An Extract Produced by Bacillus sp. BR3 Influences the Function of the GacS/GacA Two-Component System in Pseudomonas syringae pv. tomato DC3000

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The GacS/GacA two-component system is essential for virulence in many plant pathogenic bacteria, and thus represents a promising anti-virulence target. In the present study, we isolated and screened rhizobacteria that were capable of inhibiting the expression of the gacS gene in the phytopathogenic bacterium Pseudomonas syringae pv. tomato (Pto) DC3000. One candidate inhibitor bacterium, BR3 was obtained and identified as a Bacillus sp. strain based on 16s rRNA gene sequence analysis. Besides the gacS gene, the GacA-dependent small RNA genes rsmZ and rsmY were repressed transcriptionally when DC3000 was treated with an extract from strain BR3. Importantly, the extract also influenced bacterial motility, the expression of type three secretion system effector AvrPto, and the plant hypersensitive response triggered by strain DC3000. The results suggested that the extract from strain BR3 might offer an alternative method to control bacterial diseases in plants by targeting the GacS/GacA system.

Keywords: the GacS/GacA two-component system, small non-coding RNA, Bacillus sp., Pseudomonas syringae pv. tomato, type three secretion system

INTRODUCTION

To thrive and survive in complex environmental conditions, bacteria employ versatile signal transduction pathways to rapidly adapt to alterations in their surroundings. One of these pathways is the GacS/GacA two-component system, which detects and responds coordinately to external and internal stimuli, including different physiological state (Kay et al., 2005), metabolic levels (Chavez et al., 2010), and pH (Mondragón et al., 2006), and translates them into appropriate adaptive responses.

The GacS/GacA system is a conserved global regulatory system in many Gram-negative bacteria, which is comprised of the hybrid sensor kinase GacS and the cognate response regulator GacA. In the presence of an as-yet-unidentified environmental signal, the GacS sensor kinase autophosphorylates and then activates the cognate GacA response regulator via phosphotransfer
(Haas and Défago, 2005). In *Pseudomonas aeruginosa* and *Legionella pneumophila*, the activated GacA exclusively regulates the expression of several small non-coding RNAs (sRNAs) RsmY and RsmZ (Brensic et al., 2009; Sahr et al., 2009). Mutation of all these sRNAs in many bacteria resulted in the same phenotypes as mutants of the GacS/GacA system (Kay et al., 2005; Brensic et al., 2009). GacA-activated sRNAs all have multiple GGA motifs for competitive binding to carbon storage regulator (CsrA)/regulator of secondary metabolites (RsmA) family proteins (Valverde et al., 2004). In addition, the CsrA/RsmA family proteins mediate either a negative or positive posttranscriptional effect by altering the rate of translation initiation or mRNA decay (Lapouge et al., 2008). A common consensus sequence (CANGGAYG) within the loop portion of a stem–loop structure in the 5′-untranslated region (UTR) is essential for RsmA/CsrA family proteins to bind target mRNAs (Kulkarni et al., 2014). Two histidine kinases, LadS and RetS, are involved in modulating the function of the GacS/GacA system. In *P. aeruginosa* PAK, RetS affects the phosphorylation state of GacS (Goodman et al., 2009). Crystallographic studies further indicated that RetS used the reversible unfolding of a helix, or helix cracking, to control interactions with GacS (Mancl et al., 2019). In contrast to RetS, LadS in *P. aeruginosa* activates the function of GacA under high calcium conditions (Broder et al., 2016).

