples were labeled by the iTRAQ Reagent Multiplex Kit (Applied Biosystems, Foster City, CA). The samples from CK and RC were labeled with the iTRAQ tags 115 and 116, respectively.

Automated two-dimentional (2D) liquid chromatograph-mass spectrometer (LC−MS/MS) analysis was carried out using the method from Liu et al.[26] The labeled peptides were desalted through a zip Tip C18 reverse resin (Millipore corporation, Billerica, MA) and dried using a vacuum centrifuge. All of the extracted peptides were resuspended in loading buffer (10 mM KH_2PO_4, 25% ACN pH 2.6). The collected samples were separated by high-performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, CA) on a C18 reverse phase column and analyzed using an LTQ Orbitrap mass spectrometer (Thermo Electron Corp.). The spray voltage was set to 1.85 kV, and the temperature of the heated capillary was set to 200 °C. Full scan mass spectroscopy survey spectra in profile mode were acquired in the Orbitrap with a resolution of 60 000 at m/z 400 after the accumulation of 1 000 000 ions. The five most intense peptide ions from the preview scan in the Orbitrap were fragmented by collision-induced dissociation (normalized collision energy, 35%; activation, Q 0.25; and activation time, 30 ms) in the LTQ after the accumulation of 5000 ions. Precursor ion charge state screening was enabled, and all unassigned charge states and singly charged species were rejected. The dynamic exclusion list was restricted to a maximum of 500 entries with a maximum retention period of 90 s and a relative mass window of 10 ppm. The data were acquired using Xcalibur software (Thermo Electron).

Identification and quantification of proteins were performed with ProteinPilot software (version 4.0; Applied Biosystems, USA). The database used for searching was the *B. amyloliquefaciens* SQR9 CP006890 National Center for Biotechnology Information (NCBI) database (with 4106 entries). The paragon algorithm in ProteinPilot was used for peptide identification and quantification. The data search parameters were set up as follows: trypsin (KR) cleavage with two missed cleavage sites was considered along with fixed modification of cysteines by methylmethanethiosulfonate (MMTS). iTRAQ modification of peptide/protein identification and quantification was also applied with the ProteinPilot software version 4.0. The database and data search parameters were the same as above. A strict cutoff for protein identification was carried out with an unused ProtScore ≥ 1.3, which corresponds to a confidence limit of 95%, to minimize false positive results. At least two peptides with 95% confidence were considered for protein quantification. For iTRAQ quantitation, the peptide used for quantification was automatically selected by the pro group algorithm to calculate the reported peak area, p-value, and error factor (EF). The resulting data set was auto bias-corrected to eliminate any variation due to unequal mixing when different labeled samples were combined. False discovery rate (FDR) analysis was also performed using the integrated tools in ProteinPilot (FDR < 0.01).

Disruption of the *resE* Gene in *B. amyloliquefaciens* SQR9

The *resE* gene in the SQR9 genome was deleted using the method from Xu et al.[9] The flanked upstream and downstream fragments of the *resE* gene were amplified with primer sets PresE-UF/PresE-UR and PresE-DF/PresE-DR, respectively (Table S1, Supporting Information). The chloramphenicol resistance gene (*Cm*) was amplified with the primer set PresE-CmF/PresE-CmR from plasmid pNW33n, which partially overlapped with the *resE*-flanked upstream and downstream fragments. After the overlapping polymerase chain reaction (PCR), the fused fragment was transformed into competent cells of *B. amyloliquefaciens* SQR9 by electroporation. The correct mutant was verified by PCR using the primer set PresE-VF/PresE-VR and was named Δ*resE*.

Biofilm Formation Assay

To evaluate biofilm formation, SQR9 and Δ*resE* were cultivated in MSgg medium in 24-well microtiter plates. The biomass of the biofilm formed by SQR9 and Δ*resE* was evaluated as described by Hamon and Lazazzera.[27] Planktonic cells and MSgg medium were removed from the microtiter plate wells, which were then washed with distilled water. Biofilm cells in the wells were stained with crystal violet (CV). Then, the wells were rinsed with distilled water until no unbound CV was present. The bound CV was solubilized with 1 mL of 4:1 ethanol and acetone acid (v/v). Biofilms were quantified by measuring the OD_{600} for each well using the multifunctional plate reader Spectra Max M5 analysis system (Molecular Devices Corporation, CA).

Chemotaxis Assay

A modified capillary method[28] was performed for the quantitative analysis of the chemotactic responses of SQR9 and Δ*resE* toward various organic acids. Strains were incubated in Luria-Bertani (LB) culture until they reached log phase (OD_{600} = 0.8). After collection by centrifugation, the cells were washed twice with chemotactic buffer[29] and resuspended in the same buffer with an OD_{600} = 0.8. Then, 10 mL of the cell suspension was added to a Petri dish (60 mm in diameter). A standard 1 μL capillary loaded with 50 μM of various organic acids (citric acid, malic acid, oxalic acid, and fumaric acid) was immersed in the above cell suspension. After 30 min of incubation at room temperature, the cells in the capillary were transferred into a sterile Eppendorf tube by syringe and then counted by dilution plate counting.

