Identification of Adhesins in Plant Beneficial Rhizobacteria *Bacillus velezensis* SQR9 and Their Effect on Root Colonization

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Probiotic *Bacillus* colonization of plant root surfaces has been reported to improve its beneficial effect. Chemotaxis, adhesion, aggregation, and biofilm formation are the four steps of root colonization by plant growth-promoting rhizobacteria (PGPRs). Compared with the other three well-studied processes, adhesion of PGPRs is less known. In this study, using mutant strains deleted for potential adhesin genes in PGPR strain *Bacillus velezensis* SQR9, adherence to both cucumber root surface and abiotic surface by those strains was evaluated. Results showed that deletion mutations Δ lytB, Δ V529_10500, Δ fliD, Δ yhaN, and Δ sacB reduced the adhesion to root surfaces, while, among them, only Δ fliD had significant defects in adhesion to abiotic surfaces (glass and polystyrene). In addition, *B. velezensis* SQR9 mutants defective in adhesion to root surfaces showed a deficiency in rhizosphere colonization. Among the encoded proteins, FliD and YhaN played vital roles in root adhesion. This research systematically explored the potential adhesins in a well-studied PGPR strain and also indicated that adhesion progress was required for root colonization, which will help to enhance rhizosphere colonization and beneficial function of PGPRs in agricultural production.

Keywords: abiotic surface, adhesin, *Bacillus velezensis* SQR9, colonization, root surface

Biofilms are communities of surface-related microorganisms encased in the self-generated extracellular matrix. The formation of biofilm is a universal bacterial feature and is found on almost all natural and artificial surfaces, including solid surfaces and

liquid-air interfaces (Hall-Stoodley et al. 2004; Ramey et al. 2004; Stewart and Franklin 2008). Biofilms protect bacteria from environmental stress and enhance the interaction between bacteria and various biotic or abiotic surfaces, which result in the efficient colonization of bacteria on different surfaces. *Bacillus subtilis*, a nonpathogenic gram-positive bacterium, has become a model microorganism for studying biofilm formation. *B. subtilis* can form pellicle biofilms at the liquid-air interface of standing cultures and complex colony architectures on solid surfaces (Fan et al. 2011; Xu et al. 2014). The biofilm formation process includes several stages of development, maturation, and disassembly (Vlamakis et al. 2013). At the start of biofilm formation, motile cells with flagella are differentiated into nonmotile matrix-producing cells, which stop separating and form the extracellular matrix surrounded chains. In a mature biofilm, the cells that produce the matrix are sporulated. In aged biofilms, some cells secrete small molecules such as D-amino acids and polyamines to break down the extracellular matrix, allowing the cells to disperse in the environment (Vlamakis et al. 2013). The early stages of biofilm formation consist of chemotaxis, adhesion, aggregation, and biofilm formation (Aguilar et al. 2007; Smit et al. 1989).

Attachment to surfaces is the initial step for biofilm formation (Merritt et al. 2007). For example, the association of *Herbaspirillum seropedicae* with poaceous crops was initiated with the adhesion of the bacteria to root surfaces, followed by colonization at the emergence points of secondary roots (Balsanelli et al. 2010). *Azospirillum brasilense* is a plant growth-promoting rhizobacterium (PGPR); adhesion played an important role in its colonization on wheat root (Croes et al. 1993). Among factors of persistent infection in plants by *Xylella fastidiosa*, adhesion played a partial role in the initial stages of biofilm formation (de Souza et al. 2005).

The adhesion ability of microbes depends on their surface appendages anchored in the bacterial outer membrane taking part in attachment to one or both abiotic and biotic surfaces (Moreira et al. 2004). Flagella, the protrudent and filamentous appendages of some bacteria, participate in the adhesion process. *Salmonella* spp. attach to cholesterol through FliC, a flagellum protein, as the initial step for colonizing and forming biofilms on human gall stones (Crawford et al. 2010). The lipopolysaccharide (LPS) of gram-negative bacteria had an effect on cell adhesion. The lack of B-band LPS in *Pseudomonas aeruginosa* PAO1 demonstrated reduced adhesion to hydrophilic surfaces (Makin and Beveridge 1996). LPS-lacking *Escherichia coli* also

