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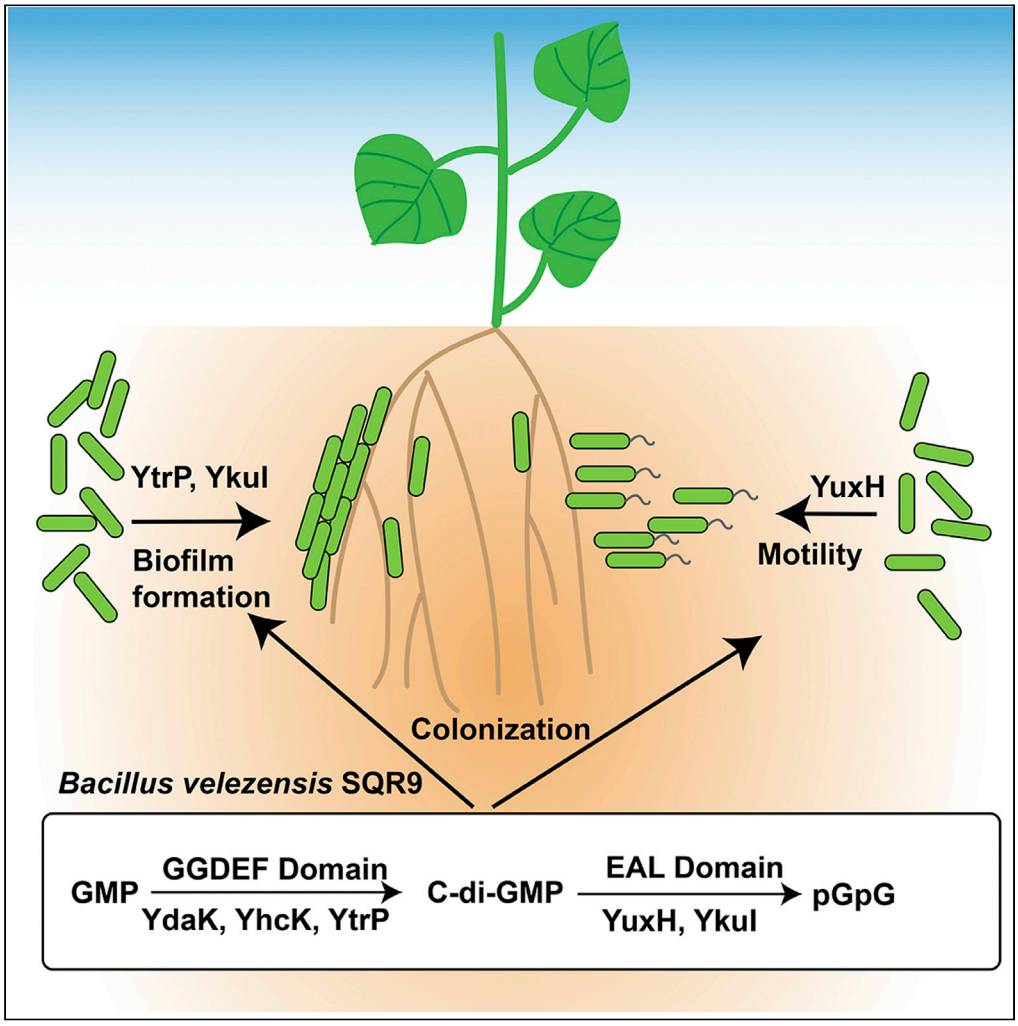

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Highlights

C-di-GMP is involved in root colonization of *B. velezensis*

YtrP and YkuI enhance the root colonization by regulating biofilm of *B velezensis*

YuxH enhances the root colonization by affecting the motility of *B. velezensis*

YtrP and YuxH are conserved in plant beneficial *Bacillus* group

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Identification of the core c-di-GMP turnover proteins responsible for root colonization of *Bacillus velezensis*

Xiaoyan Dong,[1,2] Chen Tu,[1] Yanan Liu,[1] Ruifu Zhang,[2,3] and Yunpeng Liu[2,4,*]

SUMMARY

Root colonization by beneficial rhizobacteria determines their plant beneficial effects. The messenger c-di-GMP is involved in the bacterial transition process between motility and biofilm, which are crucial to the colonization ability of the rhizobacteria. In this study, we identified three GGDEF domain-containing proteins (YdaK, YhcK, and YtrP) and two EAL domain-containing proteins (YuxH and YkuI) in beneficial rhizobacterium *Bacillus velezensis* SQR9. We found that deficiency of *ytrP* or *ykuI* in SQR9 led to impaired biofilm formation, while deficiency of *yuxH* led to weakened motility. Further investigation showed that YtrP, YuxH, and YkuI all contributed to the root colonization of SQR9 on cucumber root. Further bioinformatics analysis showed that YtrP and YuxH are conserved in plant beneficial *Bacillus* group, while they do not occur in animal pathogenic *Bacillus*. This research will be useful for enhancing the beneficial function of *Bacillus* spp. in agricultural application.

INTRODUCTION

A group of rhizobacteria has been proved to be growth-promoting and disease controlling; these beneficial rhizobacteria are widely used in microbial fertilizers for environment-friendly agricultural production (Adesemoye and Kloepper, 2009; Santos et al., 2019). Among the diverse beneficial rhizobacteria, Bacilli is one of the most important bio-agents due to its property of forming stress-tolerant spores (Aloo et al., 2019). Their beneficial functions largely depend on efficient colonization on root. The chemotactic motility toward root surface and the formation of biofilms on the root are the most important colonization processes (Feng et al., 2019; Liu et al., 2017).

3′-5′ cyclic diguanylate monophosphate (c-di-GMP), a bacterial signaling molecule, regulates several bacterial processes associated with colonization, such as motility and biofilm formation (Baker et al., 2019; Chen and Schaap, 2012; Hall and Lee, 2018; Tischler and Camilli, 2004). Matilla et al. found that overexpressing c-di-GMP producing gene in *Pseudomonas putida* KT2440 has wholly suppressed the motility and reduced the root colonization (Matilla et al., 2011). Excessive c-di-GMP also negatively regulates the root colonization of *Pseudomonas fluorescens* 2P24 (Liang et al., 2020). In summary, beneficial rhizobacteria change root colonization ability in response to variations in the concentration of c-di-GMP. Studies on the role of c-di-GMP mostly focused on Gram-negative bacteria, such as *Vibrio* spp., *Pseudomonas* spp., and *Escherichia coli*; the significance and physiological function of c-di-GMP remains largely unknown in Gram-positive bacteria, especially for the rhizosphere behavior (Baker et al., 2016; Kimbrough and McCarter, 2021; Steiner et al., 2012).