Root Colonization Assay

The bacterial suspensions of SQR9 and Δ*resE* were inoculated into sterile cucumber seedlings in $^1/_4$ MS culture. After 4 days, the cells attached to the cucumber root were collected using the method described above and quantified by dilution plate counting.

Complementation of the *resE* Gene

The P43 promoter[30] was used for *resE* gene complementation. Primers P43−F and P43-R were used to amplify the P43 promoter sequence from *Bacillus subtilis* 168 chromosomal DNA, and the *BamH*I site was introduced in the forward primer P43−F, while *Xho*I and *Pst*I were added to the reverse primer P43-R. The *resE* gene, bordered by *Xho*I and *Nsi*I sites, was amplified from SQR9 genomic DNA using the primer set PresE-FF/PresE-FR. The restriction enzymes *Pst*I and *Nsi*I were isocaudamer enzymes that could be linked together. After purification and digestion with the corresponding restriction enzymes, the P43 promoter and *resE* gene fragments were

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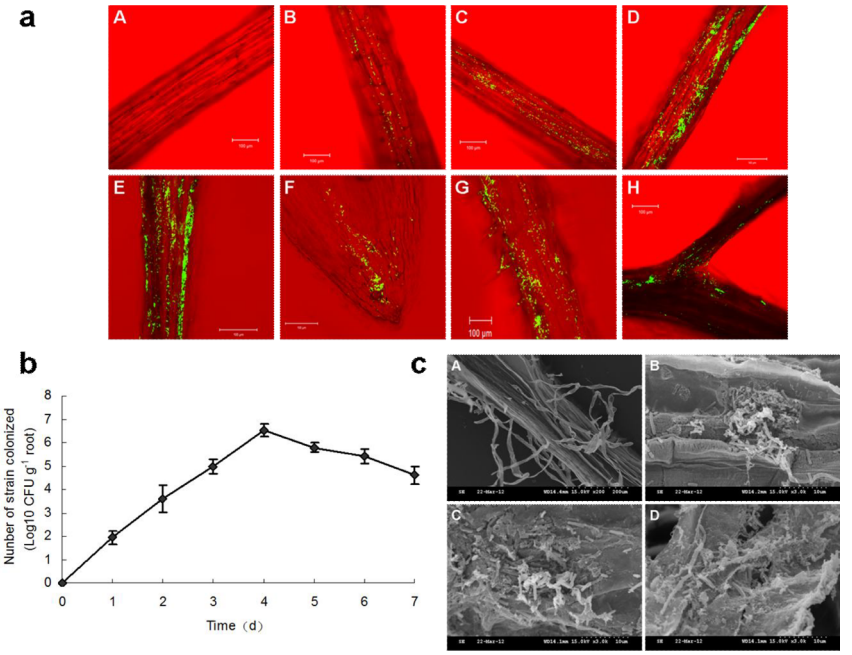

Figure 1. Qualitative and quantitative analysis of SQR9-*gfp* (SQR9) cells colonized on cucumber roots. (a) Fluorescence micrographs of cucumber roots colonized by SQR9-*gfp* cells: (A) without inoculation of SQR9-*gfp* to the cucumber roots as a control; after (B) 1, (C) 2, (D) 3, and (E) 4 days, SQR9-*gfp* colonized on the elongation zones of cucumber roots; and SQR9-*gfp* colonized (F) on the root tip, (G) root hair, and (H) junctions between the roots after an inoculation of 3 days. (b) The dynamic change of SQR9-*gfp* colonized on cucumber roots after inoculation for different times. (c) Scanning electron microscopy images of cucumber roots colonized by SQR9 cells: (A) without inoculation of SQR9 to the cucumber roots as a control; (B) after 3 days, SQR9 colonized on cucumber roots; and (C,D) SQR9 colonized on the root surfaces of cucumbers after 4 days of inoculation.

cloned into the vector pUBC19 to obtain the plasmid pUBC19-*P43-resE* (Figure S2, Supporting Information). Then, plasmid pUBC19-*P43-resE* was transformed into mutant Δ*resE* to obtain the *resE* gene complemented strain (C-*resE*).

Quantification of Gene Transcription by Real-Time PCR

To investigate the mechanism of *resE* gene regulation of biofilm formation and RC, some biofilm-related genes (*spo0A*, *epsD*, *slrR*, *slrA*, *sinI*, *sinR*, *yqxM*, *abrB*, *degU*, and *degQ*) and genes reported to be regulated by the ResD-ResE two-component regulatory system (*nasDEF*, *fnr*, *hmp*, and *ctaA*) were quantified by real-time PCR according to the protocol described by Qiu et al.,[23] and the *recA* gene was used as an internal control.