Extensive studies have demonstrated that the GacS/GacA system and its homologs play an important role in coordinating the expression of virulence factors required for successful infection of many plant- and animal-pathogenic bacteria (Heeb and Haas, 2001). In *P. syringae* pv. *tomato* (Pto) DC3000 (hereafter termed Pto DC3000), GacA acts as master regulator to control carbon metabolism, motility, and production of virulence factors, syringomycin, and quorum-sensing (QS) signals (Chatterjee et al., 2003). Furthermore, GacA positively regulates the transcription of the *pel*, *peh*, and *celV* genes that are responsible for the production of pectate lyases, pectinases, and cellulases in *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc) Z3-3, *Pseudomonas fluorescens* 2P24, and *Agrobacterium tumefaciens* NTL4 (pZLR4) were cultured in LB medium, King's Broth (KB) (King et al., 1954), or minimal medium ABM (Chilton et al., 1974) at 28°C. Type three secretion system (TTSS)-inducing minimal medium was used for immunoblotting analysis of AvrPto protein (Huynh et al., 1989). When necessary, growth media were supplemented with ampicillin (Ap) (50 µg ml⁻¹), kanamycin (Kn) (50 µg ml⁻¹), gentamycin (Gm) (5 µg ml⁻¹), tetracycline (Tet) (20 µg ml⁻¹), or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) (40 µg ml⁻¹).

**DNA Manipulation and PCR Amplification of 16S rRNA Gene**

Chromosomal DNA from strains Pto DC3000 and *Bacillus* sp. BR3, plasmid DNA extraction, and other molecular assays were performed according to standard procedures (Sambrook et al., 1989). Electroporation of Pto DC3000 and *P. fluorescens* cells with plasmid DNA was performed as described previously (Choi et al., 2006). The oligonucleotides used are listed in Table 1.

To ascertain its taxonomic position, the genomic DNA of strain BR3 was used as a template for PCR amplification using primers 63F and 1387R (Marchesi et al., 1998). The PCR product was sequenced by the Sanger method at Sangon Biotech Co., Ltd., Shanghai, China. The obtained sequences were compared with sequences deposited in GenBank using a BLASTN algorithm (Altschul et al., 1997).
TABLE 1 | Bacterial strains, plasmids, and primers used in this study.

| Strains or plasmids | Relevant characteristics | References or source |
|---------------------|--------------------------|---------------------|
| **Strains**         |                          |                     |
| *Pseudomonas syringae* |                          |                     |
| DC3000              | Wild-type                | Chatterjee et al., 2003 |
| DC3000gacA          | GacAcc3000-derivative of DC3000, Km<sup>r</sup> | Chatterjee et al., 2003 |
| DC3000ΔrncQ-U       | DC3000 ΔrncQ-U::Sp<sup>r</sup>/Sm<sup>r</sup> | Kvitko et al., 2007 |
| DC3000-GacSVSV      | DC3000 with a VSV-G epitope sequence tagged to the C terminus of GacS | This work |
| *Pseudomonas fluorescens* |                          |                     |
| 2P24                | Wild-type                | Yan et al., 2004    |
| 2P24ΔgacA           | Derivative of 2P24, gacA gene in-frame deletion, Ap<sup>r</sup> | Yan et al., 2004 |
| 2P24ΔretS           | Derivative of 2P24, retS gene in-frame deletion, Ap<sup>r</sup> | Liu et al., 2013 |
| *Pectobacterium carotovorum subsp. carotovorum* | Wild-type | Laboratory |
| Z3-3                | A. tumefaciens NT1 derivative carrying a traG–lacZ reporter fusion, AHB biosensor | Chilton et al., 1974 |
| **Plasmids**        |                          |                     |
| p970Gm-rsmYDC3000p  | Derivative of DC3000, rsmY-lacZ transcriptional fusion, Gm<sup>r</sup> | This work |
| p970Gm-rsmZDC3000p  | Derivative of DC3000, rsmZ-lacZ transcriptional fusion, Gm<sup>r</sup> | This work |
| p970Gm-gacSDC3000p  | Derivative of DC3000, gacS-lacZ transcriptional fusion, Gm<sup>r</sup> | This work |
| p970Gm-rsmYp        | Derivative of 2P24, rsmY-lacZ transcriptional fusion, Gm<sup>r</sup> | Wu et al., 2012 |
| pME6013-2P24proC    | Derivative of 2P24, proC-lacZ translational fusion, Tet<sup>r</sup> | This work |
| pME6013-phlA        | Derivative of 2P24, phlA-lacZ translational fusion, Tet<sup>r</sup> | Zhang et al., 2018 |
| p2P24Km             | Suicide plasmid with sacB used for homologous recombination, Km<sup>r</sup> | Zhang et al., 2018 |
| p2P24Km-gacSVSV     | p2P24Km with a VSV-G epitope sequence tagged to the C terminus of GacS, Km<sup>r</sup> | This work |
| pME6032             | lacI–P<sub>tac</sub> expression vector, Tet<sup>r</sup> | Heeb et al., 2002 |
| pME-gacS            | pME6032 with gacS under P<sub>tac</sub> control | This work |
| **Primers**         |                          | Restriction site     |
| rsm2pIDC3000        | ATGGATCCAGTAAAACCTCCACCG | BamHI |
| rsm2pIDC3000        | ATGGATCCGCTCGATGATAGGAAGAT | BamHI |
| RsmYBglII5p         | ATGGACATTCTTTAGCGAAACGTG | BglII |
| RsmYBglII3p         | ATGGATCCTTACCCCCCGCGCTCGGCG | BglII |
| gacS-promoterF      | GGCGATCGCTCGTTTTAGTAGTGTC | BamHI |
| gacS-promoterR      | ATGGATCCAGTAAAACCTCCACCG | BamHI |
| gacS-promoterR      | ATGGAGTACCGACAGCGTAAGGCA | BamHI |
| gacS-vsv2R          | TTTTCCTATCTTATCATATCATCTGATACACACGTCTGCTCTGCACTCCAG | BamHI |
| gacS-vsv3F          | TATACGATATTTGTAATGGTAGTTGAAACTGTTACAGAAACTGGAATGCAAG | BamHI |
| gacS-vsv4R          | ATGGATCCGCGACCCACCGGTCGACCG | BamHI |
| gacS-vsv4R          | ATGGATCCGCGACCCACCGGTCGACCG | BamHI |
| Proc-F-6013         | ATGGGCTGTAGCAGACGCGCGTGACCGACGCG | EcoRI |
| Proc-R-6013         | ATGGGCTGTAGCAGACGCGCGTGACCGACGCG | EcoRI |
| gacS-Xhol           | ATACTCGAAGCTACGCATCTGCTTACGATGCAGTCCG | XhoI |
| gacS-EcoRI          | ATCGGCGGTGATGACGCGCGTGACCGACGCG | EcoRI |
| 63F                 | CAGGCGTACACATCGAAGGC |                     |
| 1387R               | GGCGGCGTGATGACGCGCGTGACCGACGCG |                     |