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decreased the adhesive ability (Genevaux et al. 1999). *Herbaspirillum seropedicae* needed LPS to attach the maize root (Hori and Matsumoto 2010; Nevola et al. 1985). Collagen like proteins (CLPs) are located in the outer layer of bacterial cells, which are important for bacterial adhesion to host cells and biofilm formation. CLPs of *Bacillus amyloliquefaciens* FZB42 appeared to play a role in bacterial aggregation and adherence to the surfaces of abiotic materials and *Arabidopsis* roots (Zhao et al. 2015). Some polysaccharide capsules surrounding the bacteria can interact with legumes, because specific carbohydrate structures in the polysaccharide capsule can be recognized by lectin molecules produced from legume root hair tips. In *Rhizobium leguminosarum* RBL5523, there might exist two different lectin ligands, one for the clover root and the other for the pea/vetch root (Laus et al. 2004, 2006). Cell-wall hydrolases (CWHs) are surface enzymes to degrade cell wall by cleaving specific covalent bonds (López and García 2004). LytB is a CWH of *Streptococcus pneumoniae* and participates in the initial attachment to the nasopharynx; effective colonization of the respiratory tract needs the combination of CWHs LytB and LytC (Ramos-Sevillano et al. 2011).

Bacillus species are model microorganisms for biofilm study. The *B. cereus* spores have long appendages playing important roles in the surface attachment (Faillie et al. 2010; Klavenes et al. 2002). The exosporium glycoprotein BclA filaments played a major role in interaction of spores with stainless steel by providing a larger contact surface (Lequette et al. 2011). S-layer components, flagellin, and cell-bound proteases of *B. cereus* have been found to bind intestinal surface components such as mucin or fibronectin (Sánchez et al. 2009). Cell-wall peptidase CwpFM of *B. cereus* was implicated in adhesion to epithelial cells (Tran et al. 2010). In *B. anthracis*, BslA (S-layer protein A) was sufficient for adhesion of vegetative cells to human endothelial cells (Tonry et al. 2012), however, overexpression of the pleiotropic regulator CodY in *B. anthracis* decreased the attachment to sterile polystyrene (Gopalani et al. 2016). Cell-surface proteins played an important role in the initial process of attachment to stainless steel of the vegetative cells and spores of thermophilic *Bacilli* spp. (Parkar et al. 2001). The spore exosporium of *B. mycoides* has been observed to be flexible and to promote adhesion to glass surface of the spore by increasing the spore contact area on the surface (Bowen et al. 2002). Adhesion to gold surfaces by *B. subtilis* NCIB 3610 was mediated by thiol groups present in proteins of the bacterial cell membrane (Kesel et al. 2014). Filamentous hemagglutinin adhesin is a large filamentous protein, which was required in *B. licheniformis* adherence to microtiter tissue culture plate (Sadiq et al. 2019). *Bacillus* species are representative PGPRs; root colonization is a prerequisite for PGPRs to perform their plant beneficial functions. Biofilm formation of *Bacillus* on rhizoplane is well studied, but the root attachment process is less known.

In this study, the potential adhesins contributed to root attachment of a well-studied PGPR strain, *Bacillus velezensis* SQR9 (formerly *Bacillus amyloliquefaciens* SQR9), were systematically explored. *B. velezensis* SQR9 was isolated from the cucumber rhizosphere. As a strong root colonizer, SQR9 formed robust and highly structured biofilms and efficiently promoted plant growth and suppressed soil-borne diseases (Qiu et al. 2014; Weng et al. 2013). The complicated regulatory network governing *B. velezensis* SQR9 rhizosphere chemotaxis and biofilm formation has been well-elucidated (Dong et al. 2018; Feng et al. 2018; Li et al. 2018; Xu et al. 2013, 2014, 2019; Zhou et al. 2018). Here, we show that *B. velezensis* SQR9 has multiple adhesin proteins for root and abiotic surface attachment, which are required for its efficient rhizoplane colonization. These results provide guidelines for enhancing the rhizosphere colonization and beneficial function of PGPR in agricultural production.

RESULTS

Prediction of potential adhesin proteins in *B. velezensis* SQR9 and construction of adhesin mutants and corresponding complementation strains.