Bacillus possesses a multifunctional c-di-GMP signaling regulatory system that regulates motility and biofilm formation by binding to downstream receptors (Chen et al., 2012; Yang et al., 2018). Subramanian et al. demonstrated that a receptor contributes to inhibiting motility by high levels of c-di-GMP in *Bacillus subtilis* (Subramanian et al., 2017). In response to environmental signals, diguanylate cyclases (DGC), contain conserved GGDEF domain, synthesize c-di-GMP from 2 GTP molecules. Phosphodiesterases (PDE), contain conserved EAL or HD-GYP domain, hydrolyze c-di-GMP to inactive pGpG (Simm et al., 2004). Most bacteria contain numerous c-di-GMP turnover proteins, leading to complex c-di-GMP signaling systems (Römling et al., 2005). However, it is unclear whether genes for c-di-GMP production and degradation contribute to root colonization of plant beneficial *Bacillus*.

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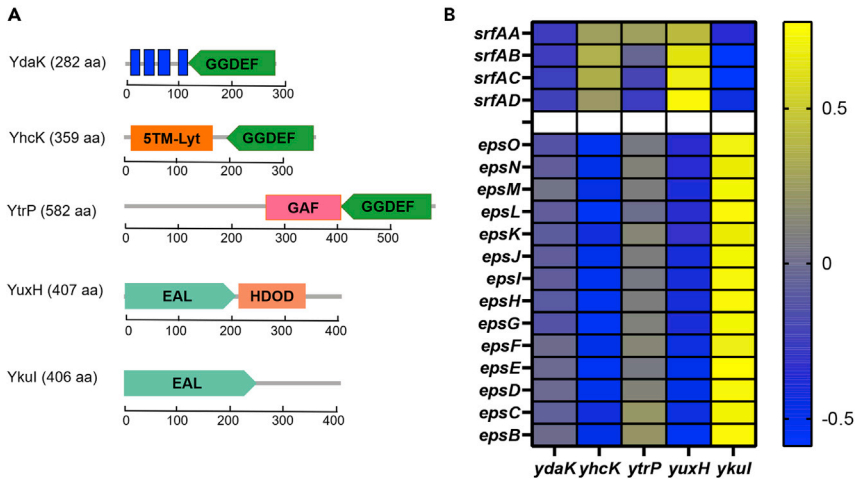

Figure 1. Five potential C-di-GMP turnover proteins in *B. velezensis* SQR9

(A) Map of the five C-di-GMP turnover proteins in *B. velezensis* SQR9 were analyzed by SMART.

(B) Transcriptional correlations analysis of five genes and extracellular polysaccharide and surfactin synthesis gene clusters. Colors indicate the R-values of the transcriptional correlations between each pair of genes: blue indicates high correlation, and yellow indicates low correlation.

In this context, we identified 5 proteins with GGDEF and EAL domain in *B. velezensis* SQR9, a well-studied plant beneficial rhizobacterium. Based on previously published RNA-seq data for SQR9 (Liu et al., 2021), we hypothesized that these 5 proteins may be involved in regulating biofilm formation and motility. We further revealed that three of them contribute to root colonization and explored their role in regulating motility and biofilm formation.

RESULTS

Identification of GGDEF and EAL motif of strain SQR9

According to the genome information of *B. velezensis* SQR9 (NCBI accession number: CP006890), the GGDEF, EAL, and HD-GYP domain-containing proteins were identified by matching the proteins to pfam database (https://pfam.xfam.org/). Briefly, amino acids sequence of each protein of SQR9 was blast against the database, and the significant hits to GGDEF, EAL, and HD-GYP domain were recorded. Totally, three GGDEF domain-containing proteins (YdaK, YhcK, and YtrP) and two EAL domain-containing proteins (YuxH and YkuI) were identified, while no HD-GYP domain-containing protein was identified in *B. velezensis* SQR9 genome. The possible domains of these five proteins were predicted by the database SMART. Based on the previously published RNA-seq data (Liu et al., 2021), we found that transcription of *ykuI* was highly correlated with *eps* operon, which responsible for producing exopolysaccharides as the major component of biofilm matrix (Murray et al., 2009), while transcription of *yuxH* was highly correlated with *srf* operon, which encode surfactin and essential for motility of *Bacillus* (Luo et al., 2015) (Figure 1B). It is possible that the function of *yuxH* and *ykuI* may relate to activating *srf* and *eps* operon, respectively. Therefore, we proposed that *yuxH* and *ykuI* may play a role in regulating chemotactic motility and biofilm formation, respectively. The five genes were then deleted from SQR9 genome to get knockout mutants (Δ*ydak,* Δ*yhcK,* Δ*ytrP,* Δ*yuxH,* and Δ*ykuI*).

YtrP and YkuI are involved in regulating biofilm formation

In our study, biofilms phenotypes at three interfaces including the agar–air interface, the liquid–air interface (pellicles), and the solid-liquid–air interface were evaluated. In LB and MSgg medium, we found that the wrinkles of biofilm formed by Δ*yuxH* at the agar–air interface are slightly weakened, and a wrinkle-less biofilm was formed by Δ*ytrP*. Deletion of *ykuI* also caused a severe defect in biofilm (Figures 2A and 2B). The biofilm formed by Δ*ydak* at the agar–air interface was weakened on MSgg medium, but not on LB medium (Figures 2A and 2B). The results presented in pellicle biofilm showed that disruption of *ytrP* and *ykuI* altered the biofilm surface microstructure, and disruption of *ykuI* resulted in the loss of surface wrinkles (Figures 2C and 2D). However, the mutant Δ*ykuI* still retained a strong ability to form adherent biofilm at solid-liquid–air (Figures 2E and 2F). The quantification of biofilm on solid-liquid–air by crystal violet staining assay showed that both deletion of *ytrP* and *ykuI* has significantly reduced biofilm formation (Figures 2G and 2H).