RNAs from biofilms formed by SQR9, Δ*resE* and C-*resE*, were extracted using the E.Z.N.A. bacterial RNA kit (OMEGA, Biotek, USA) according to the manufacturer's protocol. The RNAs were detected on a 1% agarose gel to check the quality and then reverse-transcribed using the PrimeScript RT reagent kit with a gDNA eraser (TaKaRa, Biotek, Dalian).

Statistical Analysis

Differences among the treatments were calculated and statistically analyzed with a one-way analysis of variance (ANOVA). Duncan's multiple-range test was used when one-way ANOVA indicated a significant difference ($P < 0.05$). All statistical analyses were performed with SPSS BASE version 11.5 statistical software (SPSS, Chicago, IL).

RESULTS

Colonization of SQR9-*gfp* (SQR9) on the Cucumber Roots

In the hydroponic culture system, 1 day after inoculation, there was a scattering of SQR9-*gfp* cells in the elongation zones of the cucumber roots (Figure 1 a(B)). Three days later, a great number of SQR9-*gfp* cells was observed in the elongation zones and formed microcolonies (Figure 1 a-(D)), whereas only a few cells congregated at the root tips (Figure 1 a(F)), root hair zones (Figure 1 a(G)), and junctions (Figure 1 a(H)). Four days after inoculation, more cells accumulated, and biofilm appeared in the elongation zones of the root surfaces (Figure 1 a(E)), while the SQR9-*gfp* population slightly decreased after 5, 6, and 7 days (Figure 1b). Therefore, to analyze the colonization-related proteins, SQR9 cells were recovered when the biofilm, including the abundance of SQR9 attached to the root surface and extracellular matrix, had formed (4 days after inoculation) (Figure 1 c(C,D)).

Root-Colonized and Planktonic SQR9 Cells Showed Different Proteomic Profiles

In the SDS-PAGE analysis, there were significant differences between CK and RC at the protein level (Figure S3, Supporting Information). From the proteome data, the protein ratio for the two treatments (RC:CK) was distributed in a near-Gaussian fashion, which indicates that most of the SQR9 proteins were stable in the two growth conditions. Moreover, in RC treatment, the number of up-regulated proteins was almost same as that of down-regulated proteins (Figure S4, Supporting Information).

A total of 755 common proteins of three biological repeats were identified, of which 725 were quantified (Supporting Information). In total, 78 and 95 proteins were regarded to be up-regulated (RC:CK > 2) and down-regulated (RC:CK < 0.5), respectively, with RC treatment ($P < 0.05$), and all of these proteins were classified in clusters of orthologous groups (COGs) (Tables S2 and S3, Supporting Information). The distribution in various functional categories (e.g., cell envelope and cellular processes, intermediary metabolism, information

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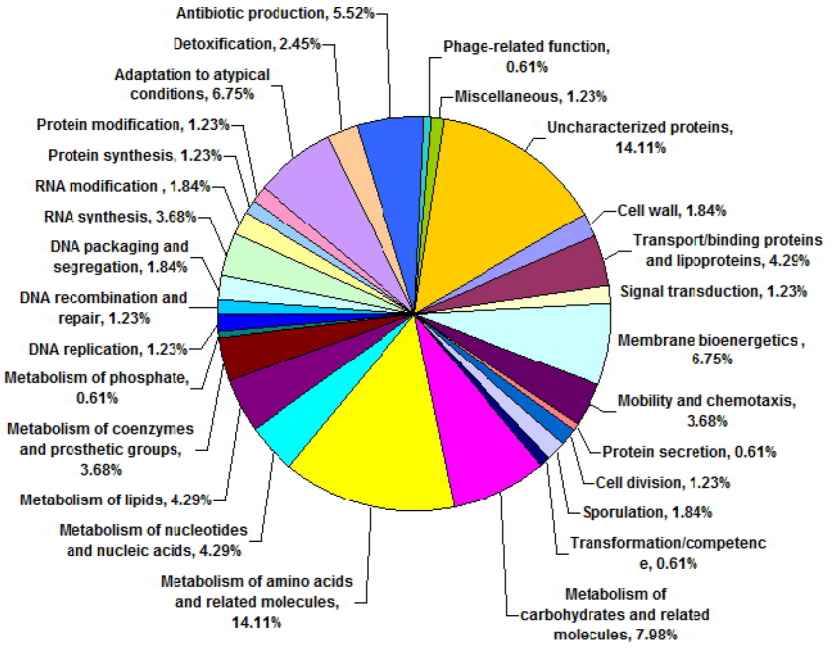

Figure 2. Distribution in various functional categories of the proteins altered in the SQR9 colonized on the cucumber rhizoplane.

pathways, and other functions) for these 173 proteins is summarized in Figure 2. SQR9 is a rhizosphere beneficial bacterium; a variety of proteins related to rhizosphere adaptation and beneficial plant functions, such as biocontrol, detoxification, biofilm formation, cell motility and chemotaxis, transport, and degradation of plant polysaccharides, were compared (Figure 3). Some proteins involved in biocontrol were enhanced with RC treatment, including SrfAA, SrfAD, BaeL, FenB, MlnG, and DfnF; however, the six proteins with a lower fold change after SQR9 colonization were associated with flagella-driven motility (FlhA, FlgE, FliL, and FliY), chemotaxis

