Current Applied Science and Technology Vol. 21 No. 4 (October-December 2021)

Medium Effect on Antagonistic Activity and Detection of Nonribosomal Peptide Synthetase Genes in Epiphytic *Bacillus* Strains

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Received: 24 July 2020, Revised: 6 January 2021, Accepted: 26 February 2021

Abstract

The biosynthesis of non-ribosomal peptides (NRPs) in biocontrol bacteria was one of the major antagonistic mechanisms for their application in agriculture. *Bacillus* spp. 1021, 2211 and 3210 were previously shown to inhibit mycelial growth of the leaf blast fungus *Pyricularia oryzae*. Here, we aimed to further study the antagonistic mechanism in those three strains. Cell-free supernatants obtained from bacteria grown in potato dextrose broth (PDB) exhibited a higher degree of inhibition against *P. oryzae* when compared to those obtained from nutrient broth (NB). This indicated the effect of culture media in the production of extracellular antibiotic compounds by these strains. Phylogenetic analysis of their partial 16S rRNA gene sequences indicated a close relationship between the three strains and *Bacillus siamensis* KCTC13613^T, *Bacillus amyloliquefaciens* DSM7^T and *Bacillus velezensis* CR-502^T. Complete genome sequences of these *Bacillus* species were analyzed on the antiSMASH server to identify the presence of NRP biosynthesis gene clusters. Non-degenerate primers were designed for the detection of the core biosynthesis genes for surfactin (*srfAA*), fengycin (*fenC*) and bacillibactin (*dhbF*). All three genes were amplified in strains 1021 and 2211, while only *srfAA* and *dhbF* were detected in strain 3210. Phylogenetic analysis of the deduced amino acid sequences indicated that the sequences of strain 1021 were distinct from those of strains 2211 and 3210. This result indirectly suggests the possibility of NRP production as the antagonistic mechanism of these three strains.

Keywords: *Bacillus*; nonribosomal peptides; nonribosomal peptide synthetases; *Pyricularia oryzae*
DOI 10.14456/cast.2021.51

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1. Introduction

Members of the genus *Bacillus* are well known for their antimicrobial activities against various groups of phytopathogens. One of the antagonistic mechanisms is the production of bioactive compounds that are collectively known as antimicrobial peptides (AMPs) [1]. These peptides consist of proteinogenic and non-proteinogenic amino acids that are linked together by peptide bonds. Based on the biosynthesis pathways, AMPs can be divided into two subgroups. The first group is ribosomally synthesized peptide antibiotics, e.g., subtilin, coagulin and entomocin [1]. The second group is known as NRPs. Members of this group, such as fengycins, lichenysins and surfactins, are synthesized non-ribosomally [1]. Several NRPs from members of the genus *Bacillus* were found to have inhibitory effects against phytopathogenic fungi. Bacillomycin D produced by *Bacillus vallismortis* ZZ185 displayed antifungal activities against *Alternaria alternata* and *Fusarium graminearum* [2]. Another study showed that iturin- and fengycin-like peptides from *Bacillus amyloliquefaciens* LBM 5006 suppressed the growth of *Aspergillus* spp. [3]. Fengycin from *Bacillus subtilis* BBG201 was implicated in the inhibition of *Botrytis cinerea* [4].

Nonribosomal peptide synthetases (NRPSs) are large enzyme complexes that biosynthesize NRPs [5]. An NRPS complex consists of multiple enzymatic modules [6]. Generally, a module contains three functionally distinctive domains including adenylation, thiolation and condensation domains. The adenylation domain acts as a gate keeper that selects a specific amino acid to be incorporated into an NRP molecule. It catalyzes two important steps. The first one is the adenylation of an amino acid using an ATP molecule. This results in the formation of aminoacyl-AMP. The second step is the transfer of the aminoacyl-AMP molecule to the pantetheine cofactor of the thiolation domain. Finally, the amino acid that is carried on the module is linked to the preceding one by the peptide bond generated by the condensation domain. Additional domains could be found on some modules [6]. For example, the thioesterase domain is present in the last module to catalyze either the release or cyclization of the NRP molecule [6]. Some modules may carry domains that modify the amino acid structure [6]. These structural and functional aspects of NRPS domains generate diverse NRPs with different biological activities. NRPSs are encoded by large gene clusters on bacterial genomes [7]. Amplification of partial NRPS gene fragments could be used as a primary indicator of NRP production. Primers are designed based on the conserved regions of NRPS genes. Antagonistic bacterial strains in major NRP-producing taxa including actinomycetes, *Bacillus* and *Pseudomonas* have been previously identified using this method [8-12]. This indicates the effectiveness of the molecular approach for identification of potential antagonistic bacteria.