The entire genome of *B. velezensis* SQR9 has been sequenced, and a gene annotation table has been made by our laboratory that contains results from Kyoto Encyclopedia of Genes and Genomes orthology, National Center for Biotechnology Information (NCBI) (accession number CP006890), and Pfam databases. To systematically explore possible adhesins in *B. velezensis* SQR9, we searched the annotation table for reported adhesins and possible adhesin-related proteins. Seven possible adhesin-related proteins have been identified. LytB (Ramos-Sevillano et al. 2011) and FliD (Tasteyre et al. 2001) are proteins that have been reported to be involved in adhesion. SacB is a levansucrase responsible for the synthesis of levan (Pereira et al. 2001), which is a polysaccharide that may be involved in adhesion (Manandhar et al. 2009). V529_10500 and YcdA are membrane lipoproteins, playing a role in cell adhesion (Knoke et al. 2020). V529_07150 contains a collagen-binding domain, which exhibits the best surface complementarity to the collagen, and the domain can be found in a *Staphylococcus aureus* adhesin (Symersky et al. 1997). These seven proteins were predicted by another database, SMART, and possible domains of each protein are listed in Table 1. These genes were deleted individually in SQR9 to obtain their mutants (Δ lytB, Δ V529_10500, Δ ycdA, Δ V529_07150, Δ fliD, Δ yhaN, and Δ sacB) as well as their corresponding complementation strains (Δ lytB/lytB, Δ V529_10500/V529_10500, Δ fliD/fliD, Δ yhaN/yhaN, and Δ sacB/sacB). Protein YcdA and V529_07150 did not show root surface adhesion function and the deleted genes were not complemented in the following research (Supplementary Table S1).

Contribution of potential adhesins to the root attachment of *B. velezensis* SQR9.

Initial attachment to the rhizoplane is an important step for PGPRs to colonize the root and perform plant-beneficial functions; bacterial adhesins and their interactions with plant roots play important roles during the attachment process. Root attachment analysis revealed that these potential adhesin genes (except for ycdA and V529_07150) significantly affected SQR9 root attachment ($n = 5$, $P < 0.05$). Compared with the wild-type strain, different mutants showed 20 to 50% reduced root attachment (Fig. 1). Mutants Δ ycdA and Δ V529_07150 showed the same adhesion ability with the wild-type strain while other mutants decreased this ability to root surfaces.

Gene complementation of these root attachment-defective mutants (Δ lytB/lytB, Δ V529_10500/V529_10500, Δ yhaN/yhaN, and Δ sacB/sacB) partially restored the impaired attachment function (Fig. 1). Confocal laser scanning microscopy (CLSM) of the cucumber root segments attached by different strains of SQR9 confirmed these observations. Although only a small number of cells were detected on the root surface, wild-type and gene-complementation strains showed more fluorescent dots compared with the corresponding mutants (Fig. 2). The combination of quantitative and qualitative results validated the adhesion function of these candidate genes in the PGPR strain *B. velezensis* SQR9, especially, Δ yhaN/yhaN greatly increased adhesion ability to cucumber root surfaces compared with Δ yhaN.

Contribution of adhesins to the abiotic surface attachment of *B. velezensis* SQR9.

To test if the surface attachment of these adhesins in *B. velezensis* SQR9 are specific to biotic material of root, attachment assays to glass and polystyrene surfaces were also performed.

The *fliD* mutant had a defect in attachment ability on abiotic surfaces, its complementation strain did not restore this ability. The adhesion ability of other mutants (Δ lytB, Δ V529_10500, Δ yhaN, and Δ sacB) to glass and polystyrene surfaces were similar to the wild type, and their complementation strains showed almost the same ability as the mutants (except for the complementation strain Δ V529_10500/*V529_10500*, whose adhesion ability on polystyrene surfaces was still defective) (Fig. 3). Thus, most of these adhesins did not take part in the attachment to abiotic surfaces. CLSM scanning of these plastic and glass surfaces attached by different strains of SQR9 revealed that there were no significant differences between the mutants and the wild type or between the mutants and their complementation strains (Supplementary Figs. S4 and S5).

Effect of adhesins on chemotaxis and biofilm formation of *B. velezensis* SQR9.

Chemotaxis is an important step of bacterial attachment (Pratt and Kolter 1998). We assessed the effect of these potential adhesins on the SQR9 chemotactic response to the known chemoattractant malic acid. Results showed that only *fliD* gene deletion affected SQR9 chemotaxis, since the motility was impaired (Supplementary Fig. S1). In addition, the biofilm formation was also tested, none of the mutant strains showed colony architecture (aggregation ability) deficiency on a solid minimal salts glutamate glycerol (MSgg) and lysogeny broth (LB) agar plate (Supplementary Fig. S2). Mutant strains could form pellicle biofilms in the air-liquid interface of liquid MSgg medium, except for Δ fliD (Supplementary Fig. S3).

