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ORIGINAL ARTICLE

Virgisporangium myanmarense sp. nov., a novel motile actinomycete isolated from an anthill soil in Myanmar

Hideki Yamamura[1], Shoya Miyazaki[1], Kodai Ikoma[1], Youji Nakagawa[1], Moriyuki Hamada[2], Misa Otoguro[2], Tomohiko Tamura[2], Katsuhiko Ando[2], Nyunt Phay[3] and Masayuki Hayakawa[1]

An actinomycete strain, designated MM04-1133^T, was isolated from an anthill soil sample collected in Bagan, Myanmar. To establish the taxonomic status of this strain, the isolate was subjected to a polyphasic approach. Strain MM04-1133^T was Gram-staining positive, aerobic, motile and formed long and narrow sporangia directly above the surface of the substrate mycelium. Whole-cell hydrolysates of the strain contained 3-OH-diaminopimelic acid, arabinose, glucose, galactose, mannose, rhamnose and xylose. The predominant menaquinones were MK-10(H_6) and MK-10(H_8). The major cellular fatty acids were iso-$C_{16:0}$ and anteiso-$C_{17:0}$. The diagnostic phospholipid was phosphatidylethanolamine. The G+C content of the DNA was 69.1 mol%. Phylogenetic analysis based on 16S rRNA gene sequence revealed that strain MM04-1133^T clustered within the genus *Virgisporangium*, with the sequence exhibiting highest similarity (98.5% identity) with *Virgisporangium ochraceum* NBRC 16418^T. The strain grew in the presence of 0–1% (w/v) NaCl, at pH 5–8 and at 20–40 °C, with optimal growth at 30–37 °C. Based on phylogenetic analysis and chemotaxonomic and phenotypic data, we propose classifying this isolate as a novel species of the genus *Virgisporangium*, to be designated as *Virgisporangium myanmarense* sp. nov. The type strain is MM04-1133^T (= NBRC 112733^T = VTCC 910008^T).

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INTRODUCTION

The genus *Virgisporangium*, established by Tamura *et al.*,[1] comprised *V. ochraceum* and *Virgisporangium aurantiacum* as motile actinomycetes belonging to the family *Micromonosporaceae*. An additional species of the genus, as reported by Otoguro *et al.*,[2] was designated *Virgisporangium aliadipatigenens*. The genus *Virgisporangium* is characterized by substrate mycelia that produce sporangia containing a single row of six or more motile spores and contains 3-OH-diaminopimelic acid in the cell wall.[3] The phospholipid pattern is type PII, which usually includes phosphatidylethanolamine as the diagnostic phospholipid. All the type strains of the genus *Virgisporangium* were originally isolated from soil samples collected from subtropical to temperate areas in Japan.[1,2] Previously, Muramatsu *et al.*[4] isolated *Virgisporangium* strains from soil samples of a tropical area in Malaysia. Similarly, members of the genus *Virgisporangium* were isolated from tropical areas in Indonesia and Vietnam by using the rehydration and centrifugation method as a selective technique for isolation for motile actinomycetes.[5,6] However, there are no data on antibiotic production in this genus, as the number of isolates is extremely small, so their potential as a pharmaceutical resource is still unknown. Recently, technological innovations have occurred in genome analysis, such as next-generation sequencing and genome editing (CRISPR/Cas system); this progress is expected to increase the value of rare actinomycetes as drug-discovery resources.[7,8] Furthermore, the discovery of additional species of this genus will contribute both to an understanding of their ecological roles and to the provision of bioresources for industrial applications.

In the course of the screening for novel actinomycetes in Myanmar, a strain, MM04-1133^T, was isolated from an anthill soil sample collected from the savanna area in Bagan, Myanmar. The aim of the present study was to determine the taxonomic position of strain MM04-1133^T using a polyphasic approach.

RESULTS AND DISCUSSION

Strain MM04-1133^T formed long and narrow sporangia directly above the surface of the substrate mycelia. Sporangia of strain MM04-1133^T were seen to contain a single row of short rod-shaped (0.8 by 1.0 μm) spores (Figure 1). After incubation at 30 °C for 30 to 90 min in 5 mM *N*-cyclohexyl-2-aminoethanesulfonic acid (CHES) buffer (pH 9.0), spores exhibited active motility.