Strains 1021, 2211 and 3210 were previously identified as members of the genus *Bacillus* based on approximately 900-bp 16S rRNA gene sequences [13]. The dual-culture test on potato dextrose agar (PDA) showed that the bacteria were among the strains that displayed the highest growth inhibition against *P. oryzae* mycelia [13]. However, the crude extracts, obtained from the bacterial culture in NB medium, were unable to suppress the fungus. It was surmised that the antagonistic activities may depend on the medium used for the bacterial culture [13]. Additionally, the antagonistic mechanisms of these three strains were not known. Thus, the first objective of the present work was to investigate the effect of the culture media PDB and NB on the production of antifungal compounds by these strains. The second aim was to indirectly study the antagonistic mechanism of these *Bacillus* strains. This was done by amplification and phylogenetic analysis of the partial fragments of genes coding for NRPSs, since members of the genus *Bacillus* were known to produce bioactive NRPs [4]. The results obtained from this study suggest the possible mechanisms of the antagonistic activity in *Bacillus* spp. 1021, 2211 and 3210.

2. Materials and Methods

2.1 Effects of culture media on antagonistic activities

Strains 1021, 2211 and 3210 were obtained from our previous study [13]. Single colonies grown on nutrient agar (Himedia, India) were inoculated in 50 ml tubes containing 10 ml of NB (Himedia, India) or PDB (Himedia, India). The tubes were placed on a rotary shaker at 180 rpm and incubated at 30ºC for 72 h. Bacterial cultures were centrifuged at 8,000 rpm for 15 min to obtain the supernatants. The supernatants were filter-sterilized using 0.2 µm diameter filters. *Pyricularia oryzae* was grown on PDA (Himedia, India) at 30ºC for 7 days and used for preparation of the mycelial plugs with a cork borer. Six *P. oryzae* mycelial plugs were submerged in 5 ml of the filter sterilized supernatants and incubated at 30ºC for 48 h. Mycelial plugs incubated in sterilized distilled water were used as controls.

2.2 Bacterial DNA isolation

Strains 1021, 2211 and 3210 were grown in 5 ml NB at 30ºC for 24 h. Total genomic DNA was isolated using a Bacterial Genomic DNA Kit (Geneaid, Taiwan), following the manufacturer's protocol. DNA concentration and purity were examined using agarose gel electrophoresis and Nanodrop spectrophotometer (Thermo Fischer Scientific, USA.).

2.3 Amplification and phylogenetic analysis of 16S rRNA gene sequences

16S rRNA gene fragments of strains 1021, 2211 and 3210 were amplified, using universal 41F (5'-GCTCAGATTGAACGCTGGCG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') [14-15]. The amplified products were purified, using a Gel/PCR Purification Kit (Favorgen, Taiwan), and subsequently sequenced using additional universal primers 337F, 785F and 800R by Macrogen Inc. (South Korea). Partial 16S rRNA gene sequences were obtained. The pairwise alignment analysis was done on EzBioCloud [16]. Sequences of related *Bacillus* strains were obtained from the GenBank database. The multiple alignment was analyzed with the CLUSTAL W program version 1.81 [17]. After manual adjustment and correction of gaps and ambiguous nucleotides, phylogenetic trees were reconstructed using the neighbour joining [18], maximum parsimony [19] and maximum likelihood [20] methods in MEGA7 [21]. The bootstrap analysis [22], with 1,000 re-samplings, determined the confidence levels of the clusters.

2.4 Amplification of NRPS genes

Complete genome sequences of related *Bacillus* strains were obtained from the GenBank database. The sequences were submitted to antiSMASH version 4.0 [23] for the prediction of NRPS gene clusters. Sequences of NRPS modules were selected and subjected to blastn analysis. Sequences of high similarity levels from different *Bacillus* species were obtained from the GenBank database and aligned using the Clustal Omega program [24]. In this study, non-degenerate primers were manually designed for the amplification of the adenylation domain of *srfAA*, *fenC* and *dhbF* genes (Figure 1). Primer sequences are provided in Table 1. The primers were used for amplification of the target genes from the genomic DNA of strains 1021, 2211 and 3210. The thermal cycles for amplification were: 94ºC for 3 min; 40 cycles of 94ºC for 30 s, 56ºC for 30 s, and 72ºC for 90 s; 72ºC for 5 min. PCR products were purified and sequenced using their corresponding primers. The amplified sequences were submitted for blastx analysis. The multiple alignment analysis was

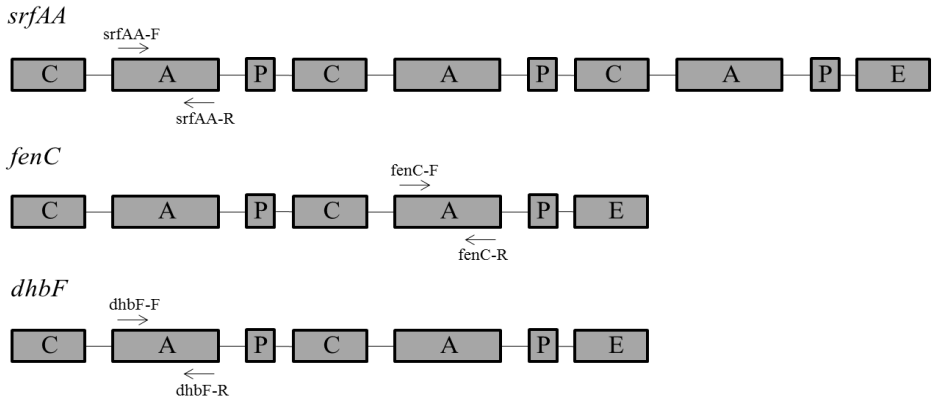

Figure 1. Schematic representation of *srfAA*, *fenC* and *dhbF* coding regions and primer-binding positions. Letters indicate the genomic regions that code for different NRPS functional domains. C, condensation domain; A, adenylation domain; P, peptidyl carrier protein domain; E, epimerization domain. Arrows indicate the primer positions and directions.

