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Research article

Genomic characterization for secondary metabolite biosynthetic genes of *Microbispora* sp. KK1-11

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Abstract

Background: Rare actinomycetes, especially genus *Microbispora*, have attracted attention because of their ability to produce various bioactive secondary metabolites. Many valuable secondary metabolites, such as bispolides, linfuranone A, and microbiaeratin, produced by *Microbispora* spp. have been reported, and there is great interest in efforts to discover new *Microbispora* spp. with the ability to produce such metabolites. The application of in silico biosynthetic predictions from genome mining data is usually used to identify promising *Microbispora* spp. in nature. In this study, we used existing genomic data to characterize the taxonomic position of *Microbispora* sp. KK1-11 and to identify biosynthetic gene clusters (BGCs) in the genome. **Methods:** An actinomycete strain KK1-11 was taxonomically characterized using a polyphasic approach. To confirm the taxonomic classification of the strain at the genus level, morphological, chemotaxonomic, and 16S rRNA gene sequence analyses were performed. Whole-genome shotgun sequencing was carried out using an Illumina MiSeq 1TB platform. The genomic data were evaluated for the production of secondary metabolites using the antiSMASH platform and the antibacterial activity of metabolites was also assessed. **Results:** Actinomycete strain KK1-11 was taxonomically characterized as a member of the genus *Microbispora.* Genomic-based identification revealed that while KK1-11 is most closely related to *Microbispora catharanthi* CR1-09T, the average nucleotide identity value was low at 94.44%. Genome analysis by antiSMASH revealed that KK1-11 contains several secondary metabolite BCGs (smBGCs), including type I and III polyketide synthase, terpene biosynthesis, non-ribosomal peptide synthetase (NRPS), and NRPS-like gene clusters. In addition, several smBGCs in the genome of KK1-11 showed no relatedness to known clusters. **Conclusion:** This study demonstrates that *Microbispora* sp. KK1-11 may represent

a novel member of the genus *Microbispora* capable of producing new secondary metabolites, representing a soil actinomycete with the potential to produce valuable bioactive compounds.

Keywords: *Microbispora*, Whole-genome sequence analysis, Actinomycete, Secondary metabolites.

Introduction

Genus *Microbispora*, of the family *Streptosporangiaceae*, is a rare actinomycete with a lower isolation frequency than the more common actinomycete strain, *Streptomyces* spp., when isolated by conventional methods. Members of this genus play important roles in soil environments as producers of humus, plant nutrients and hormones, and bioactive secondary metabolites [1,2,3,4,5], functioning as biocontrol and plant growth-promoting microorganisms. *Microbispora* spp. have been shown to produce many secondary metabolites with diverse biological activities, such as bispolides[4], linfuranone A[5], and microbiaeratin[6]. The secondary metabolite biosynthesis genes of actinomycetes are often clustered at one genomic locus, e.g., polyketide synthases (PKSs), non-ribosomal peptide synthases (NRPSs), and terpene synthase. Bioinformatics tools such as antiSMASH[7] and MultiGeneBlast[8] are used to detect and compare gene clusters in actinomycete genomes, facilitating identification of new secondary metabolite production. As *Microbispora* spp. have the capacity to produce several potential antibiotics, the practice of screening such organisms for new bioactive compounds is an important endeavor. During our investigation of novel actinomycetes from soil, we successfully isolated a *Microbispora*-like strain, KK1-11, which possesses typical morphological characteristics and antimicrobial activity against Gram-positive bacteria *Staphylococcus aureus* ATCC 25923, methicillin-resistant *S. aureus* (MRSA), and *Bacillus subtilis* ATCC6633. Here we report the genomic characterization of KK1-11 for the production of the secondary metabolites.

Materials and Methods

Isolation, cultivation, and preservation of KK1-11

An actinomycete strain KK1-11 was isolated from a soil sample collected from an herbal garden in Pathum Thani province, Thailand. The air-dried soil was heated at 120°C for 1 h and 10 g of soil sample was mixed with 90 ml sterile 0.85% NaCl solution. The soil solution was serially (10-fold) diluted with 0.01% sterile sodium dodecyl sulphate (SDS) in distilled water to a dilution of 10^{-3} and spread onto humic acid-vitamin agar supplemented with nalidixic acid (20 mg/l) and nystatin (50 mg/l). After incubation at 30°C for 21 days, a yellowish-pink colony of KK1-11 was isolated and subcultured on yeast extract-malt extract agar (International *Streptomyces* Project-2 (ISP2) medium)[9]. The pure culture was maintained in glycerol solution (20% (v/v)) at -80°C or lyophilized for long-term preservation.

