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Research article

Biocontrol Potential, Genome and Nonribosomal Peptide Synthetase Gene Expression of *Bacillus velezensis* 2211

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Colletotrichum fructicola

Abstract

Members of the genus *Bacillus* produced a diverse group of antimicrobial compounds. Here, we presented the antifungal activity and genome sequence analysis of *Bacillus* sp. 2211, a potential plant-growth-promoting bacterium. Bacterial supernatants from strain 2211 cultures in nutrient broth (NB) and potato dextrose broth (PDB) suppressed the mycelial growth of *Pyricularia oryzae*, *Colletotrichum aenigma*, *Colletotrichum fructicola* and *Fusarium oxysporum.* The supernatants were also able to suppress spore germination of these fungi, except for *F. oxysporum.* However, the supernatant from PDB displayed a significantly higher inhibition activity than NB. Additionally, the supernatant from PDB significantly reduced the disease severity caused by *P. oryzae* on rice seedlings. The genome of strain 2211 was sequenced. The highest digital DNA-DNA hybridization (80.1%) and average nucleotide identity (97.57%) levels indicated that strain 2211 was a member of the species *Bacillus velezensis*. The phylogenomic analysis showed that it clustered with *B. velezensis* NRRL B-41580^T, *B. velezensis* KACC 13105 and *B. velezensis* subsp. *plantarum* FZB42^T. The gene expression analysis showed the up-regulation of nonribosomal peptide synthetase (NRPS) genes *bmyA*, *fenB* and *dhbE* in PDB, compared to NB. This work demonstrated that the culture media affected the antagonistic activity of strain 2211 possibly through the modification of NRPS biosynthesis genes.

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1. Introduction

Plant-growth-promoting bacteria (PGPB) are known for their direct and indirect benefits to plants. The direct benefits generally involve the modification of phytohormones and improvement of the bioavailability of nutrients [1]. The indirect positive effect of these bacteria was the protection of plant hosts from phytopathogens. Three mechanisms were previously described [1]. Firstly, hydrolytic enzymes, including protease and chitinase, from PGPB targeted cell-wall structural components of the pathogens. The second mechanism was the biosynthesis of antimicrobial compounds. The third one was the induction of induced systemic resistance (ISR) in plant hosts by PGPB.

PGPB produced various groups of bioactive compounds. Many of them exhibited antimicrobial activities against phytopathogens. Much effort was dedicated to elucidation of the structure of such novel compounds, their activities, regulatory elements and biosynthesis genes. Among these, nonribosomal peptides (NRPs) represented a group of antimicrobial peptides with highly diverse structures and activities [2]. The biosynthesis of NRPs is facilitated by modular and complex enzymes that are known as nonribosomal peptide synthetases (NRPSs) [3]. The enzymes are coded by biosynthetic gene clusters (BGCs) that are prominently found in the genus *Bacillus* [4]. Initially, detection of NRPS gene clusters was achieved by partial amplification of the NRPS gene clusters [5]. However, as the cost of whole genome sequencing decreased in recent years, full sequences of NRPS gene clusters were identified and characterized in several *Bacillus* strains, using the whole genome sequencing analysis [4].

The operational group *Bacillus amyloliquefacies* belonged to the *Bacillus subtilis* species complex and consisted of *Bacillus amyloliquefaciens*, *Bacillus siamensis*, *Bacillus velezensis* and *Bacillus nakamurai* [6]. *B. velezensis* subsp. *plantarum* FZB42^T was a well-known and highly effective biocontrol agent against many phytopathogenic fungi, including *Rhizoctonia solani*, *Magnaporthe oryzae* and *Fusarium graminearum* [7]. This was mostly attributed to its production of various NRP molecules, including bacillomycin D, fengycin and surfactin [7]. The genome sequence analysis of FZB42 indicated the presence of thirteen gene clusters for the nonribosomal and ribosomal biosynthesis of antimicrobial compounds [7]. *Bacillus* sp. 2211 was previously isolated from rice roots [8]. It showed a strong inhibition activity against the mycelial growth of *P. oryzae*, the causative agent of rice blast [8]. The sequence analysis of the 16S rRNA gene indicated 99.9% sequence similarity between strain 2211 and *B. velezensis* CR-502^T [9]. The phylogenetic analysis showed that it formed a cluster with *B. amyloliquefacies* DSM7^T and *B. velezensis* CR-502^T [9]. Partial fragments of fengycin-, surfactin- and bacillibactin-biosynthetic genes were also detected in strain 2211 [9]. The aim of this study was to further investigate the antifungal compound production, biocontrol activity, whole-genome sequence and NRPS gene expression of strain 2211. This information was important for a future application of strain 2211 as a biocontrol agent.

2. Materials and Methods

2.1 Bacterial culture and supernatants

Strain 2211 was previously isolated from rice roots [8]. A single colony was grown in 10 mL NB medium (Sisco Research Laboratories, India) at 30°C for 24 h on a rotary shaker at 180 rpm. The culture was diluted to 0.1 OD_{600} with sterilized distilled water. One-hundred µL of the suspension was used as the starting inoculum in 50 mL NB and PDB (HiMedia, India). The cultures were grown at 30°C for 48 h on a rotary shaker at 180 rpm. Subsequently, two mL of the cultures were

collected for RNA extraction. Bacterial cells in the remaining cultures were removed by centrifugation at 4,500 rpm, 4°C for 20 min. The supernatants were sterilized by filtering through 0.22-µm PVDF filters (Merck, USA).

2.2 *In vivo* mycelial inhibition test

The culture of *Pyricularia oryzae* was obtained from Asst. Prof. Dr. Nonglak Parinthawong, at King Mongkut's Institute of Technology Ladkrabang. *Colletotrichum aenigma* TBRC1738 was purchased from Thailand Bioresource Research Center (TBRC). *Colletotrichum fructicola* and *Fusarium oxysporum* cultures were obtained from the Department of Agriculture, Ministry of Agriculture and Cooperatives, Thailand. All fungi were grown in PDA (HiMedia, India) at 30°C for seven days. Mycelial plugs were obtained with a 5-mm cork borer. A plug was placed on a fresh PDA plate. A well with 11-mm diameter on the PDA plate was made at 20 mm away from the mycelial plug. Two-hundred µL of the sterilized supernatant were added to the well. PDA plates were incubated at 30°C for seven days. The diameter of the fungal colonies was determined. Control plates contained only the fungal plugs. The percentage of growth inhibition was calculated, using the equation $[1 - (R_1/R_2)] \times 100$ [10]. R_1 and R_2 were the fungal colony radius of the experimental and control plates, respectively. Fungal mycelia were collected and stained with lactophenol cotton blue on a glass slide. The mycelial structure was examined under a light microscope.