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

Primer	Target gene	NRP	Primer sequence (5′→3′)
srfAA-F	*srfAA*	Surfactin	CCGATCTGACCGTGTTATTGCG
srfAA-R			CGCGGACTTTCACTTGATCGTC
fenC-F	*fenC*	Fengycin	CTGAACGAACGGGCTAACAG
fenC-R			CCGATAACCGCGGATTTTCAC
dhbF-F	*dhbF*	Bacillibactin	CTATGCCGAATTGAACAAGCG
dhbF-R			CGGCTCTGCTGATATAATCAAG

performed between the deduced amino acid sequences of strains 1021, 2211 and 3210 and their related amino acid sequences, using the program Clustal W. Gaps were manually removed, and phylogenetic trees were reconstructed, using the neighbour-joining method. The bootstrap analysis was performed with 1,000 re-samplings.

3. Results and Discussion

Strains 1021, 2211 and 3210 were originally isolated from root, stem and leaf surfaces of rice plants, respectively [13]. Their antagonism against *P. oryzae* was previously shown, using dual-culture assay on PDA plates [13]. This indicated the antagonistic activities may involve the production of extracellular antibiotic compounds. The supernatants from bacterial cultures grown in NB medium were obtained and used to prepare the crude extracts. However, they failed to suppress fungal growth when tested by disc-diffusion assay [13]. In the present work, the determination of medium effects on the antagonistic activity of these strains was carried out (Figure 2). The use of PDB for bacterial culture increased the antagonistic activity of all three

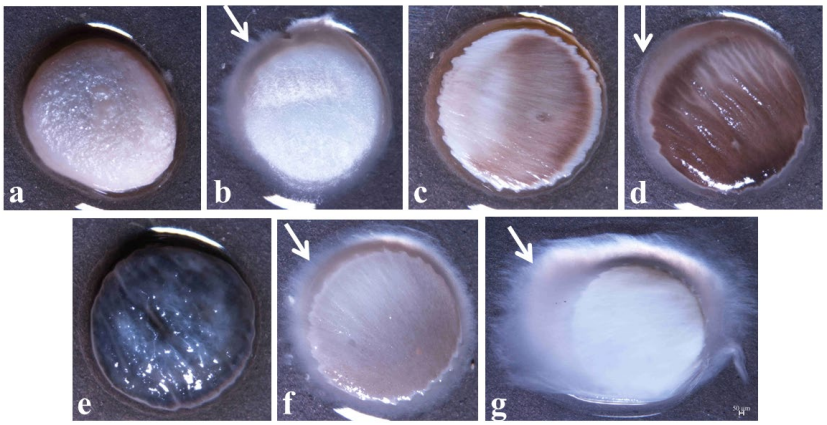

Figure 2. *Pyricularia oryzae* mycelial plugs incubated in PDB- (a, c, e) and NB-derived (b, d, f) supernatants of strains 1021 (a, b), 2211 (c, d) and 3210 (e, f), compared with sterilized distilled water (g). Arrows indicate mycelial growth of *P. oryzae* around the edge of the plugs.

strains when compared to NB. This was indicated by the absence of mycelial growth from the plugs that were incubated in PDB-derived supernatants. In contrast, mycelial growth was still visible when the plugs were submerged in NB-derived supernatants. Extensive mycelial growth of the control group that was treated with sterilized distilled water was clearly visible. This suggested PDB was more suitable for induction of the antagonistic activity in strains 1021, 2211 and 3210.

Strains 1021, 2211 and 3210 were previously tested positive for antagonistic activities against *P. oryzae*, the causative agent of leaf blast in rice [13]. The inhibition of the fungal mycelial growth by filter-sterilized supernatants suggested that the mechanism for the activity was the production of extracellular antibiotics. Additionally, the activity depended on the medium used for bacterial culture. The PDB-based supernatants exhibited a stronger inhibitory effect against *P. oryzae* than the ones obtained with NB medium. The result presented here was rather counterintuitive, regarding the purposes of both media. PDB is generally used for growing fungi, while NB is commonly used in bacterial culture. However, a previous study showed that *Bacillus pumilus*, cultured in PDB, produced antifungal metabolites that inhibited growth of several species of genera *Aspergillus*, *Penicillium* and *Fusarium* [25]. Another study showed that the use of PDB as the fermentation medium with *Bacillus natto* NT-6 yielded the highest level of surfactin production, compared to lysogeny broth, NB and Landy medium [26]. Additionally, modified PDB medium was used to induce bioactive compounds by *Bacillus subtilis* 7PJ-16 to reduce mulberry fruit sclerotiniose in a field experiment [27]. Based on these observations, PDB may be more appropriate than NB for inducing the antagonistic activity in strains 1021, 2211 and 3210.