Taxonomic characterization of KK1-11

Phenotypic characterization

Morphological properties of KK1-11 were observed by light microscopy (ECLIPSE E200; Nikon, Japan) and spore morphologies were observed by scanning electron microscopy (JSM-6610 LV; JEOL, Japan). To prepare the samples for scanning electron microscopy observation, a cultured agar block (3 × 5 mm^2) was fixed with 2% osmium tetroxide vapor for 2 h. The samples were then gently washed with sterile distilled water. The dehydration step was done through a graded ethanol series: 30%, 50%, 70%, 95%, each for 10–15 min, followed by 100% ethanol (10 min) three times. The dehydrated samples

were dried using liquid carbon dioxide in a critical-point dryer (EM CPD300; Leica, Germany). Finally, the specimens were attached to a stub and coated with gold using a sputter coater (SCD040; Balzers, Germany). Cultural characteristics were evaluated on ISP media no. 1–7 after culturing for 14 days at 30°C. Colors of aerial and substrate mycelia and any soluble pigments were assigned with the ISCC-NBS color charts[10]. Growth under various conditions, namely temperature (10–60°C), NaCl tolerance (0–7% (w/v)), and pH (4–12 at intervals of 1 pH units), were tested in ISP2 broth for 14 days. The utilization of sole nitrogen sources; decomposition of adenine, hypoxanthine, xanthine, tyrosine and cellulose; hydrolysis of starch; reduction of nitrate; peptonization; coagulation of milk; liquefaction of gelatin; and acid production from carbon sources were tested as described previously[11,12,13].

Chemotaxonomic analyses

To confirm the taxonomic classification of the strain at the genus level, several chemotaxonomic characteristics, namely the isomer of diaminopimelic acid (DAP), whole-cell sugars in cell hydrolysates, and types of menaquinones, were analyzed following protocols by Hasegawa et al.[14], Komagata & Suzuki[15], and Collins et al.[16], respectively.

Genotypic characterization

Genomic DNA of KK1-11 was extracted in accordance with a method[17] described previously. Polymerase chain reaction (PCR) amplification of partial 16S rRNA gene (1,480 bp) was conducted using the universal primers 9F (5'-GAGTTTGATCCTGGCTCAG-3')[18] and 1541R (5'-GTTACCTTGTTACGACTT-3')[19]. Sequencing of the PCR product was carried out using the 780R (5'-CTACCAGGGTATCTAATCC-3'), 350F (5'-TACGGGAGGCAGCAG-3'), 780F (5'-GATTA GATACCCTGGTAG-3'), and 1541R primers[20]. The 16S rRNA gene sequence was submitted to the EzBioCloud server[21] to calculate sequence similarity. To construct the phylogenetic tree, the CLUSTAL W multiple alignment modes within the BioEdit program version 7.1.3.0[22] were used to align 16S rRNA gene sequences obtained in this study with *Microbispora* strains from the EzBioCloud database. A neighbor tree was constructed using program MEGA version 6.0 [23], and the stability of the clades in the tree was assessed by bootstrap analysis with 1,000 resamplings[24].

Genome analysis for secondary metabolites of KK1-11

Genomic DNA was extracted at 30°C using a GeneJET Genomic DNA purification Kit (Thermo Scientific, USA). Whole-genome shotgun (WGS) sequencing was performed using an Illumina MiSeq 1TB platform (Illumina Inc., USA) and assembled de novo using SPAdes version 3.10.1[25]. The genome was annotated using the online server Rapid Annotations using Subsystems Technology (RAST) (http://rast.nmpdr.org/)[26,27]. The online web service, JSpeciesWS, was used to calculate the average nucleotide identity (ANI) and digital DNA G+C values[28]. To determine the digital DNA-DNA hybridization (dDDH) value between the genome of KK1-11 and *Microbispora catharanthi*, the Genome-to-Genome Distance Calculator version 2.1[29] was used. Program antiSMASH was used to evaluate the secondary metabolite biosynthesis gene clusters in the bacterial genome[7].

Accession number of *Microbispora* sp. KK1-11

The GenBank/EMBL/DDBJ accession number for the complete 16S rRNA gene sequence of *Microbispora* sp. KK1-11 is LC333389. The WGS projects for *Microbispora* sp. KK1-11 are deposited at DDBJ/ENA/GenBank under the accession VIRJ00000000.