2.3 *In vitro* spore inhibition test

Pyricularia oryzae was grown on rice bran agar [11] at 30°C for seven days and subsequently transferred to 25°C for 48 h for induction of conidia formation. *Colletotrichum aenigma*, *C. fructicola* and *F. oxysporum* were grown on PDA at 30°C for seven days to obtain conidia. Sterilized distilled water was added to the plates, and conidia were scraped off, using a glass spreader. The conidial suspension was filtered through a two-layer cheese cloth. Conidial suspension of *P. oryzae* was adjusted to 2×10^5 conidia mL^{-1}, using a hemacytometer. An equal volume (50 µL) of the spore suspension and the supernatants were mixed in a microtube and incubated at 30°C for 18 h. A mixture of sterilized distilled water and the spore suspension was used as the control group. The percentage of spore inhibition was calculated as the percentage of the ratio between the number of non-germinated conidia and the total number of conidia examined. For *C. aenigma, C. fructicola* and *F. oxysporum*, the concentration of conidial suspension was prepared at 1×10^7 conidia mL^{-1}. Two-hundred µL of the conidial suspension were added to 15 mL melted PDA medium. The mixture was overlaid on a PDA plate. After the top layer became solidified, an 11-mm diameter well was prepared. Two-hundred µL of the bacterial supernatant were added to the well. Sterilized distilled water was used as the control group. All PDA plates were kept at 30°C for 24 h. The presence and diameter of the inhibition zone around the well were examined. The structure of the fungal spores was examined under the light microscope.

2.4 *In vivo* biocontrol activity of strain 2211 bacterial supernatant

Strain 2211 and *Bacillus siamensis* 1021 [8] were cultured in 50 mL PDB at 30°C for 48 h on a rotary shaker at 180 rpm. The filtered-sterilized bacterial supernatants were obtained as described above. Harvested bacterial cells were resuspended in sterilized distilled water. The cell concentration was adjusted to 10 OD_{600}. Tween-20 was added to the supernatants and cell suspension to reach 0.02% concentration. Seeds of rice cultivar Khao Dok Mali (KDML) 105 were treated with 10% $NaHClO_3$ for 10 min for surface sterilization. The seeds were transferred to sterilized distilled water and shaken for 10 min. This was repeated two additional rounds. The seeds

were kept in the dark at 30°C for 24 h for germination. Five rice seedlings were transferred to a pot containing sterilized soil and grown at 30±2°C under 16-h-light/8-h-dark photoperiods with 65 µmol m^{-2} s^{-1} photosynthetically active radiation flux. Five-day-old seedlings were sprayed with 10 mL cell suspension to obtain T1 (strain 1021), T2 (strain 2211) and T3 (5 mL of strain 1021 and 5 mL of strain 2211). T4 was the control group treated with 10 mL sterilized distilled water. These groups were kept in a moist chamber and grown for an additional 48 h. On the other hand, seven-day-old seedlings were sprayed with 10 mL of the bacterial supernatant from PDB to obtain T5 (strain 1021), T6 (strain 2211) and T7 (5 mL of strain 1021 and 5 mL of strain 2211). Ten mL of sterilized distilled water were sprayed on the control group (T8). All experimental (T1-T3 and T5-T7) and control groups (T4 and T8) were subsequently sprayed with 10 mL of *P. oryzae* conidial suspension (2×10^5 conidia mL^{-1}) and kept in the moist chamber under the dark for 24 h. The seedlings (T1-T8) were allowed to grow for an additional seven days under the growth conditions. A nine-grade scale grading, according to IRRI [12] was used to obtain the disease rating. The equation $[\Sigma(r \times n_r)/(9 \times N_r)] \times 100$ [13] was used to calculate the percentage of disease severity, r represented the scale of disease rating while n_r was the number of leaves with lesions at scale r. N_r was the total number of leaves on each seedling.

2.5 Whole genome sequencing of strain 2211

Strain 2211 was grown in 50 mL PDB at 30°C for 48 h on a rotary shaker at 180 rpm. Two mL of the bacterial culture were centrifuged at 12,000 rpm for 10 min to harvest the cells. DNA extraction was carried out, using Presto Mini gDNA Bacteria kit (Geneaid, Taiwan), according to the manufacturer's protocol. The genomic DNA was submitted for whole genome sequencing at OMICS Sciences and Bioinformatic Center (Bangkok, Thailand). Genome sequencing was done on Illumina Miseq (Illumina, USA). SPADEs version 13.0 was used for the genome assembly [14]. Strain 2211 genomic sequence was deposited at the GenBank database with the assembly accession number GCA_014534715.

2.6 Genome sequence analysis

Average nucleotide identity (ANI) was obtained, using JSpeciesWS [15], while digital DNA-DNA hybridization (dDDH) was obtained from the Type (strain) Genome Server (TYGS) [16]. The phylogenomic tree was reconstructed, using Genome BLAST Distance Phylogeny approach on TYGS as well. Pseudo-bootstrap support values on the phylogenomic tree were determined based on 100 replications. Strain 2211 genome was annotated by the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) [17]. The contigs were reordered by comparing them to the complete genome of *B. velezensis* subsp. *plantarum* FZB42^T (assembly accession number: GCA_000015785), using progressiveMAUVE [18]. The circular representation of the genome was created, using Proksee (https://beta.proksee.ca/). The Venn diagrams for the analysis of the number of genes was generated, using EDGAR version 3.0 [19]. Biosynthetic gene clusters were determined using antiSMASH version 6.0 [20].