Strains 1021, 2211 and 3210 were primarily affiliated with the genus *Bacillus* based on approximately 900-bp 16S rRNA gene sequences [13]. To understand their phylogenetic positions, approximately 1,400-bp long 16S rRNA gene sequences were obtained here. The pairwise alignment of the sequences was analyzed on the EzBioCloud database. Strain 1021 showed the highest similarity (100%) with *Bacillus siamensis* KCTC 13613^T. On the other hand, the similarity levels with *Bacillus velezensis* CR-502^T for strains 2211 (99.93%) and 3210 (99.86%) were the highest. Based on the reconstructed phylogenetic tree, the three strains were phylogenetically related to *B. siamensis* KCTC13613^T, *B. amyloliquefaciens* DSM7^T and *B. velezensis* CR-502^T (Figure 3). Both the maximum-parsimony and maximum-likelihood methods showed similar matches.

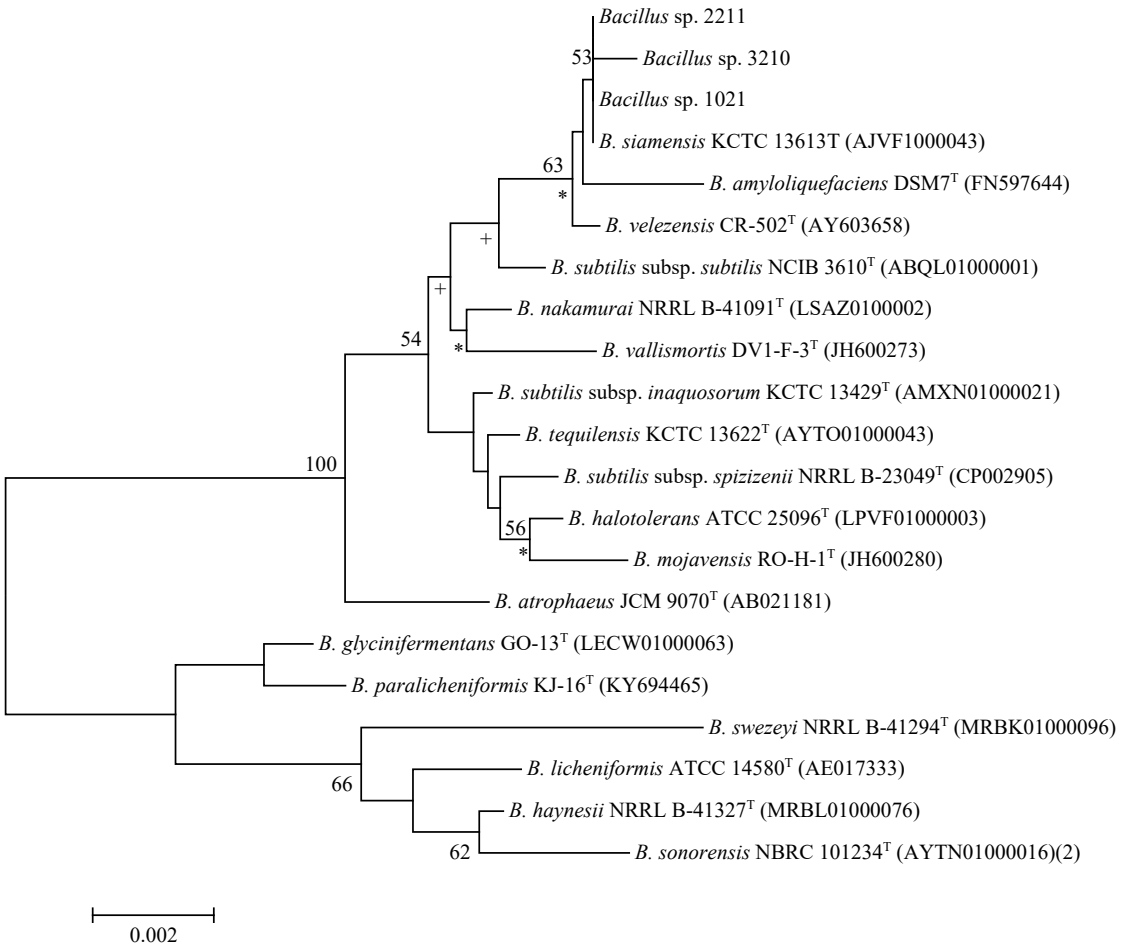

Figure 3. Phylogenetic tree reconstructed from partial 16S rRNA gene sequences of strains 1021, 2211, 3210 and other related *Bacillus* strains using the neighbour-joining method. Only bootstrap levels higher than 50% are shown on nodes. + indicates branches that were also found using the maximum-likelihood method. * indicates branches that were also found using both maximum-parsimony and maximum-likelihood methods. Bar represents 0.002 substitutions per nucleotide position.