Whole-cell hydrolysates of strain MM04-1133^T contained 3-OH-diaminopimelic acid, arabinose, glucose, galactose, mannose, rhamnose and xylose. The predominant menaquinones were MK-10 (H_6) and MK-10 (H_8). The predominant polar lipids were diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylinositol

[1]Division of Applied Biological Sciences, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Kofu, Japan; [2]Biological Resource Center, National Institute of Technology and Evaluation (NBRC), Chiba, Japan and [3]Pathein University, Pathein, Myanmar
Correspondence: Dr H Yamamura, Division of Applied Biological Sciences, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Takeda-4-4-37, Kofu 400-8510, Japan.
E-mail: hyamamura@yamanashi.ac.jp
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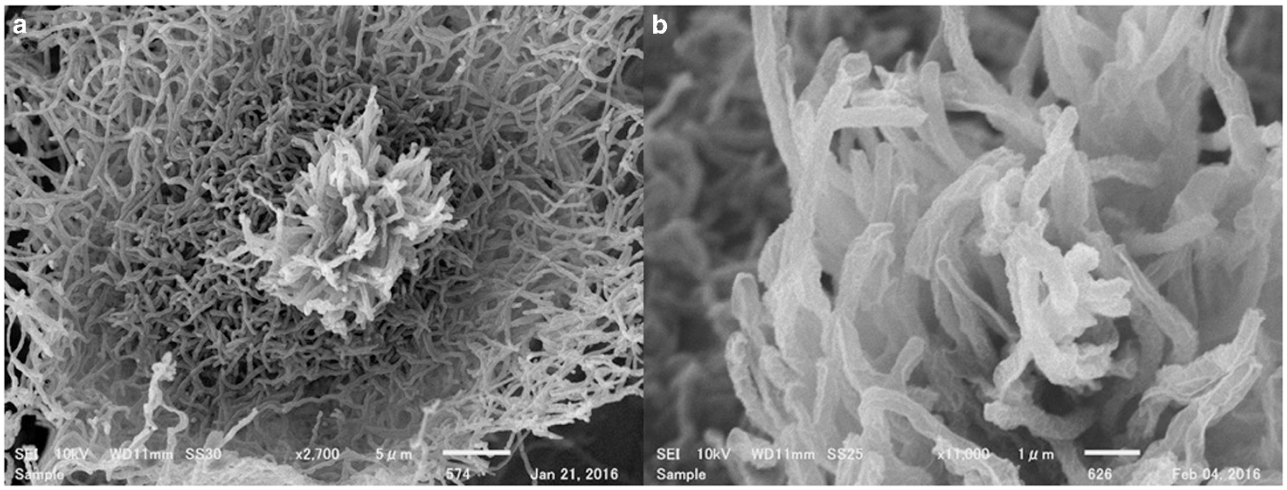

Figure 1 Scanning electron micrographs of strain MM04-1133^T grown on humic acid-vitamin gellan gum (HVG) for 5 weeks at 30 °C. Bar indicates 5 μm (**a**) or 1 μm (**b**).

(phospholipid type PII, Supplementary Figure S1).[9] In addition, trace amounts of hydroxyphosphatidylethanolamine, phosphatidylinositol-mannosides and an unidentified phospholipid were observed. The major fatty acids (>10% of the total) of the isolated strain were iso-$C_{16:0}$ (35.0%) and anteiso-$C_{17:0}$ (22.6%). The detailed cellular fatty acid content is summarized in Supplementary Table S1. According to the fatty acid profile, MM04-1133^T and *V. aurantiacum* were quite similar. The G+C content of the DNA of strain MM04-1133^T was 69.1 mol%.

The 16S rRNA gene sequence of strain MM04-1133^T exhibited similarities between 97.2 and 98.5% with those of members of the genus *Virgisporangium*. The highest similarity (98.5%) was observed with *V. ochraceum* NBRC 16418^T, followed by *V. aurantiacum* NBRC 16421^T (98.3%) and *V. aliadipatigenens* NBRC 105644^T (97.2%). The phylogenetic tree constructed by the neighbor-joining method reveal that strain MM04-1133^T fell within the cluster of the genus *Virgisporangium* (Figure 2).

Based on the phylogenetic and chemotaxonomic analyses, strain MM04-1133^T was classified in a member of the genus *Virgisporangium*. Recently, it has been suggested that 98.65–98.7% similarity among 16S rRNA gene sequences should be used as a threshold for performing DNA–DNA hybridization for species delineation because the value equates to a DNA–DNA relatedness of 70% between two strains.[10,11] The similarities between strain MM04-1133^T and the nearest phylogenetic neighbors were lower than this recommended threshold.