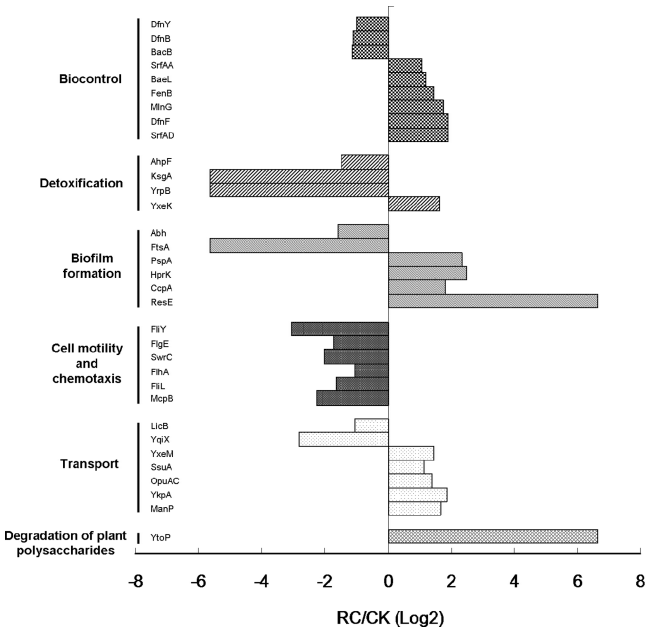

Figure 3. Distribution of the rhizosphere proteins in the presence of various treatments. The abscissa shows the logarithm of the protein ratio of two treatments (RC/CK), and the ordinate stands for various regulated proteins.

(McpB), and swarming (SwrC). For biofilm formation, there were two proteins down-regulated (Abh and FtsA) and four that were up-regulated (PspA, HprK, CcpA, and ResE). Interestingly, ResE, which is a component of a two-component regulatory system that is activated under anaerobic conditions,[31] showed a change of 100-fold increase compared to CK treatment, which indicates that this protein is possibly associated with in situ RC and biofilm formation.

Regulator ResE Was Involved in the Root Colonization and Biofilm Formation in SQR9

To confirm the function of ResE in SQR9 RC and biofilm formation, the *resE* gene was deleted, and mutant Δ*resE* was compared to the wild-type strain. After 24 h of inoculation in MSgg medium, the ability of biofilm formation in SQR9 and Δ*resE* was different, though the growth curves of the two strains were similar (Figure S5, Supporting Information). Biofilms formed by the *resE* mutant were thin and fragile compared to the robust ones made by the wild-type strain SQR9 (Figure 4A), which demonstrates that the *resE* gene had a positive influence on biofilm formation. Complementation of the *resE* gene in Δ*resE* restored the ability to form a normal biofilm based on qualitative (Figure 5A) and quantitative (Figure 5B) analysis. For RC comparison (Figure 4B), 4 days after inoculation, the number of root-colonized Δ*resE* was significantly lower than that of SQR9 and decreased approximately 10-fold.

Efficient colonization on the plant root surface was a prerequisite for plant protection by *Bacillus*, which depended on chemotactic motility and biofilm formation.[32] There were various kinds of organic acids in the plant root exudates, such as citric acid, malic acid, oxalic acid, and fumaric acid, and some of the organic acids played a key role in microorganism chemotaxis and root-PGPR interaction.[14,33] The quantitative analysis showed that the wild-type SQR9 had chemotactic responses toward citric acid, malic acid, oxalic acid, and fumaric acid; however, the chemotactic ability significantly decreased when the *resE* gene was knocked out (Figure 4C). The *resE* gene most likely promoted the rhizosphere colonization of

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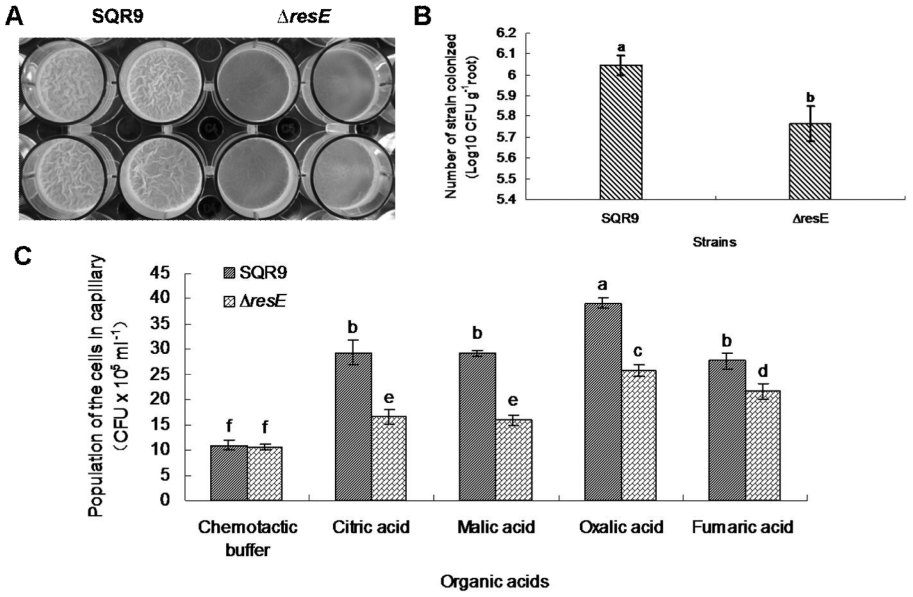