2.7 RNA extraction and reverse-transcription quantitative PCR (RT-qPCR)

Cells of strain 2211 grown in PDB and NB for 48 h were used for RNA extraction with a Favorprep Tissue Total RNA mini kit (Favorgen, Taiwan), according to the manufacturer's protocol. RNase-free DNase I (Geneaid, Taiwan) and RNA Cleanup kit (Geneaid, Taiwan) were used to remove the contaminating genomic DNA and purify the RNA solution, respectively, according to the manufacturer's protocol. Agarose gel electrophoresis of the purified RNA solution was used to

confirm the absence of genomic DNA. One µg of RNA was used for the cDNA synthesis with random hexamer primers and iScript Select cDNA Synthesis kit (Bio-Rad, USA). The temperature profile was 25°C for 5 min followed by 42°C for 30 min and 85°C for 5 min. Primers were designed from the annotated nucleotide sequences of *gyrA*, *rpoD*, *bmyA*, *fenB*, *dhbE* and *srfAA* using Primer 3 [21]. All primer sequences are provided in Table 1. cDNA samples were diluted ten folds. Two µL of the cDNA solutions were used in RT-qPCR reactions, using Luna Universal qPCR Master Mix (New England Biolabs, USA). The reaction components followed the manufacturer's protocol. The temperature profile was one cycle of 95°C for 1 min and 40 cycles of 95°C for 15 s and 60°C for 30 s. The melt-curve analysis was performed from 60°C to 95°C at a 0.5°C increment. CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) was used for the amplification and detection. Cq of each gene was determined. Relative gene expression of NRPS genes was calculated, using the ΔΔCq method [22]. The normalization was done against the reference genes *gyrA* and *rpoD*. The calibrator group was the samples of strain 2211 that had been grown in NB.

Table 1. Primers used for amplification of reference and NRPS genes in this study

Gene	Gene product	Primer sequence (5'-3')	Product size (bp)
gyrA	DNA gyrase subunit A	F: CTGACTGACGGCAAGAAACA R: AGCGTGATGCCTTTAACACC	119
rpoD	RNA polymerase sigma factor	F: GAAAGCCGTCGAGAAGTTTG R: GAACCGGGATACGGATTGTT	125
bmyA	Bacillomycin D synthetase A	F: TTATCCGAACGGGTATGAGG R: GTAAGCCGGTGCAGAAAAAG	118
fenB	Bengycin synthetase B	F: AACGGTGCTGAACCAAACGC R: CCGCCGAAAATGACATATCG	99
dhbE	2,3-dihydroxybenzoate-AMP ligase	F: AGATTGCCGCTGAAGAGGTG R: GGAATGACGAACACACAGGA	147
srfAA	Surfactin nonribosomal peptide synthetase SrfAA	F: TGAACTGTCCCGCTTGTATG R: TTGCGGTACTGCTCTGATTG	108

2.8 Statistical analysis

All experiments were done with three biological replicates. Statistically significant differences ($P<0.05$) between means were determined with either the Student's *t*-test or analysis of variance (ANOVA), followed by Tukey's post hoc test.

3. Results and Discussion

The bacterial supernatants obtained from NB and PDB were tested for their inhibitory effects against mycelia of *P. oryzae*, *C. aenigma*, *C. fructicola* and *F. oxysporum*. Reduced mycelial growth was

observed with the supernatant from NB, while an inhibition zone was clearly visible in the presence of the supernatant from PDB (Figure 1A). In contrast, fungal mycelia grew extensively on the control plates (Figure 1A). The bacterial supernatants from PDB and NB similarly induced the formation of swollen mycelial cells, when compared to the control groups (Figure 1B). Quantitatively, the supernatant from PDB exhibited significantly higher percentages of inhibition against all fungi, when compared to that from NB (Figure 2). Different results were observed when the supernatants were tested against fungal spores. The percentages of spore inhibition of *P. oryzae* conidia were insignificantly different, when treated with the supernatants from PDB (91.60±5.92%) and NB (96.91±2.12%). However, these percentages were significantly higher than that of the control group (39.23±2.90%). *Pyricularia oryzae* conidia of the control group germinated normally, while the germination of those treated with the supernatants was completely inhibited (Figure 3) When *C. aenigma* conidia were tested, the diameter of the inhibition zone of the PDA plates treated with the supernatant from PDB (26.67±1.53 mm) was significantly larger than that from NB (21.33±1.53 mm). *Colletotrichum aenigma* conidia of the control group germinated and produced normal mycelia, as opposed to the enlarged and highly vacuolated cells treated with the bacterial supernatants (Figure 3). For *C. fructicola*, a significantly larger diameter of the inhibition zone was also observed with the supernatant from PDB (26.17±0.76 mm), compared with that from NB (21.67±0.58 mm). Normal mycelia were found in the control plates, while spore germination was completely inhibited by the supernatants. In contrast, the germination of *F. oxysporum* spores was not inhibited by either supernatant. However, the supernatants still induced an enlargement of the mycelial cells, compared to the normal mycelia of the control group (Figure 3).