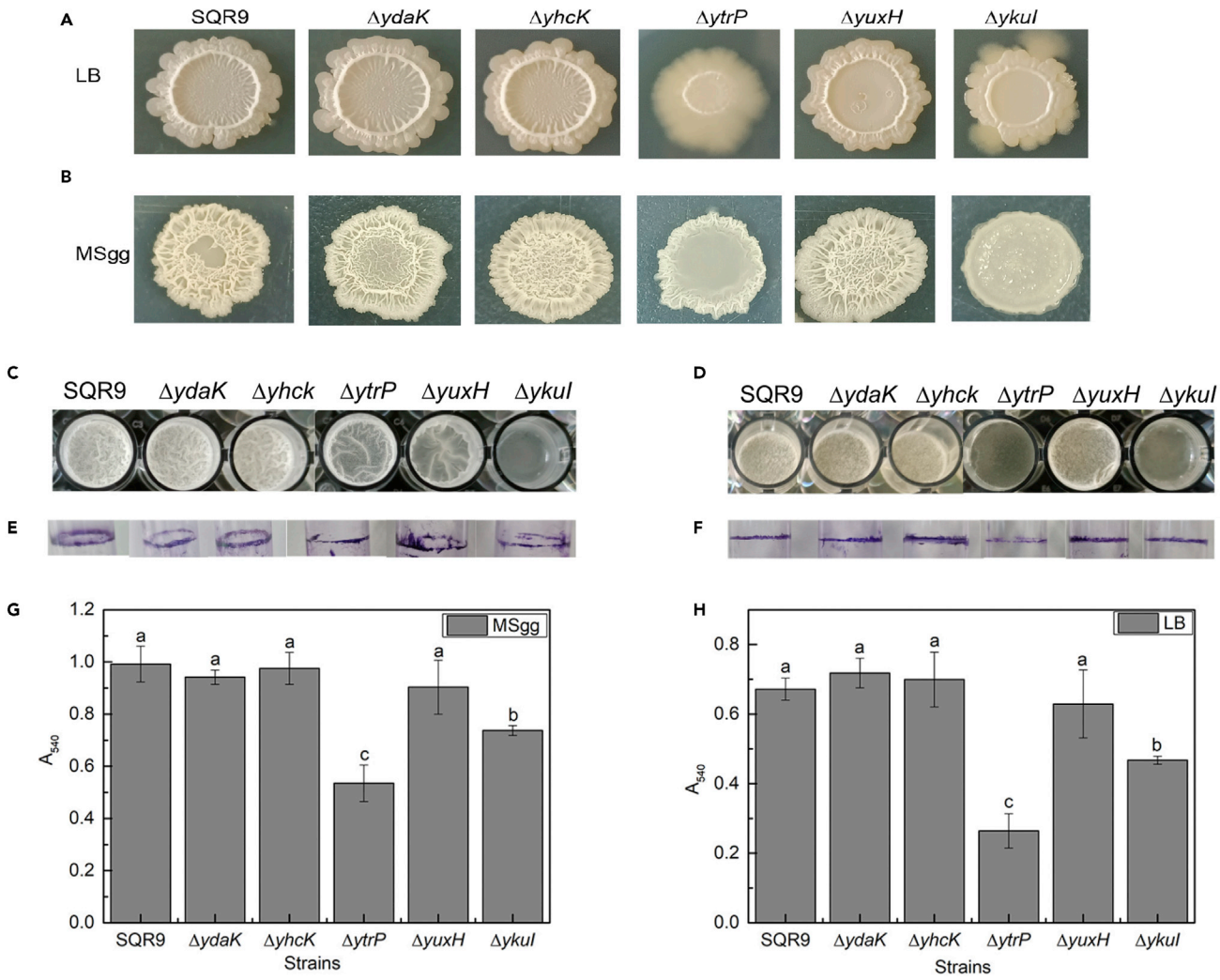

Figure 2. Biofilm formation of *B. velezensis* SQR9 and its derived mutant strains

SQR9, wild-type; Δ*ydaK*, *ydaK* mutant; Δ*yhcK*, *yhcK* mutant; Δ*ytrP*, *ytrP* mutant; Δ*yuxH*, *yuxH* mutant; Δ*ykuI*, *ykuI* mutant.

(A) The strains were incubated at the agar–air interface for 24 h in LB medium.

(B) The strains were incubated at the agar–air interface for 24 h in MSgg medium.

(C) The strains were incubated at the liquid–air interface (pellicles) for 20 h in 48-well polyvinylchloride microtiter plates containing MSgg medium.

(D) The strains were incubated at the liquid–air interface (pellicles) for 20 h in 48-well polyvinylchloride microtiter plates containing LB medium.

(E) The strains were incubated at solid-liquid–air for 20 h in glass tube containing MSgg medium.

(F) The strains were incubated at solid-liquid–air for 20 h in glass tube containing LB medium.

(G) Absorbance values of crystal violet staining of adhesive biofilm in MSgg medium.

(H) Absorbance values of crystal violet staining of adhesive biofilm in LB medium. Error bars indicated the SD based on three independently biological replicates (n = 3), different lowercase letters above the column represent significant differences ($p < 0.05$) based on one-way ANOVA.

Biofilm is a surface-associated multicellular community, in which bacteria are enveloped by polymeric substances known as the biofilm matrix. Biofilm matrix is composed of multiple components, including extracellular polysaccharides, extracellular proteins, and γ-polyglutamic acid (Cairns et al., 2014; Yu et al., 2016). The transcription level of genes involved in biofilm matrix was analyzed, and we found that the transcription of *epsD, tasA,* and *yuaB*, which are responsible for the synthesis of extracellular polysaccharides and extracellular proteins (Hobley et al., 2013; Ostrowski et al., 2011; Romero et al., 2010; Weiner et al., 1995), was significantly reduced in the Δ*ytrP* and Δ*ykuI* mutant (Figure 3). None of the mutantion has affected *ywtB*, which is responsible for producing γ-polyglutamic acid (Figure 3).