Cultivation of KK1-11 for secondary metabolite production

Microbispora sp. KK1-11 was grown on ISP2 agar at 30°C for 7 days. A loopful of substrate mycelium was inoculated in a 250 ml Erlenmeyer flask containing 100 ml of ISP2 broth. This culture was continuously cultivated for 14 days at 30°C on a rotary shaker (180 rpm).

Test microorganisms for antibacterial activity

Pseudomonas aeruginosa ATCC 27853, *Escherichia coli* ATCC 25922, *S. aureus* ATCC 25923, MRSA, and *B. subtilis* ATCC 6633, were screened for antibacterial activity.

Antibacterial activity screening

The agar-well diffusion method[30] was used for the evaluation of the antimicrobial activity of KK1-11-culture broth. Briefly, 0.1 ml of bacterial suspension (1×10^8 CFU/ml) was spread onto Mueller-Hinton agar (MHA) and allowed to dry for 5 min at room temperature. Holes 6 mm in diameter were made in the agar using a sterile cork borer. The KK1-11 culture broth (50 µl) was inoculated into each well on the culture agar. Inhibition zones were measured after incubating at 37°C for 24 h. ISP2 broth (50 µl) was used as a negative control. These studies were performed in triplicate.

Results and Discussion

Taxonomic characterization of KK1-11

Cultural, physiological, and biochemical characteristics of KK1-11

An actinomycete strain KK1-11 was isolated from a soil sample collected from an herbal garden in Pathum Thani province, Thailand. The strain formed yellowish-pink substrate mycelium and changed to white aerial mycelium after 14 days of cultivation. KK1-11 grew well on ISP2 and ISP3 agars, but showed moderate to poor growth on ISP4, ISP5, ISP6, and ISP7 media. Pale yellow diffusible pigment was observed on ISP2 medium. The longitudinally paired spores borne directly on aerial mycelia (Fig. 1) were detected easily on ISP2 and soil extract agar after cultivation for 21 days at 30°C. Spore arrangement of KK1-11

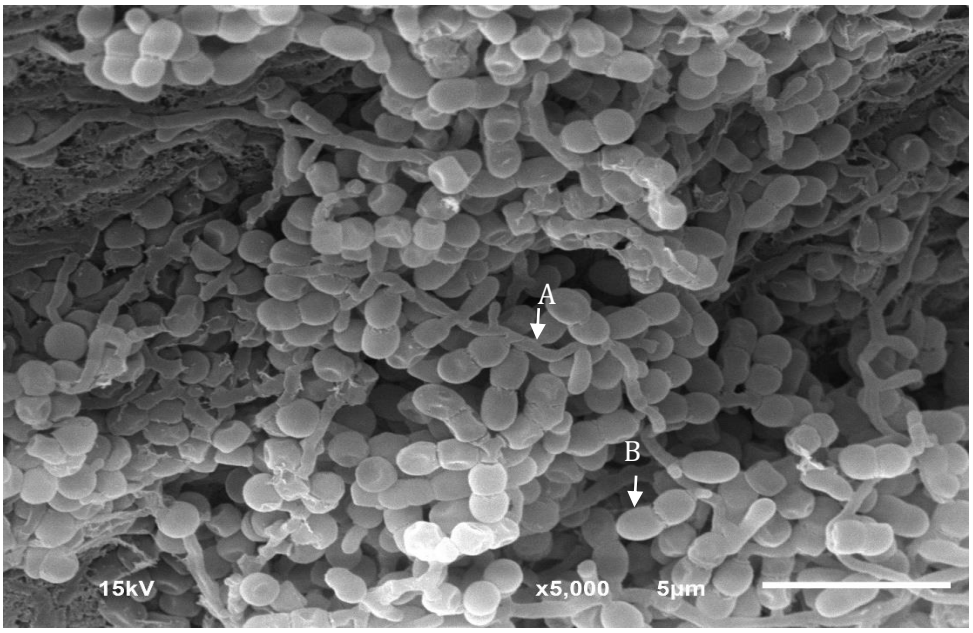


Figure 1. Scanning electron micrograph of KK1-11 grown on ISP2 agar for 21 days at 30°C. Scale bar, 5 µm. (A, mycelium; B, the longitudinally paired spores)