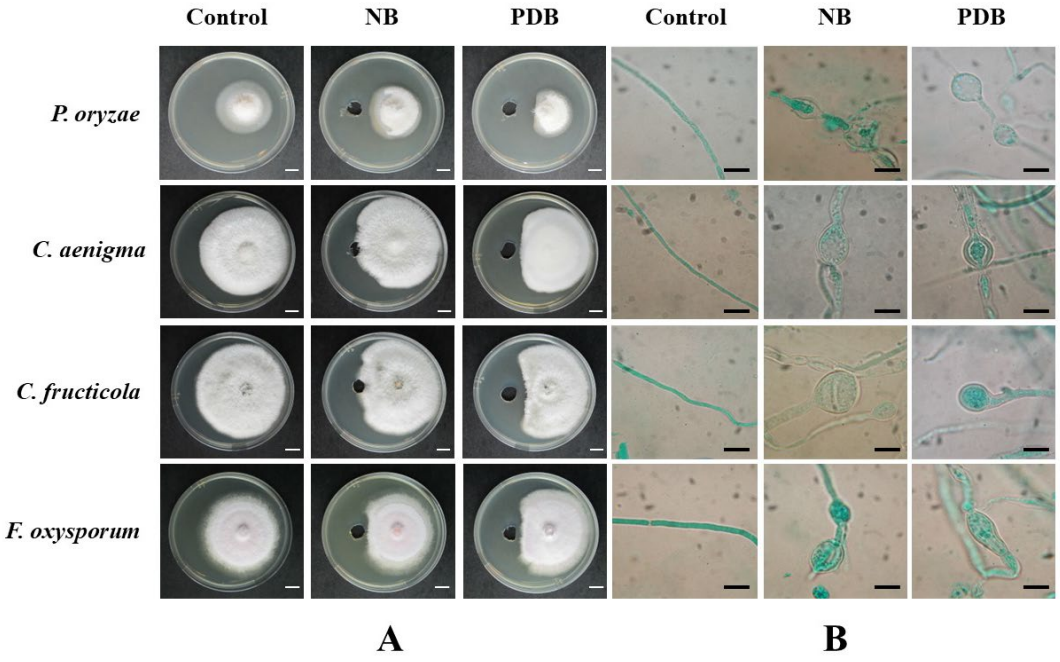

Figure 1. Growth (A) and characteristics (B) of mycelia of *P. oryzae*, *C. aenigma*, *C. fructicola* and *F. oxysporum* on the control plates and in the presence of bacterial supernatants of strain 2211 from NB and PDB. Bars indicate 2 cm (A) and 10 µm (B).

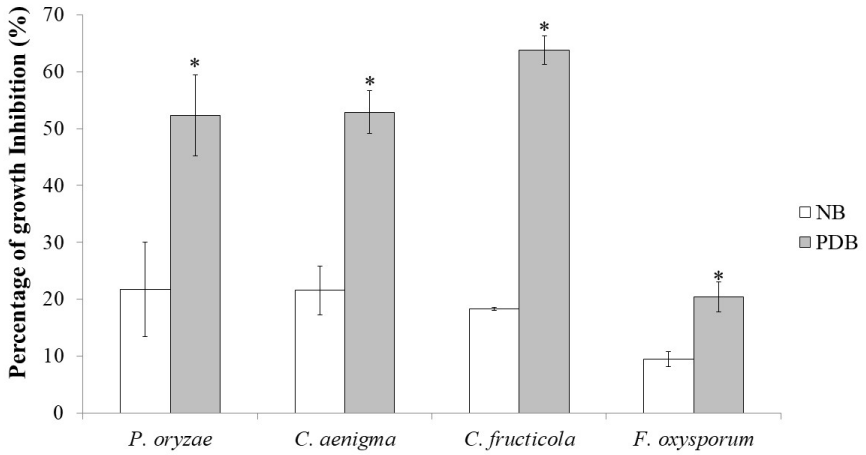

Figure 2. Inhibition activities of bacterial supernatants of strain 2211 from NB and PDB against *P. oryzae*, *C. aenigma*, *C. fructicola* and *F. oxysporum* mycelia. Error bars indicate standard deviation (n = 3). Asterisks indicate statistically significant differences (P<0.05, n=3) between the treatments, based on the Student's t-test.

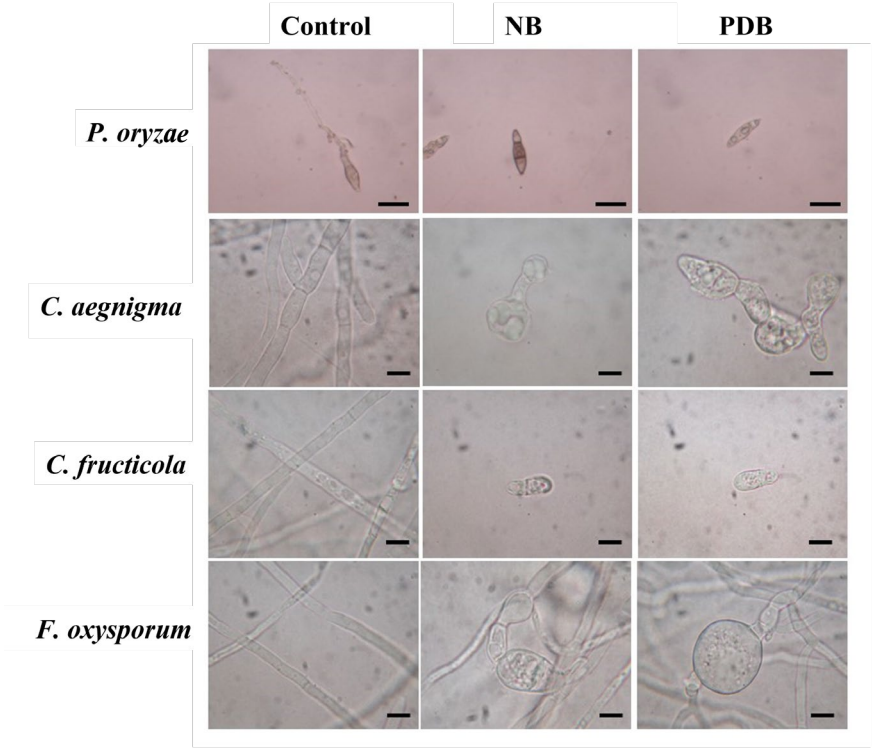

Figure 3. Characteristics of *P. oryzae*, *C. aegnigma*, *C. fructicola* and *F. oxysporum* spores in the control group compared to the treatment with bacterial supernatants of strain 2211 from NB and PDB. Bars indicate 20 µm.

The antagonistic activity of *Bacillus* spp. against phytopathogens largely involved the biosynthesis of various antibiotic compounds. Another crucial factor was the production level of these compounds. Our experiment showed that PDB likely had a higher stimulatory effect on the production level than NB. This was supported by the differences in the percentages of inhibition but similarity of characteristics of affected mycelia and spores of *P. oryzae*, *C. aenigma* and *C. fructicola*. Although the result seemed counterintuitive when considering the common usage of NB and PDB for bacterial and fungal culture, respectively, previous reports consistently showed the positive effect of PDB on the production of antifungal compounds by *Bacillus* strains. *Bacillus natto* NT-6 produced considerably higher levels of iturin and surfactin in PDB, compared to Luria-Bertani (LB) and NB media [23]. PDB was also used to grow *Bacillus* strains for the control of the causative agents of various plant diseases, including mulberry fruit sclerotiniose, green mold, and strawberry anthracnose [24-26]. A major distinction between NB and PDB compositions was the absence and presence of sugar in the media, respectively. While NB contained beef extract and peptone, PDB was made up of potato and glucose. A previous study suggested that the type of sugar was an important factor that determined the production level of fengycin by *B. amyloliquefaciens* fmb-60 [27]. This suggested that an increase of antifungal compounds by strain 2211 may be possible through the modification of sugar in PDB.