Phylogenetic analysis of the 16S rRNA gene sequences indicated that strains 1021, 2211 and 3210 were related to *B. siamensis* KCTC13613^T, *B. amyloliquefaciens* DSM7^T and *B. velezensis* CR-502^T. Consistently, strains of these *Bacillus* species were reported as antagonistic bacteria against the phytopathogenic fungus that caused the blast disease in rice [28-30]. Additionally, some strains were antagonistic against other phytopathogenic fungi, e.g., *Fusarium graminearum* [31], *Botrytis cinerea* [32], *Rhizoctonia solani* [33], *Fusarium solani* and *Fusarium oxysporum* [34]. Applications of these *Bacillus* species as biocontrol agents were effective against various plant diseases including tomato crown gall [35], potato common scab [36] and *Fusarium* head blight in wheat [37].

Several *Bacillus* species were known to carry various NRPS gene clusters [1, 7, 28]. Complete genome sequences of *B. siamensis* SCSIO 05746 (accession number: CP025001), *B. amyloliquefaciens* DSM7^T (FN597644) and *B. velezensis* FZB42 (CP000560) were obtained from the GenBank database. Based on the antiSMASH sever, all three bacterial genomes were predicted to carry biosynthesis gene clusters for three different NRPs, including surfactin, fengycin and bacillibactin. The similarity levels ranged from 82% to 100%. This information was used to investigate the presence of the core biosynthesis genes for surfactin (*srfAA*), fengycin (*fenC*) and bacillibactin (*dhbf*) in strains 1021, 2211 and 3210. Amplification of *srfAA* [38], *fenC* [39] and *dhbf* [40] was previously used as indirect indicators of antagonistic *Bacillus* strains against phytopathogenic fungi. Non-degenerate primers were designed for amplification of the adenylation domain of the genes. The amplified product was approximately 1,000 bp (Figure 4). The *srfAA* and *dhbF* genes were amplified in all three strains, while *fenC* was detected only in strains 1021 and 2211. Sequences of the DNA fragments were used for the blastx analysis (Table 2). The sequence similarities ranged from 98.4% to 100%. The sequences of strain 1021 were similar to NRPS sequences of *B. siamensis*. The sequences of strain 2211 were similar to those of *B. amyloliquefaciens*. In contrast, the *srfAA* sequence of strain 3210 was similar to *B. amyloliquefaciens*, while its *dhbF* sequence was more similar to that of *B. velezensis*.

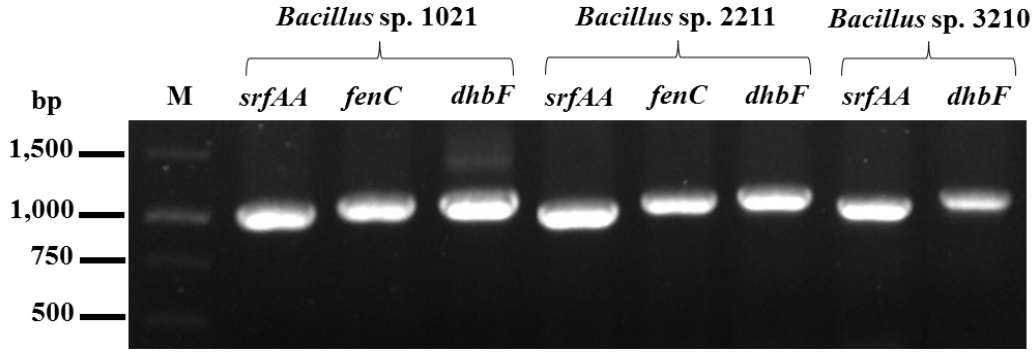

Figure 4. Amplified NRPS gene fragments of strains 1021, 2211 and 3210. M: 1kb DNA ladder

The predicted NRP products from the complete genomes of *B. siamensis* SCSIO 05746, *B. amyloliquefaciens* DSM7^T and *B. velezensis* FZB42 enabled the selection of *srfAA*, *fenC* and *dhbF* genes to be determined in the three strains. Successful amplification of *srfAA* and *dhbF* was observed in all three strains. In contrast, *fenC* was amplified only in strains 1021 and 2211, which suggested the absence of *fenC* in strain 3210 or the primers may alternatively fail to bind to the *fenC* gene of strain 3210. The blastx analysis consistently showed that all amplicons were parts of the NRPS genes. This result indicated that the complete genome sequences of *Bacillus* strains could be valuable for the study of NRPS genes in related bacteria whose genome sequences were not yet available. It also showed that non-degenerate primers could amplify the NRPS gene targets. Previously, other studies used degenerate primers for amplification of NRPS genes [41, 42], as opposed to the non-degenerate primers used here. Another previous study used non-degenerate primers to amplify five biosynthesis genes of bacyllomycin, fengycin, iturin, surfactin and bacilysin in four *Bacillus* strains [43]. However, the amplified products ranged from 269 bp to 498 bp and were relatively shorter than the ones obtained here.