showed morphology typical of members of the genus *Microbispora*[31,32]. Growth of KK1-11 was observed at a temperature range of 20–42°C (optimum = 30°C) and a pH range of 6–10 (optimum = pH 6–7). NaCl tolerance was detected between 0–3%. KK1-11 was positive for oxidase and catalase activity. Like *M. catharanthi* CR1-09^T, KK1-11 gave positive results for starch hydrolysis; acid production from D-galactose, D-lactose, D-fructose, and D-xylose; and decomposition of CM-cellulose, xanthine, and adenine. In contrast, the results of urease activity; temperature, pH, and NaCl concentration growth conditions; acid production from D-ribose, D-cellobiose, and D-mannitol; utilization of DL-2-aminobutyric acid, 4-hydroxyproline, L-cysteine, L-asparagine, L-histidine, L-serine, and L-phenylalanine; and the decomposition of tyrosine and hypoxanthine can be clearly distinguished from *M. catharanthi* CR1-09^T (Table 1).

Table 1. Phenotypic characteristics of KK1-11 and the closest phylogenetically relative, *Microbispora catharanthi* CR1-09^T. All phenotypic data were determined in this study. +, Positive; -, Negative; w, Weakly positive.

Characteristics	*Microbispora* sp. KK1-11	*Microbispora catharanthi* CR1-09^T
Starch hydrolysis	+	+
Urease activity	+	-
Maximum NaCl tolerance (%, w/v)	3	2
pH range for growth	6–10	5–10
Temperature range for growth (°C)	20–42	15–40
Acid production from:		
D-ribose	-	w
D-galactose	+	w
D-lactose	w	+
L-raffinose	-	-
D-cellobiose	+	-
D-mannitol	+	-
D-fructose	+	+
D-xylose	+	w
Nitrogen utilization:		
L-methionine	+	w
DL-2-aminobutyric acid	+	-
4-hydroxyproline	-	+
L-cysteine	+	-
L-asparagine	-	+
L-histidine	-	+
L-serine	-	+
L-valine	-	-
L-arginine	+	+
L-phenylalanine	+	-
Decomposition of:		
Tyrosine	+	-
CM-cellulose	-	-
Hypoxanthine	+	-
Xanthine	-	-
Adenine	-	-