Strain 2211 was tested for its biocontrol activity against rice blast on the blast-sensitive rice cultivar KDML105. Both bacterial cells and bacterial supernatants from PDB were examined. *Bacillus siamensis* 1021 was also included in this experiment. T1 (2211 cell suspension), T2 (1021 cell suspension), T3 (2211 and 1021 cell suspension) and T4 (sterilized distilled water) groups displayed insignificantly different disease severity levels (Figure 4). In contrast, T5 (2211 supernatant), T6 (1021 supernatant) and T7 (2211+1021 supernatants) groups considerably reduced disease severity levels, when compared to T8 (sterilized distilled water) (Figure 4). This result indicated that the bacterial supernatant from PDB was more effective for controlling rice blast in rice seedlings, compared to bacterial cell suspension. The production of NRPs was proposed as the antagonistic mechanism of members of the genus *Bacillus*. However, the bioactive compounds may be different among biocontrol *Bacillus* strains. NRPs of the iturin family from *B. safensis* B21 played a major role in the suppression of *Magnaporthe oryzae* mycelial growth [28], while fengycin from *B. subtilis* BS155 inhibited *M. oryzae* growth [29]. However, in the present study, the difference between the disease severity of the T5, T6 and T7 groups were statistically insignificant. This suggested the bioactive compounds produced by strain 2211 and *B. siamensis* 1021 were likely similar. We also found that cell suspensions of both strains were unable to reduce the disease severity in the seedlings. In contrast, *Bacillus* sp. w176, a close relative of *B. amyloliquefaciens* and *B. velezensis*, was tested for its biocontrol activity against green mold on citrus fruits [25]. The inoculation of strain w176 cells resulted in a considerably lower disease incidence than cell-free supernatant [25]. The difference between the biocontrol activity of w176 and 2211 cells may be caused by the distinction in the use of spore and vegetative cells, respectively, as the inoculum. Alternatively, based on our observation of the differential effects of PDB and NB on the antagonistic activity of strain 2211, the surfaces of rice seedlings may not be suitable for the production of antifungal compounds. This was also supported by a similar result from *B. siamensis* 1021 [11], of which the antifungal compound production was also modified by culture media.

Whole genome sequencing and genome assembly of strain 2211 yielded 24 contigs and consisted of 3,884,306 bp (Figure 5). The G+C content was 46.6%. The total number of coding sequences (CDS) was 3,879. Among these, 3,670 were annotated as protein coding CDSs. Eighty-three CDSs coded for RNAs. The strain 2211 genome was compared with those of the type strains of the genus *Bacillus*. The highest ANI level (97.57%) was observed between strain 2211 and *B. velezensis* KCTC 13012^{T}. The highest dDDH value (80.1%) was observed between the genome of strain 2211 and those of *B. velezensis* KCTC 13012^{T} and *B. velezensis* subsp. *plantarum* FZB42^{T}.

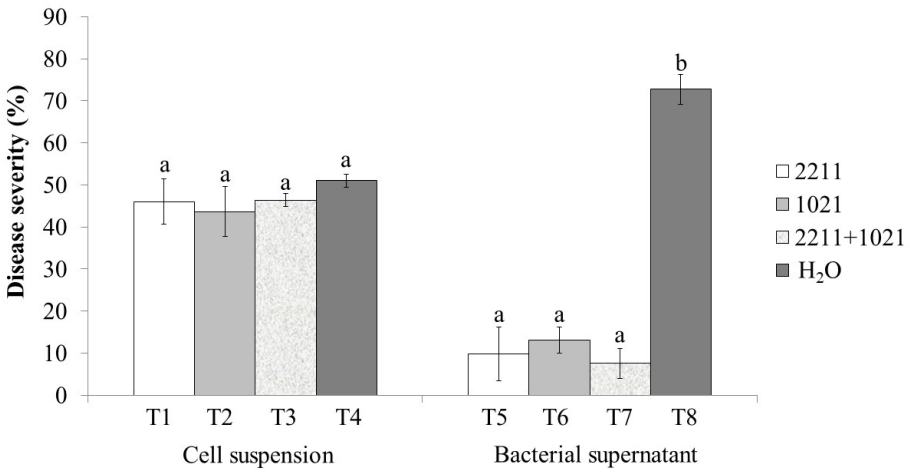

Figure 4. The rice-blast biocontrol activity of cell suspension and bacterial supernatants from strain 2211 and *B. siamensis* 1021 that were grown in NB and PDB. Error bars indicate standard deviation (n = 3). Different letters indicate statistically significant differences ($P < 0.05$) between the treatment of the same regime, using ANOVA and Tukey's test.