Figure 4. Comparison of SQR9 and Δ*resE* on (A) biofilm formation, (B) RC, and (C) chemotactic responses toward various organic acids. Letters above the bars reveal a significant difference according to Duncan's multiple range test at a $P < 0.05$ level.

SQR9, while the exact mechanism remains to be elucidated further.

ResE Affected the *spo0A-sinI-yqxM* Pathway for Biofilm Formation and Root Colonization Regulation

The transcription results showed that the biofilm-related genes *spo0A*, *sinI*, and *yqxM* were significantly reduced in Δ*resE* and restored and even enhanced in the complementary strain C-*resE* (Figure 5C), most likely because of the strong promoter of P43, while other biofilm-associated genes (*epsD*, *slrR*, *slrA*, *sinR*, *abrB*, *degU*, and *degQ*) had no significant changes (data not shown). Additionally, expression of the *resE* gene could not be detected in the Δ*resE* strain, and was significantly higher in the C-Δ*resE* strain than in SQR9 by real-time PCR (Figure 5D). Therefore, the *resE* gene was speculated to regulate biofilm formation in *B. amyloliquefaciens* SQR9 through the *spo0A-sinI-yqxM* pathway.[7]

■ DISCUSSION

PGPR promotes plant growth and disease inhibition mainly through efficient RC and robust biofilm formation on the rhizosplane.[34] In many cases, PGPR's biocontrol or growth promotion was suppressed by weak RC.[35,36] In this study, 4 days after inoculation, SQR9 was strongly colonized on the root and formed biofilm structures, consistent with previous reports,[21,37,38] that may contribute to the antagonistic and promoting functions of SQR9.[23]

Compared to previous studies of biofilm formation on the medium, this work focused on the key proteins of the PGPR strain SQR9 for in situ RC. Moreover, several proteins associated with RC and biofilm formation were classified into the following groups.

(i). Biocontrol

Surfactin, an antibiotic, was found to display antibacterial, antiviral, antifungal, antimycoplasmic, and hemolytic activities.[39] The proteome data showed that SrfAA and SrfAD, involved in surfactin synthesis, were up-regulated by RC, which is consistent with other studies. Chen et al.[3] reported that the Δ*srfAA* mutant showed a significant loss in antagonistic activity against the tomato plant pathogen *Ralstonia Solanacearum* and decreased plant protection. Furthermore, Fan et al.[40] suggested that up-regulation of *srfAD* affected maize root exudates and may contribute to a protective role of surfactin against plant pathogens.

Another two proteins, BaeL and DfnF, which are responsible for the polyketide synthesis of bacillaene and difficidin, respectively, were also increased with RC treatment. Chen et al.[41] revealed that the antibacterial polyketides difficidin and to a minor extent bacillaene acted efficiently against *Erwinia amylovora* in orchard trees. On the basis of the transcriptomic analysis of *Bacillus amyloliquefaciens* FZB42, the genes *baeL* and *dfnF* were also significantly induced by maize root exudates;[40] however, another two difficidin synthesis proteins (DfnY and DfnB) were decreased on the cucumber root surface, which is not in accordance with the results from Fan et al.[40] Most likely, these two proteins were sensitive to the components of various plant root exudates, and not all of the proteins in the cluster of difficidin synthesis were related to biocontrol.[41]

Moreover, two other induced proteins, FenB and MlnG, which lead to the biosynthesis of fengycin and macrolactin, respectively, were also detected with RC treatment. Fengycin, a lipopeptide product, has a major role in the antagonism of *Bacillus subtilis* toward *Podosphaera fusca*, and antibiosis could be a major factor involved in its biocontrol ability.[42] Furthermore, Ongena et al.[43] provided evidence for the role of fengycins in the protective effect conferred by the strain against damping-off of bean seedlings caused by *Pythium ultimum* and against gray mold of apples in postharvest disease. Macrolactin has a suppressive effect on sporulation and mycelium formation in *Streptomyces scabiei*, having inhibitory action in potato scab disease. In a study of transcriptomic profiling of *B. amyloliquefaciens* FZB42, *mlnH* and *fenE* were also up-regulated,[40] which is consistent with our proteome data.