Ap, ampicillin; Gm, gentamicin; Km, kanamycin; Tet, tetracycline.

**Construction of the Transcriptional lacZ Fusions and the Overexpression Plasmid**

To construct the reporter strain *Pto* DC3000 (p970Gm-gacSDC3000p), the gacS promoter region from *Pto* DC3000 was PCR amplified using the primers gacS-promoterF/gacS-promoterR. The amplicon was then inserted into the BamHI site of plasmid pRG970Gm to create the gacS-lacZ transcriptional fusion plasmid p970Gm-gacSDC3000p, which was introduced into *Pto* DC3000 via electroporation (Smith and Iglewski, 1989). In the same way, the promoter regions of rsmY and rsmZ from *Pto* DC3000 were amplified by PCR using primer pairs RsmYBglII5p/RsmYBglII3p and rsmZp1DC3000/rsmZp2DC3000, respectively. The products were then cloned into plasmid pRG970Gm to obtain p970Gm-rsmYDC3000p and p970Gm-rsmZDC3000p. These plasmids were then introduced into appropriate *Pto* strains by electroporation.

Plasmid pME-gacS expressing the gacS gene under the control of the isopropyl β-D-thiogalactoside (IPTG)-inducible tac promoter (P<sub>tac</sub>) was constructed as follows. The gacS gene was
amplified by PCR with the primers gacS-XhoI/gacS-EcoRI and cloned into pME6032, yielding pME-gacS. When required, 0.25 and 0.5 mM of IPTG were added to induce the Ptac promoter of the plasmid pME6032, respectively.