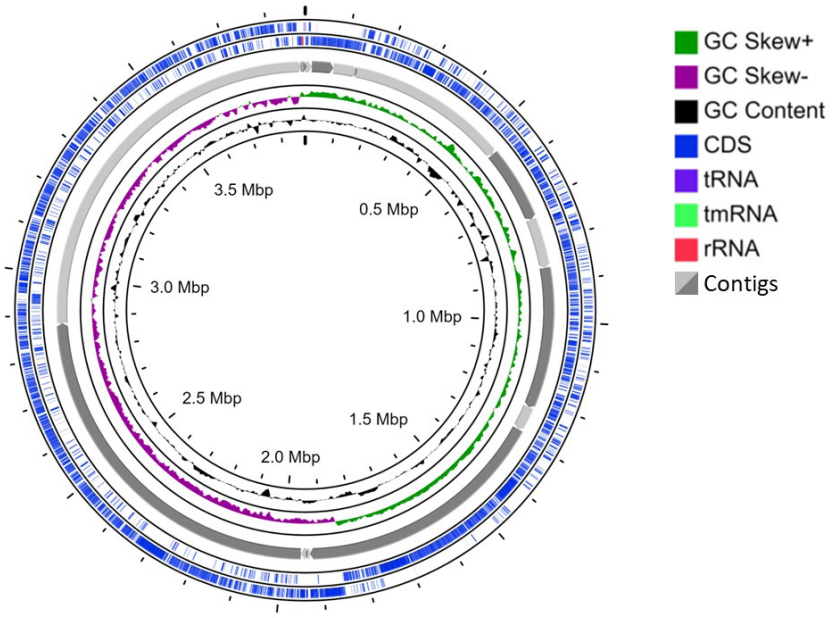

Figure 5. The circular representation of the genome of strain 2211. The outermost first and second rings represent the coding sequences on the forward and reverse strands, respectively. The third ring indicates 24 contigs of the genome. The fourth and the fifth rings show the GC skew (+/–) and the G+C content.

Both the ANI and dDDH levels were above the cut-off level at 95% and 70%, respectively, for assigning bacterial strains to the same species [30]. The phylogenomic tree showed that strain 2211 formed a cluster with *B. velezensis* KCTC 13012^T, *B. velezensis* subsp. *plantarum* FZB42^T and *B. velezensis* KACC 13105 (Figure 6). These results indicated that strain 2211 was a member of the species *B. velezensis*. Comparatively, the genome size of strain 2211 was smaller than those of strains NRRL B-41580^T, KACC 13105 and FZB42^T (Table 2). The G+C content of these strains were relatively similar and ranged from 46.3% (NRRL B-41580^T) to 46.6% (2211). Consistent with its relatively larger genome size, the highest number of protein coding CDSs was found in NRRL B-41580^T. In contrast, the number of RNAs was higher in FZB42^T, comparing to the other three strains. A Venn diagram was generated to indicate CDSs that were shared and unique among these strains (Figure 7). There were 3,370 CDSs that were commonly present in all strains. One-hundred-and-forty CDSs were specific to strain 2211. The number of CDSs that was shared between strain 2211 and NRRL B-4580^T was 3,502. This was higher than those shared between strain 2211 and KACC 13105 (3488 CDSs) and FZB42^T (3471 CDSs). This supported the genome taxonomy and phylogenomic analysis and suggested that strain 2211 was more closely related to NRRL B-41580^T and KACC 13105 rather than FZB42^T.

Genome sequencing was an important tool for the classification of bacteria in the *B. subtilis* subspecies complex, which included *B. subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, *B. amyloliquefaciens* and *B. velezensis* [31]. The type strain of *B. velezensis* was originally isolated from the mouth of the river Vélez, Spain, and it was notable for its substantial production of biosurfactant molecules [32]. Its 16S rRNA gene sequence shared 99% sequence similarity and 18.6% DNA-DNA relatedness with the type strain of *B. amyloliquefaciens* [32]. Subsequently, the species was assigned as the later synonym of *B. amyloliquefaciens*, based on a study which reported 89% DNA-DNA relatedness between the two species [33]. The discrepancy between the reports from traditional DNA-DNA hybridization led to a re-examination of *B. velezensis* using whole genome data [34]. Digital DNA-DNA hybridization revealed that the DNA-DNA relatedness between *B. velezensis* and *B. amyloliquefaciens* was 55.5% and confirmed the taxonomic distinction between these two *Bacillus* species [34]. Members of *B. velezensis* were previously studied for their potential applications in agriculture. Many strains have emerged as effective candidates as biocontrol agents against various plant diseases, including wheat powdery mildew [35], *Fusarium* head blight [36] and pepper grey mold [37]. Consistent with our study, other *B. velezensis* strains were able to suppress rice blast disease [38-40]. However, our study was the first to report the genome sequence of the *B. velezensis* strain with antagonistic activity against the mycelial growth and spore germination of *C. aenigma* and *C. fructicola*.

Biosynthetic gene clusters (BGCs) were identified in the genomes of strains 2211, NRRL B-41580^T, KACC 13105, and FZB42^T, using antiSMASH [20]. The number of reported BGCs ranged from 13 (FZB42^T) to 20 (NRRL B-41580^T). This distinction may result from the difference between the genome assembly levels of these strains. The genome of strain FZB42^T was complete and consisted of one contig, while the other genomes comprised several contigs. In strain 2211, nine BGCs displayed the similarity levels above 75% to known BGCs. This was similar to strain FZB42^T, whereas only seven and six BGCs of this similarity level were found in strains NRRL B-41580^T and KACC 13105, respectively (Table 3). Additionally, strains 2211 and NRRL B-41580^T also carried a BGC for mersacidin, which was not found in the other strains. FZB42^T was the only strain that contained a BGC for the biosynthesis of plantazolicin (Table 3). Strain 2211 carried four NRPS BGCs for bacillomycin D, fengycin, bacillibactin and surfactin (Figure 8A-C). Additionally, the BGCs for bacillomycin D (*bmy* genes) and fengycin (*fen* genes) were found next to each other. However, because the genome of strain 2211 was incomplete, fragments of the fengycin BGC was found scattered in three separate contigs (Figure 8A). This juxtaposition of the two BGCs was similar, and clearly shown in the genome of FZB42^T (Figure 8D). Similar to the fengycin BGC of

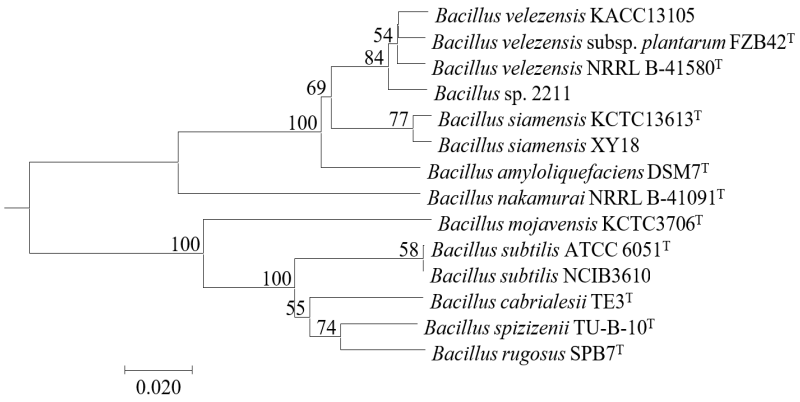

Figure 6. The phylogenomic tree based on the genome sequences of strain 2211 and other related *Bacillus* strains. The scale of branch lengths is shown in terms of GBDP distance formula d_5. GBDP pseudo-bootstrap values (>50%) from 100 replications are shown on the node of each branch. The average branch support was 73.5%.