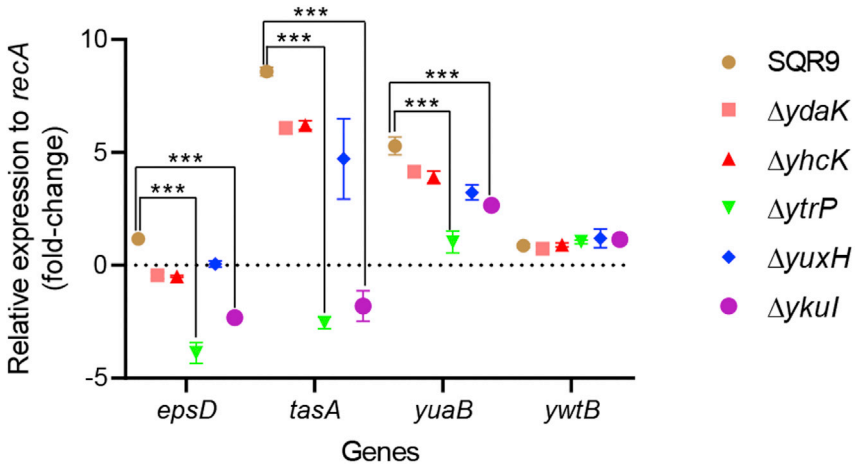

Figure 3. Transcriptional analysis of *epsD*, *tasA*, *yuaB*, and *ywtB* in *B. velezensis* SQR9 and its derived mutant strains by qRT-PCR

epsD is included in the extracellular polysaccharide synthesis gene operon *epsA-O*; The amyloid fibers are primarily composed of the TasA protein, which was encoded by the *tapA-sipW-tasA* operon; *yuaB* is responsible for the synthesis of biofilm-surface layer protein; *ywtB* is responsible for the synthesis of γ-polyglutamic acid. The housekeeping gene *recA* was used as an internal reference gene. Error bars represent the SD of three independently replicated experiments (n = 3) and asterisk (***) indicates significant differences ($p < 0.001$).

YuxH is involved in motility and chemotaxis

Swimming is the motility of a single cell in a liquid, while swarming is multicellular motility on a surface. Here, we found that YuxH protein positively regulates the swimming and swarming motility (Figures 4A and 4B), and deletion of *yuxH* reduced the motility ability of the strain by four times compared to wild-type strain SQR9 (Figures 4C and 4D). It was found that the mutant Δ*yuxH* almost lost its motor ability by microscopic observation (Video S1). In addition, deletion of *yhcK* caused a slight improvement in swarming. Malic acid and phenylalanine in root exudates are the most important chemoattractant of SQR9 (Feng et al., 2018; Zhang et al., 2014). Further experiments showed that Δ*yuxH* resulted in a significant decrease in chemotactic motility toward malic acid and phenylalanine. However, the swarming motility advantage of Δ*yhcK* did not translate into chemotaxis advantage (Figure S1). The results report above strongly suggests that YuxH plays a crucial role in the chemotaxis.

C-di-GMP regulated the rhizosphere colonization process of the strain SQR9

Biofilm formation and motility are both critical processes for root colonization by the beneficial rhizobacterium SQR9. Here, we evaluated the cells attached to the root surface and found that Δ*yuxH* has almost only half of the root attachment ability (Figure 5A), while other mutants showed the same adhesion ability to the root surfaces.

We further tested whether these genes that contribute to motility and biofilm formation (*ytrP*, *yuxH*, and *ykuI*) has influenced the root colonization. After co-culturing cucumber roots and the bacterial strains for 3 days, the number of cells colonized on the root was calculated. We found colonization by Δ*ytrP*, Δ*yuxH*, and Δ*ykuI* was significantly lower than wild-type strain, indicating that these three genes contribute to root colonization (Figure 5B). We demonstrated that YtrP, YuxH, and YkuI are the major C-di-GMP producing/degradation proteins that contribute to root colonization by *B. velezensis* SQR9.

We wondered whether these three c-di-GMP producing/degradation proteins widely occur in Bacilli. We collected the genome information from *Bacillus* strains and searched for the homologs of YtrP, YuxH, and YkuI with an identity cut-off of 0.3 (Table S3). Interestingly, the species related to *Bacillus cereus* (Gupta et al., 2020), a group of pathogens of animal, are defected with YtrP and YuxH (Figure 6). However, the species related to *B. subtilis*, which represent the most important plant beneficial *Bacillus*, possess all these three c-di-GMP producing/degradation proteins (Figure 6).