Table 2. Blastx results of the nucleotide sequences of strains 1021, 2211 and 3210

Strains	Amplicon fragments	Most similar proteins	Accession number	Sequence similarity
1021	*srfAA*	non-ribosomal peptide synthetase, partial (*Bacillus siamensis*)	WP_016936794	98.40%
1021	*fenC*	non-ribosomal peptide synthetase (*Bacillus siamensis*)	WP_081484472	100%
1021	*dhbF*	non-ribosomal peptide synthetase (*Bacillus siamensis*)	WP_095241403	98.47%
2211	*srfAA*	Surfactin non-ribosomal peptide synthetase SrfAA (*Bacillus amyloliquefaciens*)	WP_115996107	100%
2211	*fenC*	Plipastatin synthase subunit C (*Bacillus amyloliquefaciens*)	CUB46431	99.28%
2211	*dhbF*	non-ribosomal peptide synthetase (*Bacillus amyloliquefaciens*)	WP_115996640	100%
3210	*srfAA*	non-ribosomal peptide synthetase (*Bacillus amyloliquefaciens*)	WP_021494208	100%
3210	*dhbF*	non-ribosomal peptide synthetase (*Bacillus velezensis*)	WP_095273337	99.70%

The higher antagonistic activity of PDB-derived culture filtrate was similar between strains 1021, 2211 and 3210. The presence of the core biosynthesis genes indirectly indicated strains 1021 and 2211 were potential producers of surfactin, fengycin and bacillibactin, whereas strain 3210 was likely able to produce surfactin and bacillibactin. Previously, the regulation of surfactin production was shown to be regulated by quorum sensing which involved the critical cell density [44]. Thus, the greater effect of PDB over NB in this study may be derived from different growth rates in the two media. Additionally, as a preliminary study, the phylogenetic relationship between the deduced amino acid sequences of the amplified products was investigated. The sequences of strain 1021 were closely related to those of *B. siamensis* and distant from the sequences of strains 2211 and 3210 which were more related to *B. amyloliquefaciens* and *B. velezensis* (Figures 5-7). Based on this analysis, we hypothesized that the amino-acid compositions of NRP molecules produced by strain 1021 and those by strains 2211 and 3210 may be different. This was because the amplified fragments were derived from the adenylation domain that functioned in amino acid selection of the biosynthesis of NRP molecules [5]. Further characterization of the antibiotic compounds produced by these antagonistic strains is needed to confirm this hypothesis.