Table 2. Genome characteristics of *B. velezensis* strains 2211, NRRL B-41580^T and KACC 13105. The genome character of *B. velezensis* subsp. *plantarum* FZB42^T is also included.

Strain	Genome size (bp)	GC content	Protein coding CDSs	RNAs	Assembly accession number
2211	3,884,306	46.6%	3,670	83	GCA_014534715
NRRL B-41580^T	4,034,335	46.3%	3,857	90	GCA_001461825
KACC 13105	3,888,922	46.4%	3,684	91	GCA_000960265
FZB42^T	3,918,596	46.5%	3,676	121	GCA_000015785

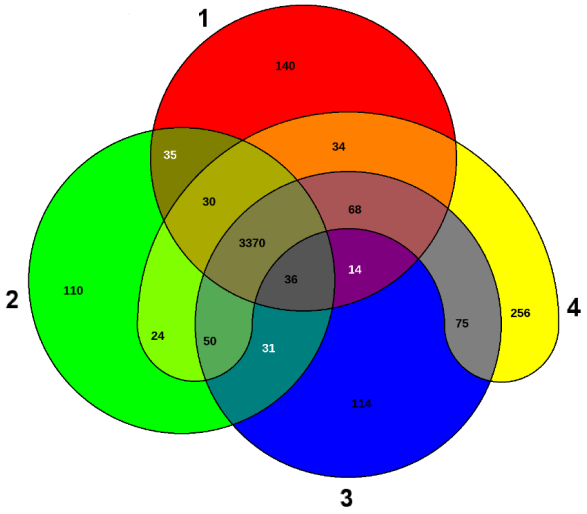

Figure 7. The Venn diagram indicates the numbers of shared and unique genes among the genomes of *B. velezensis* 2211 (1), *B. velezensis* subsp. *plantrarum* FZB42^T (2), *B. velezensis* KACC 13105 (3), and *B. velezensis* NRRL B-41580^T (4)

Table 3. BGCs with the most similar known cluster in the genomes of strains 2211, FZB42^T, NRRL B-41580^T and KACC 13105. Only the BGCs with the similarity level higher than 75% are shown.

Type	Most similar known cluster	Strain			
		2211	FZB42^T	NRRL B-41580^T	KACC 13105
NRP	fengycin	80%	100%	86%	93%
	bacillomyciin D	100%	100%	100%	100%
	surfactin	78%	91%	–	–
	bacillibactin	100%	100%	100%	100%
PKS	macrolactin	100%	100%	100%	100%
PKS+NRP	bacillaene	100%	100%	85%	100%
	difficidin	100%	100%	100%	100%
RiPP-Lanthipeptide	mersacidin	100%	–	100%	–
	plantazolicin	–	91%	–	–
others	bacilysin	100%	100%	100%	100%

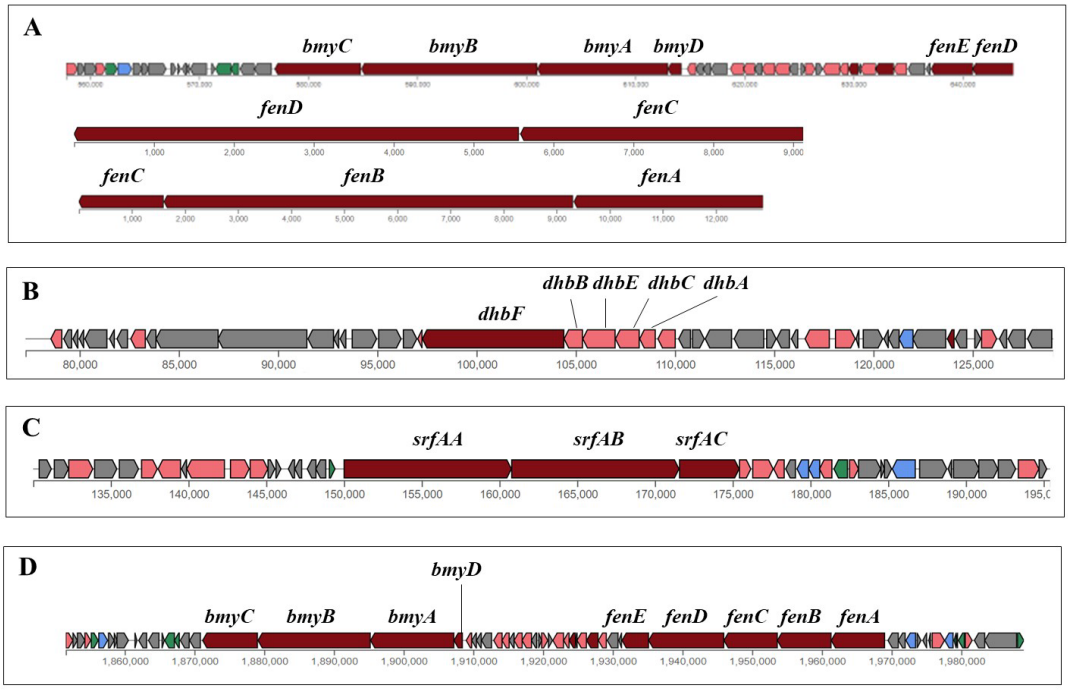

Figure 8. A diagrammatic representation of BGCs responsible for the biosynthesis of bacillomycin D (A), fengycin (A), bacillibactin (B) and surfactin (C) by strain 2211. The BGCs for bacillomycin D and fengycin biosynthesis in *B. velezensis* subsp. *plantarum* FZB42^T (D) are also shown.

strain 2211, partial fragments of surfactin BGC with a low similarity level (<75%) were found in several contigs of the genomes of NRRL B-41580^T and KACC 13105. This indicated the limitation of incomplete genomes for identification of large BGCs. To determine the effects of NB and PDB on the expression of NRPS BGCs in strain 2211, *bmyA*, *fenB*, *dhbE* and *srfAA* were selected as candidates of bacillomycin D, fengycin, bacillibactin and surfactin BGCs. Normalization of gene expression was performed against the combination of the reference genes *gyrA* and *rpoD* (Figure 9). The expression levels of *bmyA*, *fenB* and *dhbE* in NB were significantly lower (P<0.05) than those in PDB. Conversely, *srfAA* was expressed at a higher level in NB, compared to that in PDB.