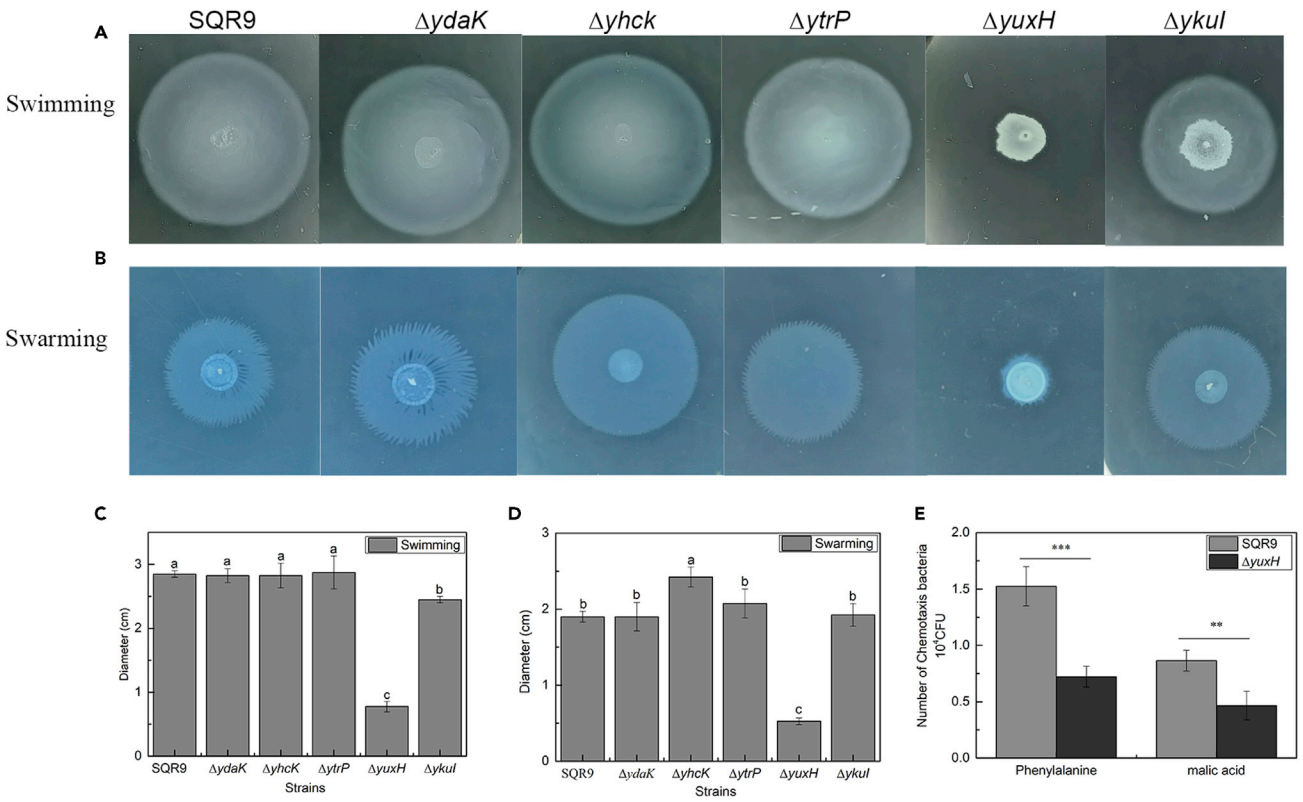

Figure 4. Motility and chemotaxis of *B. velezensis* SQR9

(A) Swimming motility of bacteria on 0.3% agar swimming medium.

(B) Swarming motility of bacteria on 0.6% agar swarming medium.

(C) The diameter of the motility zone of cells on swimming plates.

(D) The diameter of the motility zone of cells on swarming plates.

(E) Chemotaxis of SQR9 and Δ*yuxH* to malic acid and phenylalanine measured by capillary assay. Error bars represent the SD of four independently biological replicates (n = 4), different lowercase letters above the columns indicate significant differences ($p < 0.05$), asterisk (**) indicates significant differences ($p < 0.01$) and asterisk (***) indicates significant differences ($p < 0.001$).

DISCUSSION

Efficient colonization on the root is critical for *Bacillus* to benefit plant. Motility and biofilm formation are the important steps of root colonization. In this study, we found that the c-di-GMP turnover proteins YtrP containing GGDEF domain, YuxH and YkuI containing EAL domain play a predominant role in colonization of beneficial rhizobacterium *B. velezensis* SQR9. YtrP and YkuI contribute to the formation of biofilm, and YuxH contributes to chemotaxis and motility. Our findings suggest that c-di-GMP is required for its efficient rhizoplane colonization.

C-di-GMP is an important regulatory molecule that determines whether bacteria are in a motility state or a biofilm state. In soil, most bacteria survive in a surface-associated state, and when the environment changes, motility allows bacteria to disseminate and colonize a new environment (Purcell and Tamayo, 2016). *Bacillus* species are Gram-positive bacteria commonly found in the rhizosphere environment. Previous work suggests that the regulation of c-di-GMP on bacterial rhizosphere colonization has only been reported in several Gram-negative bacteria, including *Pseudomonas* and *Azorhizobium* (Liang et al., 2020; Sun et al., 2019, 2020). However, it is unclear whether c-di-GMP synthesis or degradation proteins are involved in the colonization of rhizosphere *Bacillus*. This study identified the protein YtrP for c-di-GMP synthesis and the protein YuxH and YkuI for c-di-GMP degradation responsible for root colonization of *B. velezensis* SQR9. Further bioinformatics analysis showed that YtrP and YuxH may have specificity for the root colonization by beneficial microbes. This research promoted the study of c-di-GMP in the rhizosphere environment.

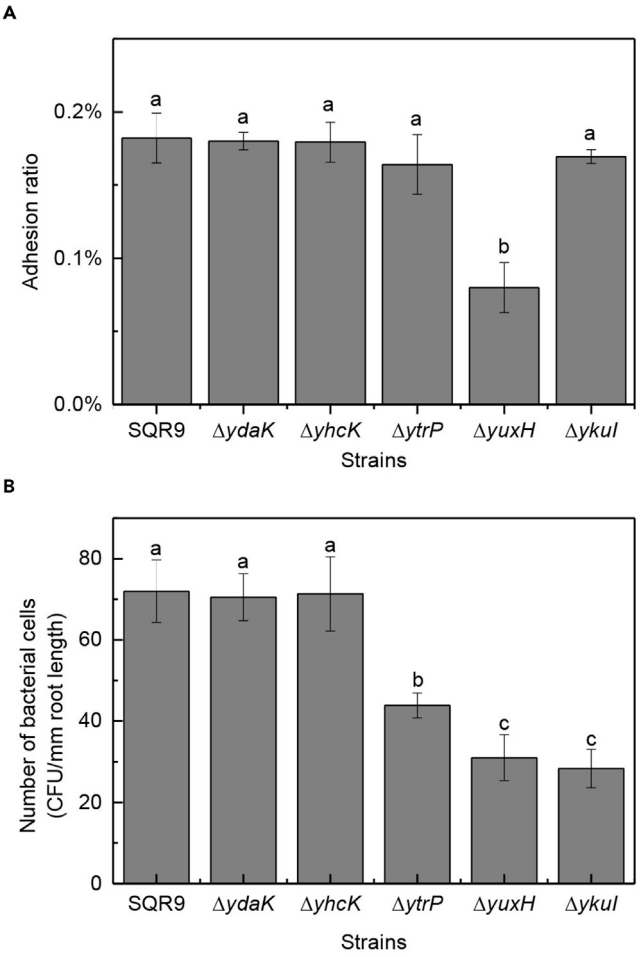

Figure 5. Adhesion and root colonization on cucumber of wild-type SQR9 and its derived mutant strains

(A) Ratio of the number of bacteria adhered to cucumber roots to the inoculated amount, Error bars indicate the SD based on five independently replicated experiments.

(B) Cucumber root surface colonization of the bacteria. Error bars indicate the SD based on six independently replicated experiments, different lowercase letters above the column indicate significant differences ($p < 0.05$) based on one-way ANOVA.