Effect of adhesins to the rhizosphere colonization of *B. velezensis* SQR9.

Adhesion is the early step for rhizoplane colonization of PGPRs. We then investigated if these adhesins in *B. velezensis* SQR9 affect its root colonization. After coculturing strains and cucumber roots for 24 h, the number of colonization cells was counted. Compared with the wild-type strain SQR9, mutants

Δ lytB, Δ yhaN, and Δ sacB showed significantly reduced colonization ability, while the root colonization of other mutants and all complementation strains had no significant differences with the wild-type strain SQR9 (Fig. 4). For glass and plastic surfaces, only Δ fliD showed obviously decreased colonization, which can be restored by *fliD* gene complementation, probably due to the effect of motility on the colonization (Supplementary Fig. S6).

Fig. 1. Adherence ability of *Bacillus velezensis* SQR9 and derived mutants or complementation strains to cucumber roots. The adherence ability was indicated by adhesion ratio, i.e., the number of bacteria attached to cucumber roots relative to inoculation amount. Error bars are standard deviation, $n = 5$. Different letters mean significant differences ($P < 0.05$, one-way analysis of variance).

Table 1. Potential adhesin-like proteins of *Bacillus velezensis* SQR9 and their structural and functional analysis

Possible adhesin-like proteins	Structures ^a	Functions
LytB	LytB contains an OmpH-like domain (67–246 aa), which interacts directly with membrane lipids and lipopolysaccharide (Walton and Sousa 2004) as well as a bacterial Ig-like domain (312–391 aa), which is present in a bacterial adhesin molecule (Kelly et al. 1999)	An enhancer protein of amidase, the cell wall hydrolase; a modifier protein of major autolysin LytC (Lazarevic et al. 1992; Ramos-Sevillano et al. 2011)
V529_10500	V529_10500 contains subunits (44–323 aa) of integrin, which is a cell adhesin molecule (Bökel and Brown 2002)	An ABC transporter substrate-binding protein (Mistry et al. 2021)
YcdA	YcdA contains an OmpH-like domain (9–228 aa) and a bacterial Ig-like domain (33–126 aa)	A putative lipoprotein that falls into the antigen superfamily (Mistry et al. 2021)
V529_07150	V529_07150 contains a bacterial Ig-like domain (370–443 aa) and a dystroglycan-type cadherin-like domain (1,102–1,197 aa) that is present in adhesin molecules (Dickens et al. 2002)	A collagen binding domain-containing protein (Mistry et al. 2021)
FliD	FliD contains a dystroglycan-type cadherin-like domain (48–135 aa), a bacterial Ig-like domain (197–275 aa), and the domain of formin homology (19–475 aa), which participates in the reorganization of the actin cytoskeleton that mediates adhesion (Tanaka 2000)	A flagellar capping protein involved in flagellin assembly (Tasteyre et al. 2001)
YhaN	YhaN includes an OmpH-like domain (126–235 aa) and a formin homology domain (511–898 aa)	A putative ABC transporter (National Center for Biotechnology Information database)
SacB	SacB contains a glyco domain that belongs to the glycosyl hydrolases family (56–464 aa)	A levansucrase that belongs to the glycosyl hydrolase family (Pereira et al. 2001)

^a The structure analysis of the seven predicated adhesin-like proteins was based on the SMART database. The position of each domain is enclosed in brackets.

Comparisons of adhesin genes in plant-associated and non-plant associated *Bacillus* strains.

To further investigate if these adhesins are related to the differentiation of plant-associated (PA) and non-plant associated (non-PA) *Bacillus* strains, 16 strains of *B. amyloliquefaciens* or *B. velezensis* (Zhang et al. 2016), which are closely related to SQR9 and of which 12 strains are PA and four strains are non-PA, were compared with SQR9 for these five adhesin genes. In addition, the whole-genome average nucleotide identity (ANI) values of SQR9 and these *Bacillus* strains were calculated to exclude the effect of phylogenetic relationship on the comparison of these adhesin genes.