**Preparation and Screening of Extracts From Soil Bacteria**

Soil samples were collected from various regions in Guangxi, China, and rhizobacteria were isolated using LB plates. The isolates were cultured in 5 ml of seed broth at 28°C for 7 days (Ling et al., 2015). One milliliter of the cultures was extracted with 200 µl of an equal volume of ethyl acetate. The ethyl acetate extracts were evaporated and dissolved in 200 µl of 100% dimethyl sulfoxide (DMSO). A portion (5 µl) of the extract was mixed with 1 ml of bacterial suspension of the reporter strain Pto DC3000 (p970Gm-gacSDC3000p). After 7 h of incubation at 28°C, the β-galactosidase activity was assayed using the Miller (1972) method.

**Swarming Assay and Hypersensitive Response Test**

Pto DC3000 and its gacA mutant were cultured overnight in KB liquid medium. Then, 5 µl of the stationary phase cultures was spotted onto SWM agar medium (0.4% agar) with BR3 extract (at final a concentration of 16 or 32 µg ml⁻¹). The plates were incubated overnight at 28°C and then imaged (Chatterjee et al., 2003).

The procedures for HR testing in tobacco leaves were previously published (Cui et al., 1996). Young, fully expanded third and fourth leaves from approximately 8-week-old Nicotiana tabacum L. cv. Samsun were used for the HR test. Bacterial cells [1 × 10⁷ colony forming units (CFU) ml⁻¹] in the absence or presence of the BR3 extract were infiltrated into tobacco leaves as indicated in the figure captions. Images were acquired at 20 h after infiltration. DMSO was infiltrated as a control.

**Mutant Strain Construction and Western Blotting Analysis**

To construct a C-terminal vesicular stomatitis virus glycoprotein (VSV-G) epitope-GacS fusion, a PCR-generated fragment with the sequence 5′-TATACAGATATTGAATGATTAG-3′ was inserted in-frame before the stop codon of gacS and cloned into p2P24Km (Zhang et al., 2018). The resulting plasmid was introduced into strain Pto DC3000 by electroporation, and the wild-type copy was replaced by the modified version after two recombinations events under high-sucrose stress. Substitution was confirmed by sequencing.

To analyze the effect of the BR3 extract on the protein levels of GacS and the TTSS effector AvrPto, Pto DC3000 and its derivatives were cultured in TTSS-inducing minimal medium at 20°C for 16 h. Cell lysates were prepared by sonication and resuspended in SDS sample buffer. The samples were separated by SDS–PAGE and protein levels were analyzed using western blotting with anti-VSV antibodies (Sangon Biotech) or anti-AvrPto antibodies (Dijk et al., 1999).

**Extraction and Detection of 2,4-Diacetylphloroglucinol and N-Acyl Homoserine Lactone**

*Pseudomonas fluorescens* 2P24 and its derivative strains were cultured in 20 ml of KBG (KB broth supplemented with 2% glucose) as described above. The antibiotic 2,4-Diacetylphloroglucinol (DAPG) was extracted from the culture supernatant and assayed using a previously described HPLC method (Shanahan et al., 1992).

Cultures of *P. carotovorum* subsp. *carotovorum* (Pcc) Z3-3 were grown overnight in LB medium with DMSO or the extract of strain BR3. One milliliter of the cultures was extracted with an equal volume of ethyl acetate. The ethyl acetate extracts were dried and resuspended in 50 µl of methanol. A portion (200 µl) of *A. tumefaciens* NTL4 (pZLR4) (OD₆₀₀ = 0.8) with 5 µl of the extract solution was incubated at 28°C for 3 h, and then β-galactosidase activities were quantified (Miller, 1972).

**Assessing the Inhibition of Pathogenicity of Pto DC3000 in Arabidopsis and Z3-3 in Chinese Cabbage by the BR3 Extract**

*Arabidopsis* plants were grown in a growth room at 23°C and 70% relative humidity under a 14-h light cycle. Strain Pto DC3000 was grown on KB agar overnight at 28°C and resuspended in water with the extract from strain BR3 or DMSO at 10⁵ CFU µl⁻¹, 20 µl of the suspension was infiltrated into 4-week-old *Arabidopsis* leaves. Bacterial populations were determined at day 0 and day 3. Three 1-cm-diameter leaf disks were collected from three independent plants, and ground in 1 ml of 10 mM MgCl₂. Bacterial colonies were then counted 2 days after plating 10 µl from serial dilutions on KB plates. For the pathogenicity test in Chinese cabbage, *Pcc* Z3-3 was grown in LB broth at 28°C for 12 h. Cells were harvested and resuspended in phosphate-buffered saline (PBS) to 10⁸ CFU ml⁻¹ with or without the BR3 extract. For each Chinese cabbage leaf, three wounds were punched and inoculated with 10 µl of bacterial suspension. Maceration symptoms were documented at 30 h post-inoculation, as described previously (Li et al., 2010).