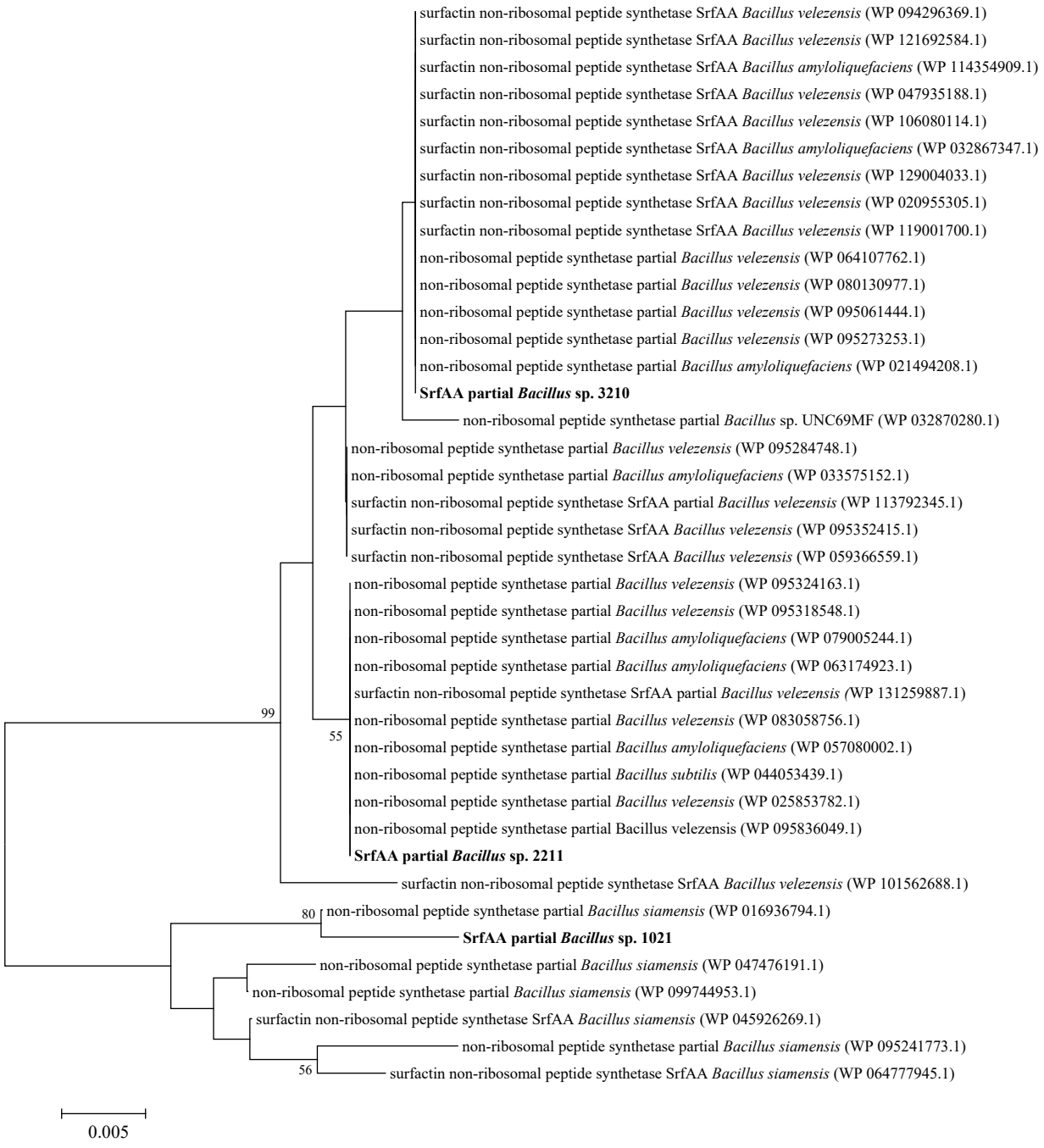

Figure 5. The phylogenetic tree based on the SrfAA sequences of strains 1021, 2211 and 3210 and other related NRPS sequences. The tree was reconstructed, using the neighbour-joining method. Only bootstrap levels higher than 50% are shown on the nodes. The bar indicates 0.005 substitutions per amino acid position.

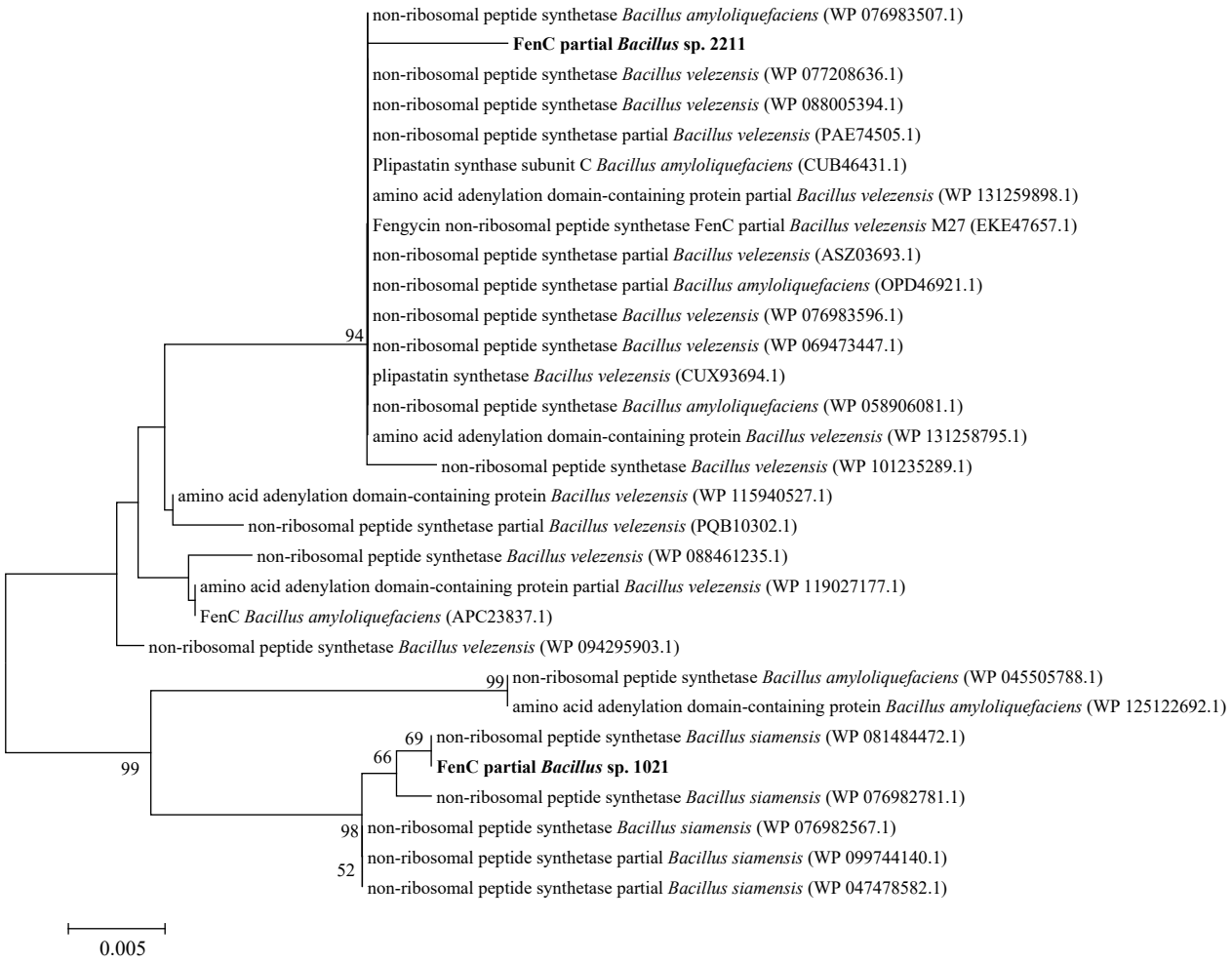

Figure 6. The phylogenetic tree based on the FenC sequences of strains 1021, 2211 and other related NRPS sequences. The tree was reconstructed, using the neighbour-joining method. Only bootstrap levels higher than 50% are shown on the nodes. The bar indicates 0.005 substitutions per amino acid position.