As shown in Table 2, the *lytB* and *V529_10500* genes in SQR9 have low identities with homologous genes in non-PA *Bacillus* strains (67 and 84%, respectively) but high identity (>97%) with PA strains. Thus, *lytB* and *V529_10500* are conserved in PA *Bacillus*. Gene *yhaN* in SQR9 has 92% identity with the homologous gene in non-PA *Bacillus* strains, which is slightly lower than the ANI (93%), while it has high identity (98 to 99%) with its homologous gene in PA *Bacillus* strains, suggesting that *yhaN* might be conserved in PA *Bacillus* strains. Gene *fliD* in SQR9 has 92% identity with its homologous gene in non-PA *Bacillus* strains, which is slightly lower than the ANI (93%); but it also has low identity (94%) with the homologous

Fig. 2. Confocal laser scanning micrographs of the adhesion of green fluorescent protein-tagged *Bacillus velezensis* SQR9, mutants, and complementation strains on the roots. The root samples were taken 30 min after inoculation. Mock represents the cucumber roots without bacterial inoculation. The results are representative of three experiments. Bar = 50 μ m.

Fig. 3. Adherence ability to abiotic surfaces by the wild-type strain *Bacillus velezensis* SQR9, adhesin mutants, and complementation strains. The adherence ability was indicated by adhesion ratio, i.e. the number of bacteria attached to abiotic surfaces relative to inoculation amount. **A**, Adherence to polystyrene surfaces. **B**, Adherence to glass surfaces. Error bars are standard deviation, $n = 4$. Different letters mean significant differences ($P < 0.05$, one-way analysis of variance).

gene in PA *Bacillus* strains. Thus, *fliD* might not be conserved in PA *Bacillus* strains, the same case as for *sacB* gene.

DISCUSSION

Root colonization is a prerequisite for PGPRs to perform their plant beneficial functions; the initial attachment and adhesion are the early stages for root colonization and biofilm formation. In this study, we demonstrated that PGRP strain *B. velezensis* SQR9 has multiple adhesin proteins for root and abiotic surface attachment, which are required for its efficient rhizoplane colonization.

Gene *fliD* has great impacts on chemotaxis, adhesion, pellicle biofilm formation, and root colonization, but not the aggregation

ability. Mutation of the *fliD* gene seemed to significantly diminish the movement of *B. velezensis* SQR9, since $\Delta fliD$ lost the chemotaxis ability. Attachment of *B. velezensis* SQR9 $\Delta fliD$ mutant to cucumber root and abiotic surfaces were lower than the wild-type strain, which indicated that *fliD* was involved in the adhesion. This phenomenon can be partially explained by the effect of motility and chemotaxis on the bacterial attachment to both biotic and abiotic surfaces (Van de Broek et al. 1998). Also, in several bacterial species, the flagellar cap protein FliD has been shown to be required for adhesion to surfaces. The FliD protein of *Clostridium difficile* could bind specifically to mucus isolated from axenic mice (Tasteyre et al. 2001). Mutant $\Delta fliD$ of *Pseudomonas aeruginosa* cannot bind mucin, but complementation with *fliD* gene restored the binding phenotype, suggesting that FliD mediates the adhesion to mucin (Arora et al. 1998). In our study, the FliD protein of *B. velezensis* SQR9 is not only related to bacterial motility but also involved in adhesion. Gene complementation of $\Delta fliD$ did not restore attachment phenotype, which may be due to the complex assembly of flagella. Flagella are complex machines assembled from over 30 different proteins organized into three structural domains, the basal body, the hook, and the filament, and assembly of flagella is strictly time-controlled. Basal bodies are assembled rapidly, followed by the assembly of the hook, then FliD acts as a cap on the end of the extending structure, and finally, the long helical filament is formed through the basal body and assembled underneath the FliD cap (Guttenplan et al. 2013; Mukherjee et al. 2013). Thus, when expressed in situ, FliD and other flagellar proteins are assembled in order, but gene complementation of *fliD* might not be as timely as in situ expression, which leads to the difficulty to synthesize flagellum filament of the last step and $\Delta fliD/fliD$ cannot restore the attachment phenotype.

It is noticed that the $\Delta yhaN$ mutant complemented with *yhaN* gene greatly increased adhesion and colonization ability compared with the wild-type strain of *B. velezensis* SQR9. In SQR9, *yhaN* and its adjacent genes *yhaP* and *yhaQ* are predicted as ABC transporter genes. The role for ABC transporters in cell adhesion and biofilm development has been proposed in other organisms. An ABC transporter was important for attachment and virulence of *Agrobacterium tumefaciens* on carrot cells (Matthysse et al. 1996). In *Pseudomonad putida*, the *potB* gene, encoding an ABC transporter component, was upregulated in the early stages of biofilm formation on silicone (Sauer and Camper 2001). An ABC transporter of *Pseudomonas fluorescens* WCS365 encoded by *lapEBC* was predicted to participate in the

Fig. 4. Colonization of cucumber roots by the wild-type strain *Bacillus velezensis* SQR9, adhesin mutants, and complementation strains. Cells were diluted in one-fourth Murashige-Skoog medium and the root of a cucumber seedling was immersed into it. After coculturing at room temperature for 24 h, cells colonized on cucumber roots were determined by the plate-counting method. Error bars are standard deviation, *n* = 5. Different letters mean significant differences (*P* < 0.05, one-way analysis of variance). Results are shown as means of Lg (number of bacteria cells per gram of fresh root).