(ii). Detoxification

Plants are often exposed to natural and synthetic toxins such as heavy metals, allelochemicals, organic contaminants, and pesticides.[44] Evidence has suggested that plant-associating bacteria have evolved the ability to metabolize aromatic

dx.doi.org/10.1021/pr500565m | J. Proteome Res. XXXX, XXX, XXX−XXX

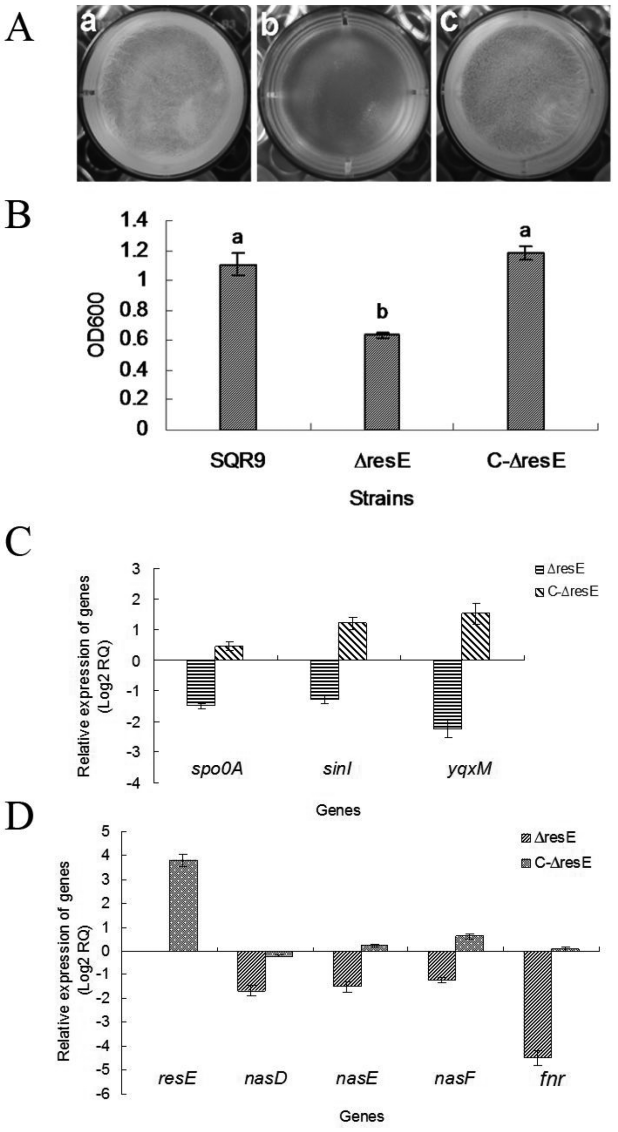

Figure 5. Comparison of the biofilm formation after complementary of *resE* gene by (A) qualitative and (B) quantitative analysis. Relative quantification of (C) genes related to biofilm formation and (D) the genes regulated by the ResD-ResE system. (A) a, SQR9; b, Δ*resE*; c, C-*resE*. (B−C) Letters above the bars reveal a significant difference according to Duncan's multiple range test at a $P < 0.05$ level.

pollutants such as naphthalene, toluene, and 2,4-dichlorophenoxyacetic acid.[45] From the proteome profiling of SQR9, some proteins related to detoxification were detected, in which YxeK was up-expressed, while YrpB, KsgA, and AhpF were down-regulated in the root-colonized SQR9 cells.

The up-regulated protein YxeK was annotated as a putative monooxygenase, and monooxygenase was reported to have a positive effect on the detoxification of carbaryl, parathion, and diazinon.[46] Consequently, in RC treatment, YxeK activation could enhance the degradation of some toxic substances on the root surface and increase the resistance of the cucumber plant. Carvalhais et al.[47] demonstrated that *yrpB*, a gene that encodes a putative 2-nitropropane dioxygenase, was down-regulated by seed exudates, which is in accordance with the proteome results. The gene *ksgA* encoded a dimethyladenosine transferase involved in resistance to kasugamycin.[47] Kasugamycin, an aminoglycosidic antibiotic, was initially reported to have potential use against *Pseudomonas*.[48] The down-expression of KsgA could, to a certain extent, decrease the repression of kasugamycin and improve the protection of plant roots. The protein encoded by the gene *ahpF* has been shown to play an important physiological role in protecting against peroxide toxicity in *Xanthomonas campestris*.[49] The reduction in AhpF expression was most likely due to less peroxide being present because of the cucumber root exudates.

(iii). Cell Motility and Chemotaxis

The processes of cell motility and chemotaxis are vital for bacterial RC.[40] After recognition of the signals emitted by plant roots, bacteria move toward the roots and attach to the surface.[50] Six proteins with a lower-fold change after SQR9 colonization were associated with flagella-driven motility (FlhA, FlgE, FliL, and FliY), chemotaxis (McpB), and swarming (SwrC); however, in the report from Fan et al.,[40] the genes required for chemotaxis and motility showed a higher-fold change in response to maize exudates, which is not consistent with our results. In this study, the samples collected within the biofilm with RC treatment were nonmotile and aggregated by an extracellular matrix composed of secreted macromolecules,[51] which may induce the down-regulation of these six proteins.