**Statistical Analysis**

All experiments were performed in triplicate. The data were analyzed and compared by performing two-sample independent t-tests using DPS v9.50¹.

**RESULTS**

**The Extract of Strain BR3 Reduced the Expression of gacS in Pto DC3000**

Extracts from 5000 isolates obtained from soil were tested for their abilities to suppress the β-galactosidase activity of the *gacS-lacZ* transcriptional fusion in the strain *Pto* DC3000

¹http://www.dpsw.cn/dps_eng/index.html
that carrying reporter plasmid p970Gm-gacSDC3000p. The extract from a strain named BR3 showed significant inhibitory activity (Figure 1A). Analysis of the 16S rRNA gene sequence of BR3 indicated that this organism belongs to Bacillus sp. (data not shown). Introduction of pME-gacS into the wild-type DC3000 improved the promoter activities of the gacS gene (Figure 1A). However, the promoter activities of gacS were considerably induced in the extract-treated wild-type DC3000 with plasmid pME-gacS, indicating that the expression of gacS gene was repressed by the BR3 extract (Figure 1A). To further verify the effect of the BR3 extract on the expression of the gacS gene, the level of GacS protein was determined. Western blot analysis of the chromosomal gacS-vsv fusion strain showed that GacS protein production was significantly decreased in cells treated with the BR3 extract (Figure 1B). Our data also showed that the BR3 extract had no effect on the growth of Pto DC3000 (Figure 1C and Supplementary Figure S1). Taken together, these results suggested that the BR3 extract suppressed the expression of gacS gene.

**The BR3 Extract Inhibited GacA-Dependent sRNA Expression in Pto DC3000**

RsmZ-like non-coding sRNAs are regulated by the GacS/GacA system (Kay et al., 2005; Moll et al., 2010); therefore, the expression of the rsmZ and rsmY genes was further analyzed in the presence or absence of the BR3 extract. The β-galactosidase activities of the rsmZ-lacZ fusion or the rsmY-lacZ fusion in wild-type Pto DC3000 were significantly higher than those in gacA mutants, suggesting that GacA positively regulated the rsmY and rsmZ genes expression (Figure 2). Addition of the BR3 extract (16 µg ml⁻¹) reduced the expression of the rsmZ-lacZ and rsmY-lacZ fusions in the wild-type Pto DC3000 compared with that in the DMSO-treated control (Figure 2). Interestingly,
The BR3 extract had no effect on the expression of rsmZ–lacZ and rsmY–lacZ fusions in the gacA mutant (Figure 2). These data indicated that the BR3 extract affected the expression of rsmZ and rsmY via the GacS/GacA system.

The Effect of the BR3 Extract on Bacterial Swarming Motility, Effector AvrPto Production, and HR on Tobacco

Previous studies indicated that the swarming motility of the gacA mutant was much reduced compared with that of wild-type Pto DC3000 (Chatterjee et al., 2003). Therefore, we checked whether the BR3 extract influenced the swarming motility of Pto DC3000. As expected, when treated with the extract, the wild-type displayed a swarming deficiency resembling that of its gacA mutant. While the swarming motility of the gacA mutant was not further affected upon the treatment of the BR3 extract (Figure 3A).