BGCs for NRP biosynthesis were commonly found among *B. subtilis* species complex. However, the distribution of these BGCs among members of these groups was different, according to a previous study. Consistent with our study, fengycin BGCs were mainly found in *B. velezensis* and *B. amyloliquefaciens* strains, while plipastatin was found in *B. subtilis* and *B. atrophaeus* [41]. Bacillomycin D BGCs were also found among *B. velezensis* strains [41]. On the other hand, BGCs for the biosynthesis of bacillibactin and surfactin were widely spread among the genomes of *B. subtilis*, *B. velezensis*, *B. amyloliquefaciens* and *Bacillus atrophaeus* [41]. We also found a positive correlation between *bmyA*, *fenB* and *dhbE* expression patterns and the antagonistic levels of the bacterial supernatants. The expression levels of these genes were higher in strain 2211 grown in PDB, which also yielded a higher level of antagonistic level against the tested fungi, compared to NB. In contrast, the *srfAA* expression pattern was inversely related to the antagonistic levels. A relatively similar result was also found in strain 1021 [11]. Bacillomycin D and fengycin were previously reported for their antifungal activities against various fungi [42]. However, there was little information regarding the role of bacillomycin D in suppression of rice blast. On the other hand, fengycin was demonstrated in several previous studies for its suppression of *P. oryzae* growth [43-45]. Fengycin injured fungal cells by increasing the permeability of the cell membrane [46]. This was consistent with the swelling of affected mycelial cells in this study. Bacillibactin was a siderophore and originally considered for its function in iron scavenging [47]. Surfactin was known for its indirect suppression of plant diseases by the induction of induced systemic resistance (ISR) [42]. Based on the results obtained in our study and the previous reports, we proposed that fengycin from strain 2211 is the bioactive compound that suppresses rice blast in KDML105 seedlings.

Regulation of NRP production may be differentially and specifically regulated by various regulatory genes. A previous study showed that *codY*, *comA*, *degU* and *spo0A* were likely the positive regulators of bacillomycin D production in *B. amyloliquefaciens* fmbJ [46]. Fengycin production was not affected by *codY* deletion but highly reduced in *spo0A*, *comA* and *degU* deletion mutants [46]. In contrast, surfactin production was negatively regulated by *codY* and *degU* and positively regulated by *comA* and *spo0A* [48]. *degQ* was another regulator that was previously demonstrated for its positive effect on fengycin production in *B. subtilis* NCD-2 [49]. It phosphorylated downstream DegQ kinase which subsequently stimulated DegU [50]. *codY*, *comA*, *degU*, *spo0A* and *degQ* were also annotated in the genome of *B. velezensis* 2211. Whether their functions were similar to those of other *Bacillus* species still remains unknown and requires further investigation using deletion mutants.

4. Conclusions

In this study, we demonstrated the broad-range antagonistic activity of *B. velezensis* 2211 against *P. oryzae*, *C. aenigma*, *C. fructicola* and *F. oxysporum* mycelia. Except for *F. oxysporum*, spore germination of the tested fungi was also inhibited. The use of culture media likely affected the production level of the bioactive antifungal compound. This suggested an increase of antifungal

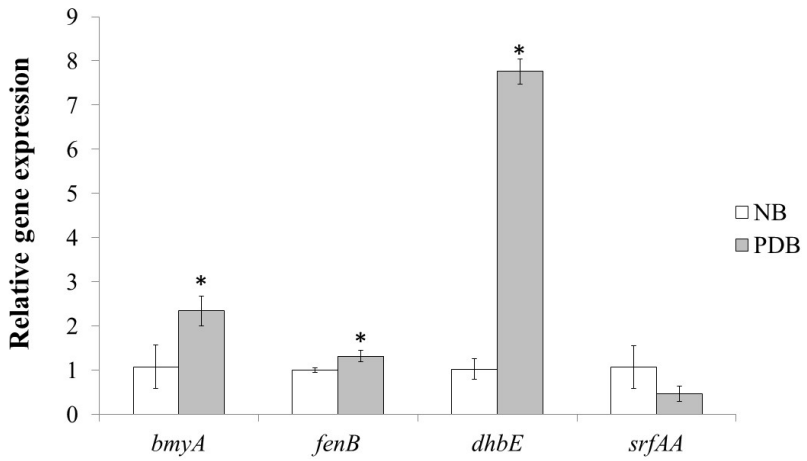

Figure 9. Relative gene expression of selected core biosynthesis genes of the BGCs responsible for the biosynthesis of bacillomycin D (*bmyA*), fengycin (*fenB*), bacillibactin (*dhbE*) and surfactin (*srfAA*). Error bars indicate the standard deviation ($n = 3$). Asterisks indicate statistically significant differences ($P < 0.05$), based on the Student *t*-test.

activity could be achieved through the modification of the culture media. Coupled with the antagonistic activity analysis, genome sequencing and gene expression analysis suggested that fengycin was likely the bioactive compound that suppressed rice blast disease in cultivar KDML105 seedlings. This study demonstrated the potential and the possible antagonistic mechanism of *B. velezensis* 2211 as a biocontrol agent. However, since the genome sequence of strain 2211 was still incomplete, the core biosynthetic genes of the fengycin BGC were found in three separate contigs. This suggested the requirement of the complete genome for a thorough understanding of BGC structure and function.

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