Table 2. Identity (%) between SQR9 and *Bacillus amyloliquefaciens* plant-associated or *B. subtilis* non-plant associated strains in adhesin genes or whole genomes

Gene ^a	Plant-associated strains ^b											
	NJN-6	LFB112	Y2	CAU B946	NAU-B3	YAU B9601-Y2	CC178	UCMB5033	UCMB5036	UCMB5113	FZB42	IT-45
<i>lytB</i>	98	98	98	98	98	98	98	98	98	98	98	98
<i>fliD</i>	94	94	94	94	95	94	99	94	96	94	99	94
<i>sacB</i>	98	98	99	98	99	99	99	99	99	99	99	98
<i>yhaN</i>	98	98	99	98	98	99	99	99	99	99	99	98
<i>10500</i>	97	97	98	97	98	98	98	98	98	98	98	97
SQR9	97	97	97	97	97	97	98	98	98	98	98	97
	Non-plant associated strains ^b											
	DSM7	LL3	TA208	XH7								
<i>lytB</i>	67	67	67	67								
<i>fliD</i>	92	92	92	92								
<i>sacB</i>	95	95	95	95								
<i>yhaN</i>	92	92	92	92								
<i>10500</i>	84	84	84	84								
SQR9	93	93	93	93								

^a The nucleotide identity (%) of adhesin genes was calculated using BLAST from the National Center for Biotechnology Information database.

^b The whole-genome average nucleotide identity (%) was calculated by FastANI from KBase.

secretion of LapA, a large cell-surface protein required for biofilm formation and attachment to quartz sand (Hinsa et al. 2003). These results suggest that the role of ABC transporters in biofilm development may be conserved across organisms, perhaps for the secretion of cell surface adhesins. In SQR9, we postulate that *yhaNPQ* encode an ABC transporter participating in the secretion of YhaL, a sporulation protein. Some sporulation proteins might be involved in biofilm formation, for example, *comER* gene of both *B. subtilis* and *B. cereus*; *comER* gene mutation resulted in defects in biofilm formation and a delay in spore formation in these two *Bacillus* species (Yan et al. 2016). Therefore, *yhaN* gene may contribute to adhesion and colonization in an indirect way as an ABC transporter.

LytB is known as a peptidoglycan hydrolase, the role of LytB in *Streptococcus pneumoniae* colonization of the nasopharynx was well-studied (Gosink et al. 2000; Moscoso et al. 2006). Our results showed that Δ *lytB* of *B. velezensis* SQR9 decreased the ability to attach and colonize cucumber root. As a peptidoglycan hydrolase, LytB plays a role in daughter cell separation (García et al. 1999), which might be related to root attachment and colonization.

SacB is a levansucrase, responsible for the synthesis of levan. Levan activity may contribute to cell aggregation and biofilm maturation. For example, levan acted as binding sites for *Streptococcus mutans* adhesion (Rozen et al. 2004). Levan also might act as an energy storage for *Pseudomonas syringae* due to the accumulation of levan in biofilm matrix, implying a function as a nutrient storage source for later stages of biofilm development (Laue et al. 2006). The levansucrase of *Gluconacetobacter diazotrophicus* is encoded by the *lsdA* gene, its deficiency decreased the ability to form cell aggregates on abiotic surfaces, and complementation of the mutant with the *lsdA* gene restored this ability (Velázquez-Hernández et al. 2011). Thus, SacB in *B. velezensis* SQR9 might contribute to adhesion and colonization ability by adhering to surfaces or forming biofilm structure.

The mutants of *B. velezensis* SQR9 with poor adhesion ability to root surfaces showed a deficiency in colonization on the rhizosphere. Besides, we were interested in the possible correlation between attachment or colonization of *B. velezensis* SQR9 to biotic and to abiotic surfaces and whether the mutants isolated in our screen showed a general deficiency in all surfaces or were just altered in functions specific for attaching to roots and rhizosphere colonization. Since the roles of adhesin genes involved in rhizosphere adhesion are just listed by hypothesis, the detailed analysis of the mechanism of isolated genes of *B. velezensis* SQR9 in adhesion needs future studies.