(iv). Transport

The transport system plays a key role in the communication and adaptation of bacteria with their environment.[52,53] In the SQR9 proteomics study, the expression of seven proteins, including transport/binding proteins and lipoproteins, was significantly altered in the root-colonized SQR9. Four ATP-binding cassette (ABC) transporters (YxeM, OpuAC, SsuA, YkpA, and ManP) showed elevated expression. YxeM is a binding protein for amino acids in *Bacillus*, and amino acids are involved in rhizosphere competence. The chemotactic response of *Bacillus* to amino acids is a common phenomenon and provides them with a competitive advantage for RC and the detection and consumption of available nutrients in a natural environment. Kappes et al.[54] noted that as an extracellular substrate-binding protein anchored in the cytoplasmic membrane, OpuAC was involved in the uptake of glycine betaine in *B. subtilis*. *B. subtilis* can face drastic changes in the osmotic strength of their environment by synthesizing or accumulating any of a variety of osmolytes, including amino acids, various ectoines, and glycine betaine. The protein encoding the aliphatic sulfonate ABC transporter SsuA functions in the assimilation of sulfate, and van der Ploeg et al.[55] revealed that for *B. subtilis*, disruption of *ssuA* and *ssuC* decreased the utilization of a range of aliphatic sulfonates, such as alkanesulfonates, taurine, isethionate, and sulfoacetate, as a source of sulfur. Inorganic sulfate is involved in the synthesis of cysteine and methionine in *Bacillus*. YkpA, a nucleotide binding domain protein, may work as a drug efflux pump in conjunction with a certain transmembrance protein not yet identified. In addition, two other ABC transporters, LicB and YqiX, showed depressed expression in response to the cucumber root secretions as described in Table S3 of the Supporting Information.

(v). Degradation of Plant Polysaccharides

Plant polysaccharides consist of plant cell wall polysaccharides (cellulose, hemicelluloses, and pectin) and storage polysaccharides (e.g., starch, inulin, gums),[56] including many different monomers associated with each other by a diversity of linkages.[57] The protein YtoP, similar to endo-1,4-beta-

dx.doi.org/10.1021/pr500565m | J. Proteome Res. XXXX, XXX, XXX−XXX

glucanase, was activated in situ in the biofilm on the cucumber root surface. Enhanced expression of YtoP in the plant roots could improve the utilization of cellulose and hemicelluloses.

(vi). Biofilm Formation

Upon root efficient colonization, a stable, extensive biofilm could be formed and provide the plant root with a protective barrier against attack by pathogenic microbes.[50] The expression of two proteins, FtsA and Abh, was much lower in the colonized SQR9 than in the planktonic cells. Hu et al.[58] found that the *ftsA* mutants significantly reduced the cell growth rate and biofilm formation compared to the wild-type *Riemerella anatipestifer*. Yoon et al.[59] proved that cell elongation, which likely occurred as a consequence of defective cell division (encoded by the gene *ftsA*), contributed to the biofilm formation of *Pseudomonas aeruginosa*; therefore, the decreased FtsA expression in our study was reasonable because of the biofilm formation on the root surface. The protein Abh, a sequence and structural homologue of AbrB, has been shown to regulate the biofilm architecture of *B. subtilis*.[60] Furthermore, Murray and Stanley-Wall[61] reported that Abh was a transition state regulator of *B. subtilis* that controlled biofilm formation and the production of several diverse antimicrobial compounds. The SQR9 cells were in the stationary stage after they colonized the cucumber root and had a lower activity compared to that of the CK. Abh has been shown to regulate gene expression during the transition from active growth to the stationary phase,[62] which could explain why the expression of *abh* decreased when the SQR9 biofilm formed on the root surface.

However, another four proteins (PspA, HprK, CcpA, and ResE), which influence biofilm formation, were enhanced after SQR9 colonization on the root surface. Moscoso et al.[63] reported that deficiency of the gene *pspA* in *Streptococcus pneumoniae* decreases the capacity to form biofilms. Bacteria have the ability to selectively uptake and metabolize carbon sources that allow them to grow at the maximal rate and offer the best success in competition with other microbes. This phenomenon is collectively called carbon catabolite repression (CCR).[64] Gram-positive bacteria, such as *Bacillus subtilis*, rely mainly on HPr for the regulation of CCR. HPr can be phosphorylated at the serine-46 residue by HPr kinase/phosphatase HPrK under energy-replete conditions. HPr-Ser46-P, after it is bound to the transcriptional regulator CcpA, can repress genes associated with secondary carbon source utilization.[65] Stanley et al.[66] identified that CCR was a physiological regulator of biofilm formation by *B. subtilis* using DNA microarrays and indicated that glucose inhibited biofilm formation through the catabolite control protein CcpA. Carbon nutrients, such as glucose, fructose, and maltose, were present in various plant root exudates.[67,68] The proteins CcpA and HprK with RC treatment were up-regulated with a fold change of 3.5 and 5.6, respectively. These CCR proteins were activated by the carbon source in the cucumber root exudates.