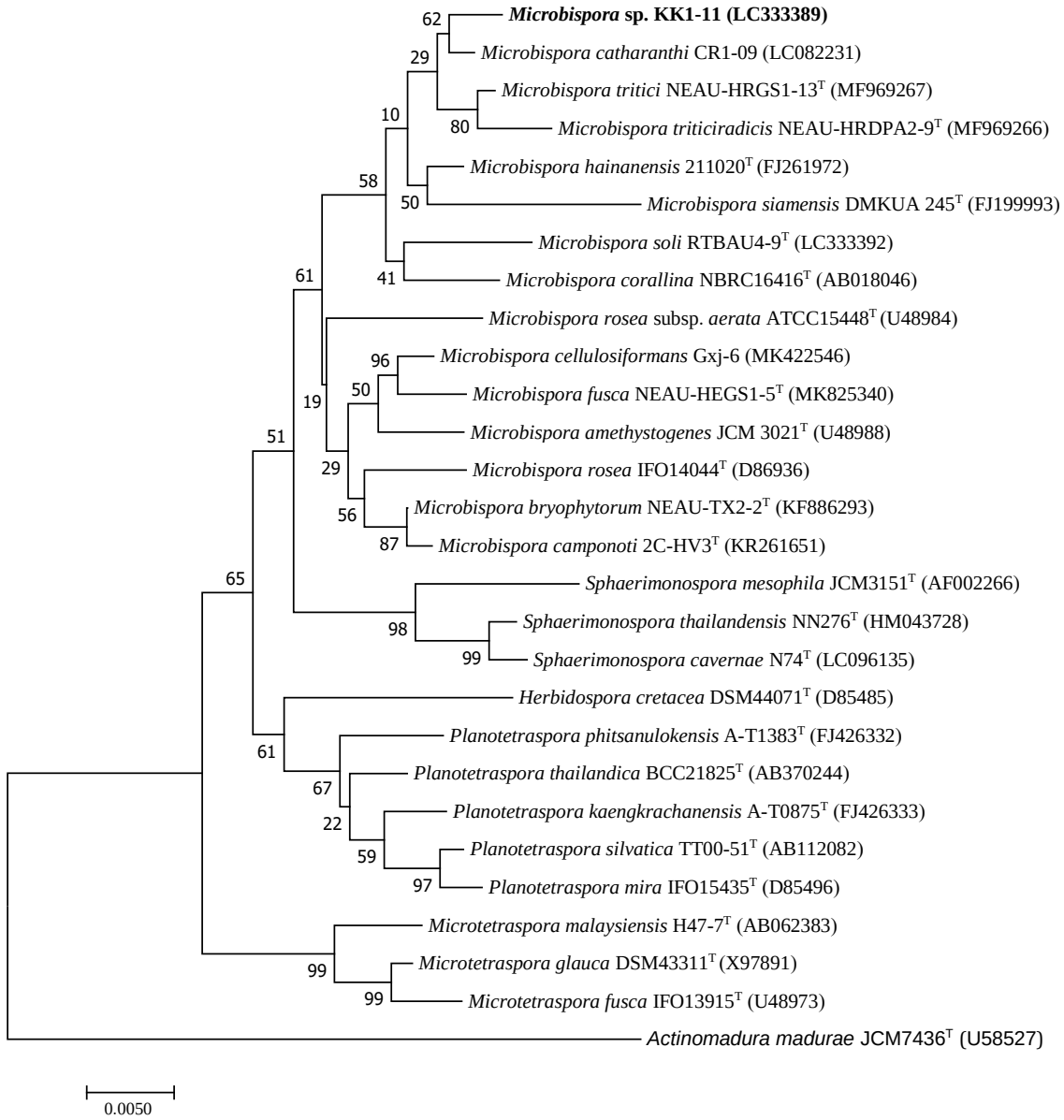


Figure 2. Neighbor-joining (NJ) tree based on almost-complete 16S rRNA gene sequences (1,480 nt) showing the relationships between the strain KK1-11, the *Microbispora* species with validly published names, and members of the genera *Sphaerimonospora, Planotetraspora, Herbidospora,* and *Microtetraspora*. *Actinomadura madurae* JCM7436^T was used as an outgroup.

Chemotaxonomic properties of KK1-11

To confirm the taxonomic position of KK1-11 at the genus level, several chemotaxonomic characteristics, such as isomer of diaminopimelic acid, whole-cell sugars, and types of menaquinones, were analyzed. Chemotaxonomic data of KK1-11 revealed that *meso*-diaminopimelic acid exists in its cell wall peptidoglycan. Madurose (3-*O*-methyl-D-galactose), the diagnostic reducing sugar found in almost all members of the genus *Microbispora*, was also detected in KK1-11 cell hydrolysates. KK1-11 was also shown to contain menaquinones MK-9(H_2), MK-9(H_0), and MK-9(H_4), which are similar to the types of menaquinones found in other *Microbispora*

sp. The results of chemotaxonomic analyses indicate that the taxonomic position of KK1-11 is consistent with its classification as a member of the genus *Microbispora*[18,33-36].

Genotypic characteristic of KK1-11

Analysis of 16S rRNA gene sequence of KK1-11 revealed that it is a member of the genus *Microbispora*. KK1-11 showed the highest 16S rRNA gene sequence similarity value (99.51%) to *M. catharanthi* CR1-09^T, followed by *Microbispora bryophytorum* NEAU-TX2-2^T (99.24%), and *Microbispora tritici* NEAU-HRGS1-13^T (99.17%). The taxonomic position of KK1-11 in the neighbor-joining tree confirmed that KK1-11 is a member of the genus *Microbispora*, forming a phyletic branch with *M. catharanthi* CR1-09^T (Fig. 2). The draft genome sequence analysis showed that the ANI value of KK1-11 obtained from the JspeciesWS server was 94.44%. This value is lower than the ANI *threshold* range (95%–96%) for *species demarcation*[37]. Additionally, the dDDH value between the genomes of the two strains, KK1-11 and *M. catharanthi* CR1-09^T, was 62.30%, which is lower than the 70% cut-off point for indicating strains to be the same species[38]. The results of phenotypic, chemotaxonomic, and genotypic tests indicate that KK1-11 can be clearly distinguished from its closest *Microbispora*-type strain, *M. catharanthi* CR1-09^T. Further taxonomic studies are needed to describe KK1-11 as a new species of *Microbispora*.