MATERIALS AND METHODS

Strain and growth condition.

Bacillus velezensis SQR9 (CGMCC accession number 5808 [China General Microbiology Culture Collection Center]) was cultivated at 37°C in LB medium (1% tryptone, 0.5% NaCl, and 0.3% yeast extract) solidified with 2% agar for routine growth (Cao et al. 2011).

Construction of mutants and complementation strains.

Mutants of *B. velezensis* SQR9 deficient in LytB, V529_10500, YcdA, V529_07150, FliD, YhaN, or SacB were constructed with marker-free strategy as described previously (Zhou et al. 2017). To complement the deleted genes in these mutants, the *amyE* gene was used as an insertion site, and 100 mg of spectinomycin per milliliter was used to select the gene complementation strains. Plasmid pNW33N containing the green fluorescent protein (*gfp*) gene (Zhou et al. 2018) was transformed to construct GFP-tagged strains. All these mutants and gene complementation strains are listed in Supplementary Table S1.

Plant growth.

Cucumber (*Cucumis sativus* L. variety Jinchun 4) was used in this study. The seeds of cucumber were surface-sterilized in 2% NaClO for 8 min and in 75% ethanol for 30 s and were then washed by distilled water three times. The seeds were germinated on a sterilized wet filter paper in a Petri dish and were grown at 30°C in the dark until the radicle reached 1.5 to 2.0 cm. Next, the seedlings were moved out, were washed, and were placed into 50-ml flasks, containing one-fourth Murashige-Skoog medium, for growth at 30°C under 16 h of illumination with regularly changing of solution.

Chemotaxis assays.

Chemotaxis was tested with a drop assay (Grimm and Harwood 1997). Bacterial cells in the logarithmic phase of growth were harvested and were resuspended in an equal volume of chemotaxis buffer. Aqueous solution of 1% hydroxypropyl methylcellulose was added to the cell suspension to restrict the spread of the chemoattractant. The viscous cell suspension was then layered on the bottoms of 60-mm-diameter Petri dishes, to a depth of about 3 mm, and 10 μ l of 100 mM malic acid was added to the center of a dish as an attractant. The chemotactic response of cells to the added compound resulted in the formation of a turbid ring near the center of the Petri dish after 10 min. Three replications of this experiment were conducted.

Adherence to cucumber roots.

The root attachment assay was described by Smit (1986) with modification. Bacterial cultures were centrifuged at 4,000 $\times$ g for 5 min, the pellets were washed three times with phosphate buffer (PBS) (pH 7.5) and were suspended to a final optical density at 600 nm (OD₆₀₀) of 0.002, which corresponds to 5 $\times$ 10⁵ bacterial cells per milliliter (Supplementary Fig. S9A), the number of cells was analyzed by the plate counting method. This bacterial suspension was used to immerse roots. Young cucumber lateral roots were chosen to be cut into segments approximately 3 cm long. These segments were immersed in 10 ml of bacterial suspension for 30 min at room temperature in static after mixing. Then, root segments were washed eight times in PBS to remove free and weakly attached bacteria. After sonication for 30 s and vortexing until the adhered bacteria were detached from roots, the number of attached bacterial cells was analyzed by the plate counting method. Five replications of this experiment were conducted.

Adherence to polystyrene or glass surfaces.

Bacterial cultures were centrifuged at 4,000 $\times$ g for 5 min. The pellet was washed three times with PBS and was suspended in the same solution. The cells were diluted to a final OD₆₀₀ of 0.0004 in PBS in a well of a 48-well polystyrene plate, corresponding to 10⁵ bacterial cells per milliliter (Supplementary Fig. S9B). The cells were diluted to a final OD₆₀₀ of 0.004 in PBS in a glass tube, corresponding to 10⁶ bacterial cells per milliliter (Supplementary Fig. S9C). The number of inoculation cells was analyzed by the plate counting method. The bacterial suspension was then injected into a plate or glass tube for 30 min at room temperature in static after mixing. Then, bacterial cultures in wells or tubes were discarded. Wells or tubes were gently washed five times in PBS to remove free and weakly attached bacteria. Attached cells were suspended in the sterile PBS. After sonication for 30 s and vortexing until the adhered bacteria were detached from wells or tubes, attached cells were counted by the plate counting method. Four replications of this experiment were conducted.