Strain MM04-1133^T grew well on International *Streptomyces* Project (ISP) media 2, 3, 4, 5 and 7, and on yeast extract-starch (YS) agar. However, the other tested *Virgisporangium* strains showed weak growth on ISP media 4 and 5. Strain MM04-1133^T grew weakly on ISP medium 6, whereas *V. aurantiacum* NBRC 16421^T showed good growth on this substrate, meaning that the two strains were distinguished based on growth on this medium. Aerial mycelia were not observed on any of the tested media. The reverse color of all strains was yellow or orange. Production of soluble pigment by strains MM04-1133^T and *V. ochraceum* NBRC 16418^T was observed only when the strains were cultured on ISP medium 7; in contrast, strain *V. aurantiacum* NBRC 16421^T produced soluble pigments during growth on ISP media 2 and 7, and on YS agar. Strain MM04-1133^T exhibited growth when cultured at temperatures ranging from

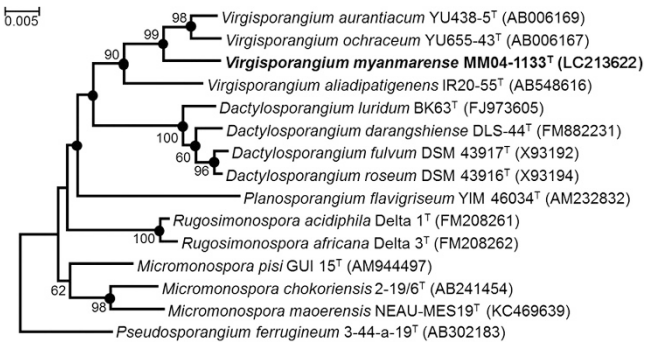

Figure 2 Phylogenetic tree derived from 16S rRNA gene sequences showing the relationship of strain MM04-1133^T to the genus *Virgisporangium* and related taxa. The tree was constructed using the neighbor-joining method and K_{nuc} values.[35] Only bootstrap values above 50% are shown (1000 resamplings) at the branching points. Solid circles indicate that the corresponding nodes were also recovered using the maximum-parsimony and maximum-likelihood algorithms.[36] Bar, 0.005 K_{nuc}.

20–40 °C, but not when cultured at 15 °C or 45 °C; in contrast, the other strains showed weak or good growth at 15 °C. Optimal growth of the strain MM04-1133^T was observed at 30–37 °C. Growth was observed at pHs ranging from 6.0 to 8.0, with an optimum at pH 7.0; the other type strains were capable of growth even at pH 9.0. Strain MM04-1133^T was positive for production of H_2S and degradation of gelatin, but was negative for reduction of nitrate, production of urease and decomposition of elastin, gelatin and casein. In the API ZYM enzyme assays, strain MM04-1133^T was negative for valine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities. Strain MM04-1133^T utilized D-fructose, D-mannose, D-xylose, L-arabinose and L-rhamnose. Thus, based on the above results, there are some cultural characteristics that can be used to differentiate MM04-1133^T from other species of the same genus (Table 1).

Strain MM04-1133^T exhibited no antimicrobial activity against any of the tested strains. However, a PCR-based genetic survey revealed that the strain contain genes capable of encoding polyketide synthase-II and nonribosomal peptide synthetase proteins.

Table 1 Differential characteristics of strain MM04-1133^T, *V. ochraceum* NBRC 16418^T and *V. aurantiacum* NBRC 16421^T

Characteristic	*1*	*2*	*3*
Utilization of sole C-source			
L-Arabinose	+	–	+
D-Fructose	+	–	–
D-Mannose	+	w	–
L-Rhamnose	+	w	–
L-Xylose	+	w	–
API ZYM			
Esterase lipase (C8)	–	w	+
Valine arylamidase	–	+	+
Cystine arylamidase	–	+	–
Acid phosphatase	–	+	+
Naphthol-AS-BI-phosphohydrolase	–	+	+
β-Galactosidase	+	–	+
N-acetyl-*β*-glucosaminidase	–	+	+
Degradation of			
Gelatin	+	+	–
Decomposition of			
Elastin	–	+	+
Casein	–	+	+
Growth at (°C)			
15	–	w	+

Abbreviations: +, positive; w, weakly positive; –, negative.
Strains: 1, *V. myanmarensis* MM04-1133^T; 2, *V. ochraceum* NBRC 16418^T; 3, *V. aurantiacum* NBRC 16421^T.
All data were generated in the present study.