GacA regulates the expression of hrpL, a master regulator of TTSS (Chatterjee et al., 2003); therefore, we further analyzed the protein level of the TTSS effector AvrPto using western blotting and observed a significant reduction in the level of AvrPto in the extract-treated wild-type (Figure 3B). Pto DC3000 could elicit a typical HR in tobacco leaves when infiltrated at 1 × 10⁷ CFU ml⁻¹ of bacterial cells, whereas the BR3 extract-treated Pto DC3000 failed to trigger HR at the same cell concentration (Figure 3C). In addition, the hrcQ-U mutant was also incapable of showing HR with or without the BR3 extract (Figure 3C). Pto DC3000-triggered HR on tobacco leaves is TTSS-dependent, these data suggested that the BR3 extract possible impaired the function of the TTSS in Pto DC3000.

The BR3 Extract Influences rsmY2P24 and phlA Expression in P. fluorescens 2P24

The GacS/Rsm system is conserved in many Gram-negative bacteria, and globally regulates the production of a large number of secondary metabolites, including antibiotic 2,4-DAPG in Pseudomonas spp. (Laville et al., 1992). We measured the influence of the BR3 extract on the expression of the sRNA gene rsmY2P24 and 2,4-DAPG biosynthetic gene phlA in P. fluorescens 2P24. The results showed that the BR3 extract reduced rsmY2P24 transcription and strongly inhibited the expression of the phlA–lacZ translational fusion (Figure 4). 2,4-DAPG production in the extract-treated cells reduced by approximately twofold (Figure 4C). In addition, the expression of rsmY2P24 and phlA was significantly decreased in the gacA2P24 mutant, but no further decrease was found when treated with the BR3 extract (Figures 4A, B). The expression of a housekeeping gene proC, which encodes the constitutive delta 1-pyrroline 5-carboxylate reductase (the third enzyme of proline biosynthesis), was not affected by the addition of the BR3 extract (Figure 4D; Savioz et al., 1993). In conclusion, our data suggested that the BR3 extract strongly inhibited the genes of the GacS/GacA regulon in P. fluorescens 2P24, and this inhibitory effect is GacA-dependent.

Effect of the BR3 Extract on the Virulence of Pto DC3000 and Pectobacterium carotovorum subsp. carotovorum Z3-3

In phytopathogenic bacteria, the Gac/Rsm system is associated with pathogenicity. The observation that the BR3 extract suppressed the activity of the Gac/Rsm system prompted us to determine whether it interfered with the GacS/GacA system-dependent virulence process. As predicted, the extract-treated Pto DC3000 displayed a significantly reduced in planta population compared with that of the Pto DC3000 strain treated with DMSO (Figure 5A).

Previous data indicated that the GacS/GacA system positively regulates the Expl/ExpR QS system, which participates in the regulatory pathway for exoenzyme production in Pcc (Cui et al., 2001; Whitehead et al., 2002). Therefore, we further examined the
influence of the BR3 extract on the pathogenicity of Pcc toward Chinese cabbage. The BR3 extract significantly inhibited QS signal molecules [N-acyl homoserine lactone (AHL)] production and the virulence of Pcc on Chinese cabbage (Figures 5B, C). Thus, the GacS/GacA system antagonist present in the BR3 extract is capable of disrupting GacS/GacA system-directed pathogenicity in phytopathogenic bacteria.

DISCUSSION

The interaction between pathogens and plants plays a critical role in plant development and fitness, including cellular mechanisms, growth, reproduction, hormonal signaling, and tolerance to environmental stresses are involved in this process (Saleem et al., 2017a). Furthermore, the interactions of bacterial species in the phyllosphere influence plant development and fitness (Saleem et al., 2017b). It is challenging to elucidate the detailed mechanisms of the effects of the microbiota on plant function (Haas and Défago, 2005).

Many plant growth-promoting rhizobacteria suppress plant disease by producing one or several antibiotic compounds to inhibit the growth or directly kill the pathogens (Haas and Défago, 2005). However, the resistance of pathogens developed quickly under the antibiotic stress and is becoming a potential challenge for the biological control of soil-borne pathogens (Spellberg and Shales, 2014; Deising et al., 2017). Other bacterial virulence-related factors, such as two-component systems, QS systems, disulphide bond forming enzymes, and TTSSs, have been considered as attractive targets of the chemical therapy.
FIGURE 4 | Regulation of *rsmY*, *phlA*, and *proC* of strain 2P24 in the presence or absence of the BR3 extract. The β-galactosidase activities of transcriptional *rsmY–lacZ* (A), translational *phlA–lacZ* (B), and *proC–lacZ* (D) fusions were determined in *P. fluorescens* 2P24 in the absence or presence of the BR3 extract. Biosynthesis of 2,4-DAPG of strain 2P24 in the absence or presence of the BR3 extract was assayed by HPLC (C). All experiments were performed in triplicate, and the mean values ± standard deviations are indicated, *P* < 0.05.