From the iTRAQ data, there were seven 100-fold highly up-regulated proteins (ResE, YkuR, Csd, RsbT, SigF, YrrK, and YtoP). We disrupted these seven genes of SQR9; only the mutant of *resE* significantly influenced the biofilm formation (data not shown). Additionally, it is intriguing that the proteins that showed up-regulation were either under 10-fold change or 100-fold change and that nothing was between 10 and 100, which was in accordance with the previous report.[69] The reason for this phenomenon probably was the collection time of SQR9 attached to the roots or the environment of cucumber rhizoplane. ResE, which is component of a two-component regulatory system that is activated under anaerobic conditions,[31] showed a change of 100-fold up-regulation compared to the CK treatment. Stanley et al.[66] revealed that ResE appeared to regulate gene expression under biofilm formation conditions. An investigation of whether ResE is active in planktonic cells and can regulate the transition from the planktonic to biofilm stage would be interesting. The increased fold change in ResE implied that this protein was possibly stimulated by cucumber root exudates or related to in situ RC and biofilm formation.

From the quantification results of biofilm genes, *spo0A*, *sinI*, and *yqxM* were significantly influenced by the expression of the *resE* gene. The *resE* gene was suggested to participate in the *spo0A-sinI-yqxM* pathway for the biofilm regulation of SQR9 in cucumber roots. Beauregard et al.[70] provided evidence that biofilm genes were crucial for *Arabidopsis* RC by *B. subtilis*, and that neither *spo0A* nor *sinI* mutants were able to colonize the roots. Chen et al.[3] also proved that the Δ*sinI* mutant of *B. subtilis* exhibited severely diminished cell attachment to tomato roots; however, the transcriptional evidence was not sufficient; the accurate, detailed mechanisms of *resE* gene regulation of biofilm formation and RC need more investigation.

ResE, the kinase in the ResD-ResE two-component regulatory system, has been shown to phosphorylate ResD to activate downstream genes, such as *fnr*, *nasDEF*, *hmp*, and *ctaA*,[71] which indicates that the expression of these genes has a positive correlation with ResD phosphorylation. Compared to wild-type SQR9, the expression of *fnr* and *nasDEF* were significantly lower for the mutant Δ*resE* (Figure 5D), which implies that the ResD phosphorylation level was different between SQR9 and Δ*resE* strain. It will be interesting to determine whether *resE* regulates the biofilm formation of SQR9 through ResD phosphorylation or not. Kobayashi[72] demonstrated that the *resD* gene regulated the transcription factor *cwlS* to influence biofilm formation in *Bacillus subtilis*. From another point of view, oxygen limitation is a common condition in the biofilm architecture.[73] During biofilm formation, the oxygen content in the microecological environment of biofilm has been shown to gradually decrease.[66] For the thinnest biofilm for Δ*resE* compared to other strains (SQR9 and C-*resE*), the oxygen content was the most adequate at this time. This result can to some extent be explained by increased expressions of *fnr* and *nasDEF* within the enhanced biofilm, because these genes are anaerobically regulated.[71] However, the *ctaA* gene, which is regulated under aerobic conditions,[74] did not show the opposite result; namely, the expression of the *resE* mutant was significantly higher than that of other strains, which might be worth further study.

CONCLUSION

High-throughput proteomic approaches identified the key proteins involved in the in situ RC of the PGPR strain *Bacillus amyloliquefaciens* SQR9 that are related to rhizosphere competition and adaptation and belong to the functional categories of biocontrol, detoxification, biofilm formation, cell motility and chemotaxis, transport, and degradation of plant polysaccharides. On the basis of the proteomic data and further gene knockout experiments, the regulator protein ResE was found to positively influence biofilm formation and rhizosphere colonization, which has not been reported before. This study enhanced the understanding of the PGPR RC mechanism and

will help promote the application of PGPR strains in agricultural production.

■ ASSOCIATED CONTENT

Supporting Information

Primers used in this study. List of up-regulated proteins of RC treatment and the related fold-change (RC:CK > 2), and list of down-regulated proteins of RC treatment and the related fold-change (RC:CK < 0.5). Visual model that represents the sterile culture of cucumber seedlings. Construction of the expression vector pUBC19-P43-*resE* of *resE* gene. The difference of cell proteins between treatments by SDS-PAGE. The statistic image of the distribution of the protein ratio of two treatments (RC:CK). Growth curves of wild type SQR9 and Δ*resE*. All data including the list of identified and quantified proteins. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was financially supported by the National Natural Science Foundation of China (41271271), National Key Basic Research Program of China (973 program, 2015CB150505), and Specialized Research Fund for the Doctoral Program (SRFDP) of Higher Education (20110097110002). R.Z. and Q.S. were also supported by the Priority Academic Program Development (PAPD) of Jiangsu Higher Education Institutions and the 111 Project (B12009). Additionally, we thank Guangzhou FitGene Biotechnology Co., Ltd., China, for the technical assistance.

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