Based on these chemotaxonomic, phenotypic, and phylogenetic characteristics, strain MM04-1133^T represents a novel species within the genus *Virgisporangium*, for which the name *V. myanmarense* sp. nov. is proposed.

Description of *Virgisporangium myanmarense* sp. nov

V. myanmarense (my.an.mar.en'se. N.L. neut. adj. *myanmarense* pertaining to Myanmar, where the organism was originally isolated).

Cells are aerobic and Gram-staining positive. Aerial mycelia are absent and sporangia develop on humic acid vitamin (HV)-gellan gum. Slender sporangia are formed directly from substrate mycelia. Several spores are present per sporangium and the spores are short rods (0.8 to 1.0 mm); spores are motile when suspended in 5 mM *N*-Cyclohexyl-2-aminoethanesulfonic acid buffer (pH 9.0) and incubated at 30 °C for 30–90 min. The substrate mycelium is yellow or orange and soluble pigment is produced on ISP medium 7. Growth occurs in the presence of 0–1% NaCl (w/v) and at 20–40 °C, but not at 15 or 45 °C. Growth occurs at an initial pH of 6–8, with an optimum at pH 7. Nitrate is not reduced and catalase reaction is positive. Production of urease is positive. Tyrosine is hydrolyzed, but not casein, elastin, hypoxanthine or xanthine. L-arabinose, cellobiose, D-fructose, D-mannitol, D-mannose, L-rhamnose and D-xylose can be used as sole carbon sources, but adonitol, dextrin, D-maltose, D-melezitose, raffinose and sodium acetate cannot. In the API ZYM, tests for alkaline phosphatase, leucine arylamidase, *β*-galactosidase, α-glucosidase and *β*-glucosidase are positive. Weakly positive for esterase (C4), trypsin and α-chymotrypsin. Activities of esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, *β*-glucuronidase, *N*-acetyl-*β*-glucosaminidase, α-mannosidase and α-fucosidase are negative.

The type strain, MM04-1133^T (= NBRC 112733^T = VTCC 910008^T), was isolated from an anthill soil sample collected from Bagan, Myanmar. The DNA G+C content of the type strain is 69.1 mol%.

MATERIALS AND METHODS

Isolation and maintenance of organism

Strain MM04-1133^T was isolated from an anthill soil sample collected from Bagan, Myanmar, using the rehydration and centrifugation method[12] and humic acid–vitamin agar[13] containing nalidixic acid (20 mg l^{-1}) and cycloheximide (50 mg l^{-1}) after incubation at 30 °C for 2 weeks. The rehydration and centrifugation method facilitates the isolation of motile actinomycetes from natural substances and has been employed by several researchers.[5,6,14] *V. ochraceum* NBRC 16418^T and *V. aurantiacum* NBRC 16421^T were used as known controls for cultural, physiological, biochemical and chemotaxonomic characterization of the novel isolate.

Observation of morphological characteristics

Strain MM04-1133^T was grown on humic acid-vitamin gellan gum plates for 5 weeks at 30 °C.[15] The morphological features were observed by light and scanning electron microscopy (model JSM-6010; JEOL, Tokyo, Japan). To prepare samples for scanning electron microscopy, the microorganism was fixed with formaldehyde vapor and dehydrated through a graded series of ethanol solutions (80 and 100%); the ethanol was then replaced with *t*-butyl alcohol. After freeze drying (model JFD-320; JEOL), the sample was subjected to platinum coating (model JFC-1600; JEOL). Spore motility was evaluated by light microscopy using the hanging drop method.[13] Briefly, the strain was grown on a humic acid-vitamin gellan gum plate for 5 weeks at 30 °C and then suspended in 5 mM *N*-Cyclohexyl-2-aminoethanesulfonic acid buffer (pH 9.0) at 30 °C for 30–90 min. The resulting suspension was carefully observed by light microscopy.

Comparison of cultural, physiological and biochemical characteristics.