YtrP negatively regulates motility and positively controls biofilm formation in Gram-negative bacteria such as *Pseudomonas* sp. (Aragon et al., 2015; Nicastro et al., 2020). Deletion of this gene also inhibits the synthesis of the c-di-GMP in *Bacillus amyloliquefaciens* (Yang et al., 2018). However, its effects on the factors related to rhizosphere colonization still lack systematic research. In *B. amyloliquefaciens* PG12, it was reported that deletion of *ytrP* did not display defects in biofilm formation, but overexpression stimulated biofilm formation (Yang et al., 2018). In the present results, deletion of *ytrP* reduced biofilm formation. YtrP also is a conserved protein in plant-associated *Bacillus* (Figure 6). It has been demonstrated that the EAL domain of YkuI protein is inactive; it mainly regulates other processes than motility by combining with GMP (Bedrunka and Graumann, 2017; Minasov et al., 2009). But our research shows that deletion of *ykuI* caused a severe defect in biofilm formation. We suggest that because SQR9 contains fewer GGDEF domain- and EAL domain-containing proteins, so the effect of single gene mutation could not be covered by the redundancy effect. It was reported that YuxH contributed to decreasing c-di-GMP in cells and thus led to increasing motility in *Bacillus*, *E. coli*, and *Salmonella* (Amikam and Galperin, 2006; Chen et al., 2012; Paul et al., 2010; Povolotsky and Hengge, 2016; Pultz et al., 2012). Here, we report a similar observation that disruption of *yuxH* results in the loss of motility and altered the biofilm surface microstructure. We further showed that YuxH plays a crucial role in rhizosphere colonization.

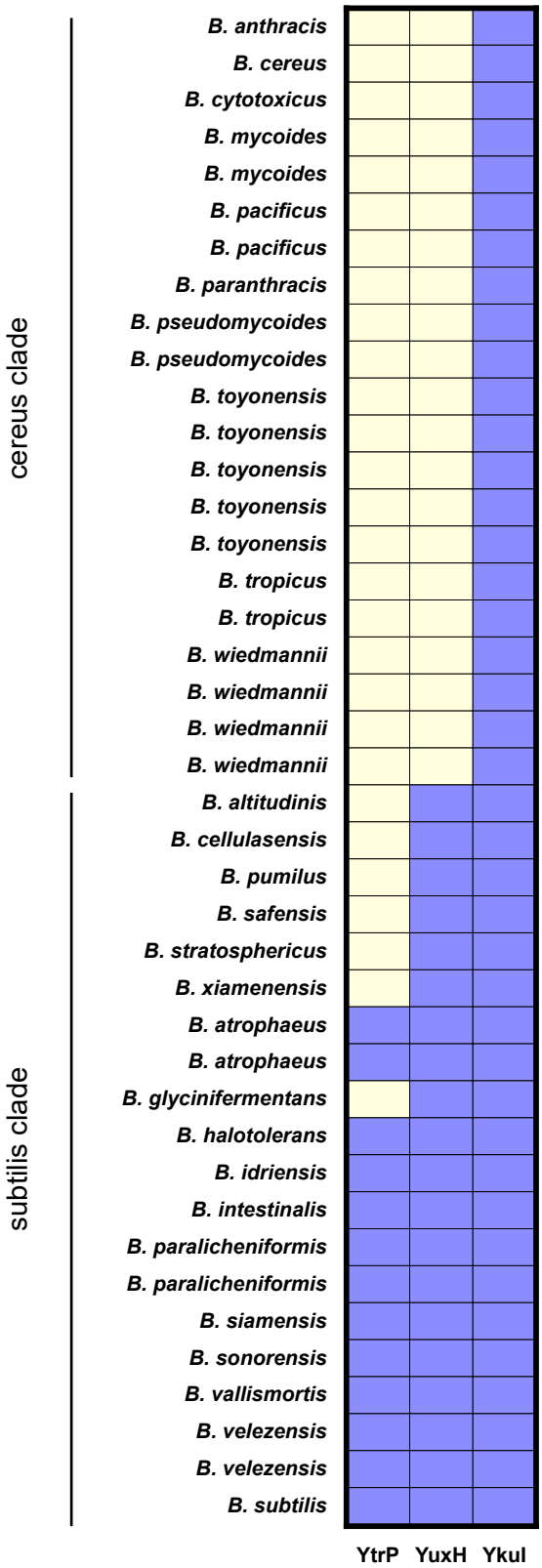

Figure 6. Distribution of YtrP, YuxH, and YkuI in Bacilli

The genome information from *Bacillus* strains was collected and searched for the homologs of YtrP, YuxH, and YkuI with an identity cut-off of 0.3.

Limitations of the study

In this study, we demonstrated the role of several c-di-GMP turnover proteins in regulating biofilm formation and motility of *B. velezensis*, but it is still to be clarified how these proteins regulate different bacterial behaviors. The function of the different domains of these proteins in signaling transduction was not investigated.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105294.

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AUTHOR CONTRIBUTIONS

X.D. performed experiments, analyzed data, and wrote the manuscript. C.T. conceived and designed research. Y.L. executed part of biofilm formation experiments. R.Z. designed research and modified the manuscript. Y.L. designed research, analyzed data, and wrote the manuscript. All authors read and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
B. velezensis SQR9	Laboratory stock	N/A
B. velezensis SQR9 Δ*ydaK*	This study	N/A
B. velezensis SQR9 Δ*yhcK*	This study	N/A
B. velezensis SQR9 Δ*ytrP*	This study	N/A
B. velezensis SQR9 Δ*yuxH*	This study	N/A
B. velezensis SQR9 Δ*ykuI*	This study	N/A
Chemicals, peptides, and recombinant proteins		
Zeocin	Invitrogen	Cat#BC64828
Chloramphenicol	Sigma-Aldrich	Cat#C0378
DL-chloro-phenylalanine	Sigma-Aldrich	Cat#C6506
DNA polymerase	Vazyme	Cat#P525
Reverse Transcriptase	Vazyme	Cat#R333
Critical commercial assays		
ChamQ Universal SYBR qPCR Master Mix	Vazyme	Cat#Q711
Bacteria DNA Isolation Kit	Vazyme	Cat#DC103
Gel DNA Extraction Kit	Vazyme	Cat#DC301
RNA Isolation Kit	Vazyme	Cat#RC101
Deposited data		
B. velezensis strain SQR9 genome sequence	Zhang et al., 2015	NCBI Accession number:CP006890
Recombinant DNA		
Plasmid: pNW33N	Laboratory stock	N/A
Plasmid: p7S6	Laboratory stock	N/A
Software and algorithms		
GraphPad Prism 7.0	GraphPad Software, La Jolla, CA	https://www.graphpad.com/scientific-software/prism/
SnapGene 5.1	Insightful Science	https://www.snapgene.com/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Yunpeng Liu (liuyunpeng@caas.cn).