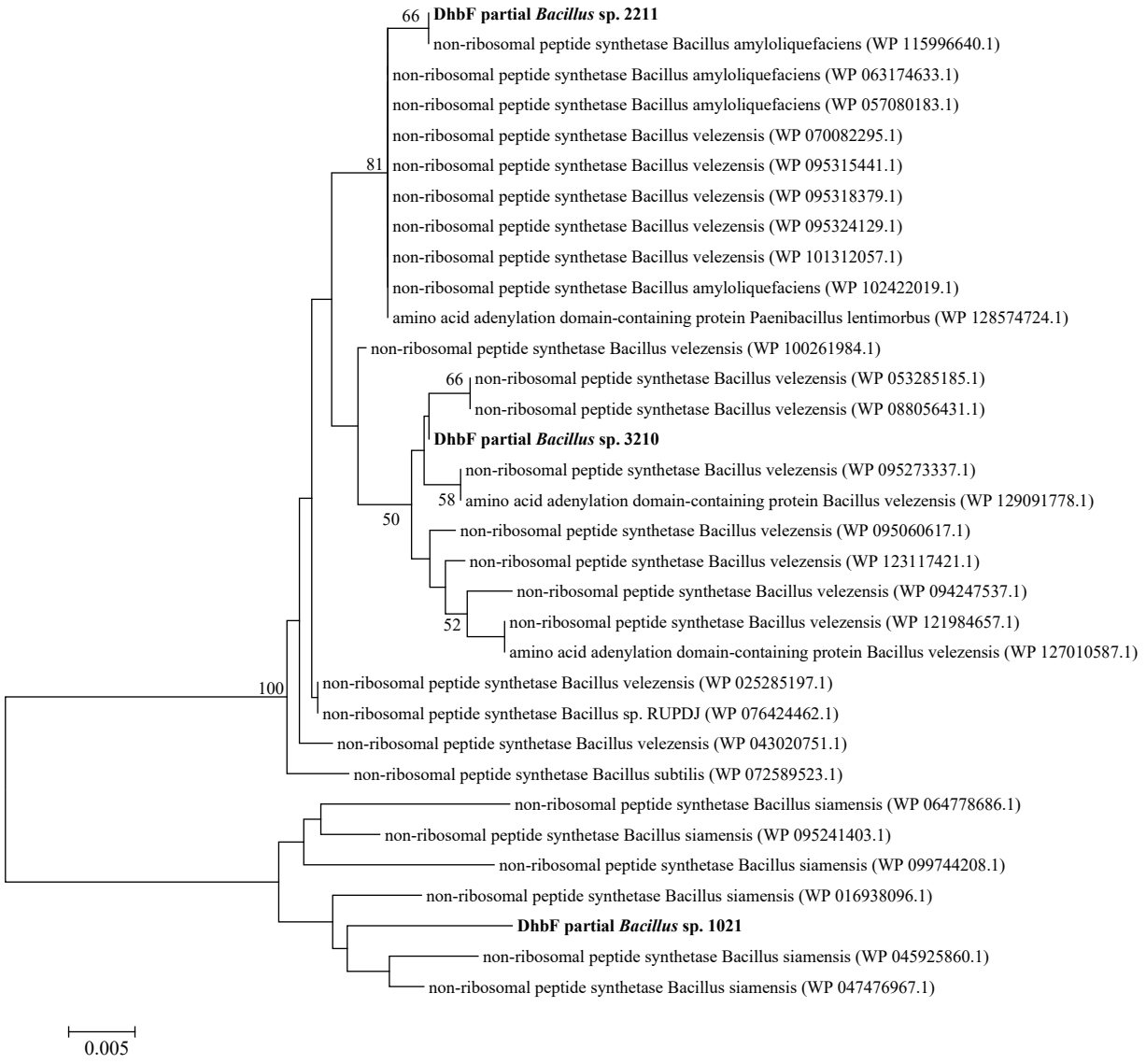

Figure 7. The phylogenetic tree based on the DhbF sequences of strains 1021, 2211, 3210 and other related NRPS sequences. The tree was reconstructed, using the neighbour-joining method. Only bootstrap levels higher than 50% are shown on the nodes. The bar indicates 0.005 substitutions per amino acid position.

4. Conclusions

The present study showed that the antibiotic production of *Bacillus* spp. 1021, 2211 and 3210 grown in PDB was likely higher than NB. The phylogenetic analysis of the 16S rRNA gene sequence indicated that the three strains were closely related to *B. siamensis* KCTC13613^T, *B. amyloliquefaciens* DSM7^T and *B. velezensis* CR-502^T. Non-degenerate primers of the core biosynthesis genes *srfAA*, *fenC* and *dhbF* were designed and used for detection of the partial fragments of these genes in strains 1021, 2211 and 3210. All three genes were detected in strains 1021 and 2211, while only *srfAA* and *dhbF* were amplified in strain 3210. The phylogenetic analysis of the deduced amino acid sequences indicated the distinction between the sequences of strain 1021 and those of strains 2211 and 3210.

5. Acknowledgements

The culture of *P. oryzae* was kindly provided by Asst. Prof. Dr. Nonglak Parinthawong of the Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang.

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