Genome analysis for secondary metabolites of KK1-11

De novo assembly of the genome sequencing data of KK1-11 resulted in 195 contigs, N50 of 217 kb, and genome coverage of 120×. The draft genome of KK1-11 was a single linear 9 Mb chromosome with a G+C content of 71.3%. The genome contained 8,619 predicted genes. There were 84 genes that were assigned to RNA genes (72 tRNA, 10 rRNA, and 2 tmRNA genes). Others (8,535) were identified as protein-coding sequence genes involved in cellular metabolism (28.3% protein, 16.0% carbohydrate, 7.7% lipid, and 4.8% nucleotides and nucleosides metabolism) and are responsible for cellular processes and information storage and processing. Moreover, genome analysis showed that KK1-11 has 12 protein-coding sequence genes involving secondary metabolites. Analysis results obtained from the antiSMASH server revealed that KK1-11 contains five terpene biosynthesis gene clusters, three lanthipeptide, two type I polyketide synthase (T1PKS), two thiopeptide, two siderophore, and six NRPS genes. Other clusters are associated with the synthesis of type III polyketide synthase (T3PKS), bacteriocin, PKS-like, NRPS-like, heterocyst glycolipid synthase-like PKS (hglE-KS), and TfuA-related RiPPs genes (Table 2). The secondary metabolite biosynthetic gene clusters (smBGCs) exhibited genetic relatedness to known clusters producing alkylresorcinol (100%) in *Streptomyces griseus* subsp. *griseus* NBRC 13350[39], geosmin (100%) in *Nostoc punctiforme* PCC 73102[40], desferrioxamine (83%) in *Streptomyces argillaceus*[41], guangnanmycin (78%) in *Streptomyces* sp. CB01883[42], and actinokineosin (63%) in *Actinokineospora spheciospongiae*[43]. The remaining biosynthesis genes exhibited low similarity (5%) to the funisamine biosynthetic gene cluster from *Streptosporangium* sp.[44], 6% similarity to the friulimicinA biosynthetic gene cluster from *Actinoplanes friuliensis*[45], and 13% to the indigoidine biosynthetic gene cluster from *Streptomyces aureofaciens*[46]. Interestingly, we found that the genome of KK1-11 contains several smBGCs showing no relatedness to any known clusters in antiSMASH, e.g., thiopeptide (positions 59,264–87,841 nt), bacteriocin (positions 314,933–325,088 nt), terpene (positions 91,447–113,483 nt), siderophore (positions 72,870–86,100 nt), and thiopeptide (positions 108,076–135,048 nt). These data indicate that KK1-11 has the potential to produce new secondary metabolites and could be a source of valuable bioactive compounds.

Table 2. Number of secondary metabolite gene clusters in the genomes of *Microbispora* sp. KK1-11 and *M. catharanthi* CR1-09^T

Secondary metabolite gene cluster	*Microbispora* sp. KK1-11	*M. catharanthi* CR1-09^T
Type I PKS	2	1
Type III PKS	1	2
NRPS	6	4
NRPS-like fragment	1	2
Bacteriocin	1	2
Thiopeptide	2	1
Siderophore	2	4
Terpene	5	6
Lanthipeptide	3	1
Heterocyst glycolipid synthase-like PKS (hglE-KS)	1	0
TfuA-related RiPPs	1	0
Total	25	23

Screening for antibacterial activity of KK1-11 culture broth

To prepare the broth culture for anti-bacterial activity tests, KK1-11 was cultured in ISP2 broth on a shaker (180 rpm) at 30°C for 14 days. After cultivation, the broth was centrifuged at 6,000 rpm to separate actinobacterial cells. The results obtained from the agar-well diffusion assay showed that the culture broth exhibited moderate anti-bacterial activity (~8 mm inhibition zone) against three Gram-positive bacteria: *S. aureus* ATCC 25923, MRSA, and *B. subtilis* ATCC 6633. However, no activity was observed against Gram-negative bacteria *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922.

Conclusions

In the present study, we report the taxonomic characterization and genome properties of *Microbispora* sp. KK1-11. 16S rRNA gene sequence analysis showed that KK1-11 can be identified as the member of the genus *Microbispora* and is closely related to *M. catharanthi* CR1-09^T. Genome-based taxonomic characterization of KK1-11 revealed low ANI (94.44%) and dDDH (62.30%) values between KK1-11 and *M. catharanthi* CR1-09^T, implying that KK1-11 may represent an undescribed *novel species* of the genus *Microbispora*. Draft genome analysis for secondary metabolite production of KK1-11 revealed the presence of an abundance of smBGCs, with terpene, lanthipeptide, T3PKS, T1PKS, hglE-KS, NRPS-like fragment, and bacteriocin gene clusters of particular interest in the search for novel antibiotics. The present work suggests that KK1-11 is a potential source of bioactive metabolites.

Acknowledgments

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Competing interests

The authors declare no competing interests.

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