Materials availability

Bacterial strains and plasmids generated in this study are available from the lead contact upon request.

Data and code availability

The *B. velezensis* SQR9 genome have been deposited in the NCBI database (Accession number CP006890). RNA-seq data could be found in refered paper (Liu et al., 2021). This paper dose not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

B. velezensis SQR9 was isolated from the rhizosphere of a cucumber plant, and the entire genome has been sequenced (NCBI accession number CP006890). *B. velezensis* SQR9 was cultivated in the medium of

autoclaved LB nutrient media (10 g tryptone, 5 g yeast extract and 5 g NaCl per liter) at the pH of 7.0. For biofilm formation assay, *B. velezensis* cells were grown in solid medium surface, 48-well polyvinylchloride microtiter plates and glass tube at 37°C. Motility of bacteria was tested by swimming and swarming assay. A combination tip and syringe device was used to analyze the chemotaxis of the strains. For root colonization assay, the cucumber was incubated in a climatic chamber at 12 h light (28°C)/12 h dark (22°C) and 70% relative humidity.

METHOD DETAILS

Microbial strains and related culture conditions

The strains and plasmids used in our study are described in Table S1. *B. velezensis* SQR9 (NCBI accession NO. CP006890) was incubated in Lysogeny broth (LB) medium. The biofilm formation of strain SQR9 and its derived mutant strains were measured in LB medium and MSgg medium (Dong et al., 2018; Xu et al., 2014). The motility of strains was measured with swimming medium (1 g tryptone, 0.25 g NaCl, 0.3 g agar per 100 mL) and swarming medium (1 g tryptone, 0.5 g yeast extract, 0.5 g glucose, 0. 5 g NaCl, 0.6 g agar per 100 mL) (Hall et al., 2018).

Bacillus genomic analysis

Previously published RNA-seq data (Liu et al., 2021) was used for analyzing the correlation of the genes in SQR9. The FPKM values were then normalized by the sum and transformed to relative log expression (RLE) values. The Pearson correlation of the transcription between each gene pair was analyzed by R (v3.6).

For genomic analysis of the distribution of *ykuI*, *ytrP* and *yuxH*, 41 genomes of *Bacillus* species (NCBI accession number: GCA_000022865.1, GCA_000011625.1, GCA_002250885.2, GCA_005217685.1, GCA_005217805.1, GCA_009884315.1, GCA_006349595.1, GCA_009884335.1, GCA_002579385.1, GCA_002568975.1, GCA_002589595.1, GCA_009799785.1, GCA_001275045.2, GCA_002557005.1, GCA_002571765.1, GCA_006457285.1, GCA_006349625.1, GCA_005217915.1, GCA_002552205.1, GCA_005217695.1, GCA_002557675.1, GCA_004563755.2, GCA_001908475.1, GCA_004006455.1, GCA_008244765.1, GCA_004569515.1, GCA_000300535.1, GCA_000264395.1, GCA_002243495.1, GCA_002443095.1, GCA_002993765.1, GCA_009674765.1, GCA_002027305.1_, GCA_009497935.1, GCA_002068155.1, GCA_002754795.1, GCA_003473165.1, GCA_002113805.1, GCA_000769555.1, GCA_000583065.1, GCA_000789275.1) were downloaded from the NCBI database (https://www.ncbi.nlm.nih.gov/), and the Table S3 contains the detailed information of all *Bacillus* species. We collected the genome information from these strains and searched for the homologues of YtrP, YuxH and YkuI by Blast, with an identity cut-off of 0.3.

Gene knockouts

According to the whole genome sequence of *B. velezensis* SQR9, the potential DGC and PDE were predicated based on the conserved GGDEF and EAL or HD-GYP domain. The gene *ydaK* (V529_04130), *yhcK* (V529_08740) and *ytrP* (V529_29480) were individually deleted using an unmarked genetic manipulation that depend on a counter-selectable *pheS* marker (Zhou et al., 2017). 800 bp fragments upstream, 400 bp downstream of the target gene and 800 bp target gene were amplified from SQR9 genome, and the 2.3 kb *pheS* marker and spectinomycin (spc) resistance gene fused fragment was amplified from p7S6 plasmid. The upstream, downstream, *phes*-spc, and target gene fragments were overlapped. All PCR products were gel purified using an AxyPrep DNA gel-purification and extraction kit (Axygen, Hangzhou, China). The transformation of DNA into SQR9 was performed following the method described by Li (Li et al., 2018). The overnight cultured SQR9 was inoculated to fresh LB medium. The bacteria was grown until OD_{600} reached 0.5. After them, xylose was added to the medium with a final concentration of 0.5% (W/V). The bacteria was then cultured with shake at 37°C for 1 h to obtain competent cells. Subsequently, 10 μL of DNA fragments was added to 200 μL of competent cells and cultured at 37°C 110 rpm for 8 h. The transformants were selected on LB agar plates containing 100 μg mL^{-1} spectinomycin. Transformants were validated by PCR. In the second homologous recombination, the correct transformants were cultured in LB liquid medium. The mutants were selected on LB agar plates containing 10 mM DL-chloro-phenylalanine. *yuxH* (V529_31410) and *ykuI* (V529_13420) were disrupted using a chloramphenicol resistance gene. 1 kb fragments upstream and downstream of the target gene were amplified from SQR9 genome, and the 1.1 kb chloramphenicol (Cm) resistance gene was amplified from the plasmids pNW33n, respectively. The upstream, Cm, and downstream fragments were overlapped. The fused fragments were individually

transformed into SQR9 competent cells, and the transformants were selected on LB agar plates containing 5 μg mL^{-1} chloramphenicol. The mutants were verified by PCR with the primers listed Table S2.