Adhesion observation by fluorescent microscopy.

Sterilized cucumber roots were prepared as mentioned above, the capillary and plastic filament were chosen as glass and plastic material, respectively. GFP-tagged strains were grown in LB

medium containing 5 mg of chloramphenicol per milliliter for 12 h. The bacterial cultures of different strains were harvested, were washed with PBS, and were suspended to the same concentration. Roots, capillaries, and plastic filaments were cut into segments approximately 3 cm long. These segments were immersed in 10 ml of bacterial suspension for 30 min at room temperature in static. Then, segments were washed in PBS to remove free and weakly attached bacteria and the segments were observed immediately by CLSM (Leica Model TCS SP2; Heidelberg, Germany). Excitation wavelengths of 488 nm and emitted wavelengths of 500 to 600 nm were collected for the GFP. Three replications of this experiment were conducted.

Aggregation and pellicle biofilm formation.

For aggregation ability analysis, strains were inoculated on solid MSgg medium (5 mM potassium phosphate, 100 mM morpholine propanesulfonic acid, 2 mM MgCl₂, 700 M CaCl₂, 50 M MnCl₂, 50 M FeCl₃, 1 M ZnCl₂, 2 mM thiamine, 0.5% glycerol, 0.5% glutamate, 50 g of tryptophan per milliliter, 50 g of phenylalanine per milliliter, and 50 g of threonine per milliliter) and were cultured at 37°C for 12 h, and aggregation architectures were recorded by the stereoscope. For colony formation, each strain was grown overnight in LB broth, and a 3-μl sample of the culture was spotted onto LB agar and was incubated at 37°C. To assess pellicle biofilm formation, 10 μl of suspension was inoculated into standing culture consisting of 1 ml of MSgg in a microtiter plate well, and the cultures were incubated at 37°C without shaking. Three replications of each experiment were included.

Colonization on the rhizosphere.

Bacterial suspension was inoculated into one-fourth Murashige-Skoog medium to make the final OD₆₀₀ value of 0.1, into which sterile cucumber seedlings with three true leaves were immersed. After slowly shaking, cells colonized on cucumber roots were determined by plate colony counting 24 h after inoculation at room temperature. In detail, roots were washed eight times in PBS to remove free and weakly attached bacteria. After vortexing for 5 min until colonized bacteria were detached from roots, 100 μl of the bacterial suspension was analyzed by the plate counting method and were incubated at 37°C for 24 h. Five replications of this experiment were included.

Colonization on polystyrene or glass surfaces.

For the colonization assays, bacterial cells were cultured in 3 ml of LB, containing an appropriate antibiotic, at 37°C with shaking. When OD₆₀₀ reached 1, the cells were diluted to 1:10 in one-fourth Murashige-Skoog medium in a 48-well polystyrene plate or glass tube and were incubated at 37°C. The colonization ability was determined after 24 h by the crystal violet staining method. In detail, pellicles in wells or tubes were gently removed with PBS before drying up, biofilms that adhered to wells or tubes were stained with 1 ml 0.1% crystal violet for 30 min and were washed three times with PBS. Next, 1 ml of 70% ethanol was added to dissolve the biofilm for 20 min at room temperature before measuring the OD₅₇₀ values. Three replications of this experiment were included.

Analysis of nucleotide sequence identity (%).

Sixteen strains of *B. amyloliquefaciens* or *B. velezensis* (Zhang et al. 2016) have been chosen, 12 PA strains and four non-PA. They were compared with SQR9 for these five adhesin genes and for the whole genome. Nucleotide BLAST from NCBI was used for comparisons of adhesin genes in PA and non-PA *Bacillus* strains. In detail, FASTA sequence of each SQR9 adhesin gene was input as query sequence and the accession number of each *B. amyloliquefaciens* or *B. subtilis* strain was input as subject sequence. The results have been optimized

for “somewhat similar sequences”. FastANI from KBase was used for ANI (%) of the whole genome of SQR9 and PA and non-PA *B. amyloliquefaciens* and *B. subtilis*, respectively, strains. FASTA sequence of the whole genome of each strain was uploaded to this website for FastANI results between SQR9 and other *B. amyloliquefaciens*/*B. subtilis* strains.

AUTHOR-RECOMMENDED INTERNET RESOURCE

KBase: <https://narrative.kbase.us/narratives>

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