Although the GacS/GacA system plays a critical role in the production of extracellular factors, ecological fitness, and even primary metabolism (Heeb and Haas, 2001; Takeuchi et al., 2012), the chemical signals that stimulate the GacS/GacA system remain obscure. Dubuis and Haas (2007) found that extracts from culture supernatants of *Pseudomonas* sp. and *Vibrio* sp. induced the expression of sRNAs and the production of antibiotic compounds, indicating that the signals could be self-produced. Our study further demonstrated that the BR3 extract could interfere with multiple targets of the GacS/GacA regulon in different bacterial species. In addition, overexpression of the *gacS* gene could attenuate the inhibition effect of the extract BR3 on the promoter activities of *gacS* (Figure 1A). These data suggested that the BR3 extract inhibits the function of the GacS/GacA system by modulating the expression of *gacS* gene. To date, few regulatory elements have been identified to influence the expression of *gacS* (Hrabak and Willis, 1992). Our data showed that the BR3 extract could influence the GacS-dependent phenotypes in strains DC3000, 2P24, and Z3-3, whereas no
A conserved motif was found in the promoter regions of the \textit{gacS} gene (data not shown), suggesting that the effect of the BR3 extract on \textit{gacS} transcription may involve a potential regulator.

Although the method for isolation of the BR3 extract was similar to that of the AHL, the activity of the BR3 extract was not affected at pH 12 and 30°C for 1 h (data not shown). Under the same conditions, AHLs are be degraded (Swift et al., 2001). Moreover, the typical reporter system \textit{A. tumefaciens} with the \textit{traG–lacZ} fusion failed to show any reaction with the BR3 extract (Supplementary Figure S2A). These data suggested that the active ingredient from the BR3 extract does not belong to the AHL signals. Isolation of active ingredient from the BR3 extract showed that a pure compound named CX03 (MW = 413.2659) has the same inhibitory activity with the BR3 extract (Supplementary Figures S2B,C). Plant immunity is modulated by complex regulatory networks in response to abiotic and biotic factors (Jones and Dangl, 2006). Interestingly, our data showed that HR was restricted by the BR3 extract (Figure 3C). However, the BR3 extract had no effect on the transcript level of \textit{PR1} (At2g14610) in \textit{Arabidopsis} (data not shown). Further studies are needed to solve the chemical formula of compound CX03 and to investigate the mechanism of CX03 on plant immunity.

Rhizobacteria deploy several strategies, such as the production of antibiotics, QS, and type six secretion systems, to disrupt or otherwise manipulate commensal bacteria to facilitate their colonization and survival (Quiñones et al., 2004; Vacheron et al., 2019). Our data explored the possibility that some bacteria produce specific secondary metabolites to influence the signal transduction of others. \textit{Bacillus} species are ubiquitous in the soil environment, and some of them are beneficial to plant growth or suppression of plant diseases (Saleem et al., 2017b); therefore, our
findings deepen our understanding of the biocontrol mechanisms used by root-associated Bacillus spp.

Collectively, the extract from strain BR3 caused dysfunction of the GacS/GacA system, leading to attenuated activity of virulence factors, thus it may form the basis of a new anti-virulence strategy.

DATA AVAILABILITY

The datasets generated for this study can be found in NCBI accession MK864268, https://www.ncbi.nlm.nih.gov/nuccore/MK864268.

AUTHOR CONTRIBUTIONS

BZ, YZ, FL, and XW conceived and designed the experiments. BZ, YZ, FL, and YM conducted most of the experiments. XW wrote the manuscript and all authors reviewed the manuscript critically.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.02005/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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