Biofilm formation assay

The biofilm at the agar–air interface was carried out in LB and MSgg agar medium. The strains were cultured in LB and MSgg medium to an OD_{600} of 0.8, and then 5 μL of bacterial suspension was added to the surface of the corresponding solid medium. The plates were cultured at 37°C for 24 h. The biofilm formation at the liquid–air interface (pellicles) was carried out in 48-well polyvinylchloride microtiter plates (Hamon and Lazazzera, 2002). *B. velezensis* SQR9 and its mutants were cultured in LB medium and MSgg medium until the OD_{600} reached 0.8, and then the strains were transferred to the 48-well cell culture plate containing the LB medium or MSgg medium according to 1% of the inoculation amount. The biofilm was grown at 37°C for 20 h. Adherent biofilm at solid-liquid–air was measured in a glass tube in accordance with the above operation method. Medium was removed from the glass tube, the adherent biofilms were stained in 1% crystal violet for 20 min. Subsequently, the unbound crystal violet was eluted with distilled water. Crystal violet combined with biofilm was solubilized with 1:4 acetone acid and ethanol(v/v), and adherent biofilm was quantified by the value of A_{570} (Li et al., 2018).

Gene transcription analysis

B. velezensis strains were cultured in MSgg liquid medium for 16 h, the cells were centrifuged at 5500 rpm for 5 min, and then total RNA was obtained using a Vazyme kit. Transcription of genes encoding extracellular matrix was detected by RT-qPCR using a SYBR Green kit (Vazyme) with the primers described in Table S2. The transcription of the housekeeping gene *recA* was used as an internal control. Housekeeping genes *rpoD* and *gyrA* were used additional to gene *recA*. All *recA*, *rpoD* and *gyrA* are not differentially expressed in tested strains, indicating stable transcription of *recA* which can be used as internal control (Figures S2 and S3). The real-time data were analyzed by the $2^{-\Delta CT}$ method. The CT value represents the number of PCR cycles when the fluorescence signal reaches the threshold, and the $2^{-\Delta CT}$ value represents the ratio of the relative expression quantity of the target gene to the housekeeping gene *recA*.

Swimming and swarming of strain

Swimming assay was performed to measure the single cell motility. Briefly, 2.5 μL of bacterial culture with OD_{600} of 0.8 were inoculated to the center of a Petri dish containing swimming medium (1 g tryptone, 0.25 g NaCl, 0.3 g agar per 100 mL). The Petri dishes were then incubated at 37°C for 6 h. Swarming assay was performed to measure the multicellular motility of bacteria. Briefly, 2.5 μL of bacterial culture with OD_{600} of 0.8 were inoculated to the center of a Petri dish containing the swarming medium (1 g tryptone, 0.5 g yeast extract, 0.5 g glucose, 0. 5 g NaCl, 0.6 g agar per 100 mL). The swarming plates were incubated for 4 h at 37°C. The diameter of the motility zone was measured.

Chemotaxis assays

Chemotaxis experiment was performed following the modified capillary assay (Liu et al., 2014). Overnight cultures of *B. velezensis* strains were transferred into 100 mL liquid LB medium at 1% inoculum and incubated at 37°C and 170 rpm until OD_{600} value of 0.8. Bacterial cells were harvested at 5,500 rpm, washed in chemotaxis buffer for 3 times, and then resuspended in an equal volume of chemotaxis buffer. Then, 100 μL of bacterial suspension and chemoattractant (malic acid and phenylalanine) were loaded to 200 μL-tip and 1 mL syringe, respectively. The tip and syringe were connected together head-to-head to allow the chemotactic swimming to the chemoattractant for 30 min. The cells in the syringe were measured by plate counting.

Adherence to cucumber roots

The experiment was performed following the adherence assay described by Huang et al. (2021). Cells with OD_{600} value of 0.8 were centrifuged at 5500 rpm for 3 min. The pellets were washed with sterile PBS buffer three times and suspended to a 1.5×10^5 CFU/mL bacterial cells concentration. Several cucumber lateral roots with a length of 3 cm were cut out. These lateral roots were dipped into 2 mL of bacterial suspension with a cell concentration of 1.5×10^5 CFU/mL for 30 min. Then, these lateral roots were washed with PBS buffer 8 times to remove bacteria that failed to adhere. The washed lateral roots were immersed in sterile PBS buffer. After sonication for 30 s and vortex for 5 min, the number of attached cells on roots was measured by plate counting.

Root colonization of *B. velezensis* SQR9 strains

Root colonization was conducted according to previously reported methods (Liu et al., 2017; Tian et al., 2021). Briefly, the cucumber seeds were surface sterilized with 75% ethanol for 2 min, washed with sterile water 3 times, and then soaked in sodium hypochlorite solution (2% active chlorine) for 5 min, and washed with sterile water 3 times. The surface sanitized cucumber seeds were deposited onto the Petri dish, which was covered by a piece of sterile moist filter paper, and germinated in a 30°C incubator. The germinated seeds were transplanted to plastic pots filled with sterilized vermiculites and incubated until 3-leaf-stage in a climatic chamber at 12 h light (28°C)/12 h dark (22°C) and 70% relative humidity. Overnight cultured *B. velezensis* SQR9 or the mutants were transferred into 100 mL liquid LB medium and incubated at 37°C, 170 rpm until an OD_{600} value reached 1.0. Cells were collected and washed in 1/4 MS medium 3 times and inoculated to cucumber rhizosphere with a final concentration of 10^4cells/g vermiculites. After another 7 days of incubation, roots of cucumber were collected and washed with sterilized water, and the cells colonized on the root were collected by shaking and measured by plate counting.

QUANTIFICATION AND STATISTICAL ANALYSIS

Results are expressed as Mean ± SD (standard deviations). Data were analyzed using one-way ANOVA as specified in the Figure legends. Statistical significance of differences between two experimental groups were carried out using t test in GraphPad Prism 7.0 software and a two-tailed (ns, $p > 0.05$; **, $p < 0.01$;***, $p < 0.001$) was taken to indicate statistical significance.