The physiological characteristics were examined using methods described previously.[16,17] The ranges of the growth temperature and pH were investigated by incubation for 3–21 days on YS agar at temperatures of 5, 10, 15, 20, 25, 30, 37, 40 and 45 °C, and at pHs ranging from 4 to 12 in 1 pH-unit intervals. Specifically, pH was buffered at pH 4.0–5.0 using 0.1 M citric acid, 0.1 M sodium citrate; at pH 6.0–8.0 using 0.1 M KH_2PO_4, 0.1 M NaOH; at pH 9.0–10.0 using 0.1 M $NaHCO_3$, 0.1 M Na_2CO_3; at pH 11.0 using 0.05 M Na_2HPO_4, 0.1 M NaOH; and at pH 12.0 using 0.2 M KCl, 0.2 M NaOH. Cultural characteristics on media according to the ISP[18] and on YS agar[19] were observed after 14 days of incubation at 30 °C. The Guide to Color Standard (Japan Color Research Institute, 1954) was used for color determination. The biochemical characteristics were determined according to the standard methods.[20] The production of H_2S was tested using ISP medium 6 as a basal medium.[21] The reduction of nitrate was tested using ISP medium 8. The utilization of carbon sources at a final concentration of 1% (w/v) was tested using ISP medium 9 as a basal medium.[18] The production of urease was tested using urease broth as a basal medium.[22] The degradation of gelatin was tested on YS medium containing 1% gelatin instead of agar. The decomposition of elastin and casein at final concentrations of 0.4 and 0.1% (w/v) (respectively) was tested using YS agar as a basal medium. API ZYM biochemical kits (bioMerieux, Tokyo, Japan) were used to investigate several physiological and biochemical characteristics according to the manufacturer's instructions. Antimicrobial activity was assayed using an overlay method[12] to test activity against the following 8 microorganisms: *Aspergillus niger* ATCC 9642, *Bacillus subtilis* NBRC 3134, *Candida albicans* NBRC 1385^T, *Colletotrichum orbiculare* NBRC 33130, *Escherichia coli* NBRC 3044, *Pseudomonas fluorescens* NBRC 14106 and *Staphylococcus aureus* NBRC 3061.

Chemotaxonomy

Freeze-dried cells for chemotaxonomic analyses were obtained from cultures grown in YS broth on a rotary shaker at 30 °C for 7 days. Cells were collected by centrifugation and the resulting pellet was washed twice with distilled water. Diaminopimelic acid isomer and sugars in whole-cell hydrolysates were analyzed based on the methods established by Hasegawa *et al.*[23] and Tamura *et al.*,[24] respectively. The cellular fatty acids were processed and analyzed as methyl esters, following the protocol of the MIDI Sherlock Microbial Identification System.[25] Standard procedures were also used for the extraction and analysis of isoprenoid quinones and polar lipids,[26] and the results were compared with the appropriate controls. The isoprenoid quinone content was determined using liquid chromatography/mass spectrometry, as described by Hamada *et al.*[27] The polar lipids were identified by two-dimensional thin-layer chromatography, followed by spraying with appropriate detection reagents according to the method of Yassin *et al.*[28] Genomic DNA of MM04-1133^T was obtained by the method of Saito and Miura.[29] The G+C content of the DNA was determined by high-performance liquid chromatography, as described by Tamaoka and Komagata.[30]

Phylogenetic analysis based on 16S rRNA gene

The 16S rRNA gene of MM04-1133^T was amplified by PCR and the resulting products were directly sequenced using the methods previously described by Tamura and Hatano;[31] direct sequencing of the PCR product was performed using an ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and an automated DNA analyzer (model 3730; Applied Biosystems). The obtained 16S rRNA gene sequence was compared with published 16S rRNA gene sequences of bacterial type strains using the EzBioCloud (http://www.ezbiocloud.net/).[32] The obtained 16S rRNA gene sequence was aligned with those of members of the genus *Virgisporangium* and phylogenetically related species, obtained from EMBL/GenBank/DDBJ, using the CLUSTAL_X program.[33] The phylogenetic trees were constructed using Molecular Evolutionary Genetics Analysis software, version 6.0,[34] with the neighbor-joining,[35] maximum-parsimony,[36] and maximum-likelihood[36] methods. The topology of the phylogenetic tree was evaluated by bootstrap analysis with 1000 iterations of the respective (neighbor-joining, maximum-parsimony and maximum-likelihood) methods.[37]

Detection of secondary metabolite synthase gene fragments

In order to establish the potential for antibiotic productivity by actinomycete isolates, genetic assessment was performed. The amplification of the genes encoding polyketide synthases I and II, and nonribosomal peptide synthetases were performed with the relevant gene-specific degenerate primers described by Metsä-Ketelä *et al.*[38] and by Ayuso-Sacido and Genilloud.[39]

Nucleotide sequence accession number

The 16S rRNA gene sequence of strain MM04-1133^T determined in this study has been deposited in the DDBJ database under the accession number LC213622.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)