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ORIGINAL ARTICLE

Microbispora thailandensis sp. nov., an actinomycete isolated from cave soil

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The taxonomic position of actinomycete strain NN276^T, isolated from cave soil, was studied using the polyphasic taxonomic approach. A phylogenetic tree based on 16S ribosomal RNA (rRNA) gene sequences showed that the isolate formed a distinct evolutionary linage with the genus *Microbispora*, with *M. mesophila* JCM 3151^T as its closest phylogenetic neighbor (97.9% similarity). The organism contained *meso*-diaminopimelic acid and the *N*-acetyl type of peptidoglycan. Madurose was detected in the whole-cell hydrolasate. The predominant menaquinones were MK-9(H_4), MK-9(H_2) and MK-9. Mycolic acids were not detected. Major phospholipids were diphosphatidylglycerol, hydroxy-phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannoside. The major cellular fatty acid was iso-$C_{16:0}$ and G + C content 70 mol%. DNA–DNA hybridization demonstrated that the isolate was distinct from *M. mesophila* JCM 3151^T. On the basis of phenotypic and genotypic data, it is proposed that strain NN276^T represents a novel species of the genus *Microbispora*, hence the name *Microbispora thailandensis* sp. nov. The type strain is strain NN276^T (= BCC 41490^T = NRRL B-24806^T = NBRC 107569^T).

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INTRODUCTION

The genus *Microbispora* was proposed for actinomycetes that formed aerial hyphae bearing longitudinal pairs of spores[1] with *M. rosea* as type species. In 1998, Zhang *et al.*[2] reclassified the genus *Thermomonospora* and proposed to transfer mesophilic actinomycete that produces spores singly borne on aerial hyphae, *Thermomonospora mesophila*, to *M. mesophila* owing to their related chemotaxonomic properties and 16S ribosomal RNA (rRNA) sequences. The genus *Microbispora* was then emended with a change in morphological description by having a single spore in the species *M. mesophila*. The genus *Microbispora* currently contains five species, namely *M. amethystogenes*,[3,4] *M. corallina*,[5] *M. mesophila*,[2,6] *M. rosea* (containing *M. chromogenes*, *M. diastatica*, *M. indica*, *M. karnatakensis*, *M. rosea*, which were reduced to a single taxon as *M. rosea* subsp. *rosea*, and *M. aerata*, *M. thermodiastatica* and *M. thermorosea*, which were also combined as *M. rosea* subsp. *aerata*)[3,4] and *M. siamensis*.[4] Representatives of this genus form a distinct monophyletic group within the evolutionary radiation encompassed in the family *Streptosporangiaceae*, the family that included the following genera: *Acrocarpospora*, *Herbidospora*, *Microbispora*, *Microtetraspora*, *Nonomuraea*, *Planobispora*, *Planomonospora*, *Planotetraspora*, *Sphaerisporangium*, *Streptosporangium* and *Thermopolyspora*.[7–9] During our investigation of cultivable actinomycetes from tropical limestone caves in Nakorn Sawan province, Thailand, an actinomycete strain, NN276^T, was isolated from cave soil. The present polyphasic study was designed to establish the taxonomic status of the strain. On the basis of polyphasic resultant data, isolate NN276^T represents a novel species of the genus *Microbispora*.

MATERIALS AND METHODS

Strain NN276^T was isolated from a soil sample collected from tropical limestone cave. Soil suspension was pretreated with an electromagnetic wave according to a modified method from Bulina *et al.*[10] by irradiated soil suspension with the microwave oven at a frequency of 2460 MHz and a power setting of 100 W for 45 s. The pretreated samples were further diluted with 0.85% NaCl solution. One hundred microliters of an appropriate dilution was spread over the surface of humic acid–vitamin (HV) agar[11] supplemented with nalidixic acid (25 μg ml^{-1}) and ketoconazole (100 μg ml^{-1}) to inhibit the growth of bacteria and fungi, respectively. Plates were incubated at 28 °C for 2 weeks. The strain was then purified and maintained on glucose yeast extract (GYE) agar[12] at room temperature. Suspensions of spores or mycelium were stored in glycerol (20%, v/v) at –20 °C and lyophilized for long-term preservation.

Cultural characteristics of the strain NN276^T were determined using a 14-day culture on ISP media 2, 3, 4, 5 of Shirling and Gottlieb[13] (Difco, Detroit, MI, USA) and GYE agar at 27 °C. The color of mycelium and the

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soluble pigment were determined by comparing with the color chips from the Color Harmony Manual.[14] The morphological characteristics were observed by light microscopy and scanning electron microscopy (SEM, JEOL-JSM 5600 LV; JEOL Ltd., Tokyo, Japan) of 15-day-old cultures grown on ISP medium 3. The utilization of a variety of substrates as sole carbon sources was tested using the basal inorganic nitrogen medium[15] supplemented with a final concentration of 1% (v/v) of the filter sterile-tested carbon sources. Catalase activity was determined with 3% (v/v) hydrogen peroxide solution and oxidase activity was tested with 1% tetramethyl *p*-phenylenediamine dihydrochloride solution. Growth at various pH values and tolerance of NaCl were examined on ISP medium 2. The temperature range for growth was determined on ISP medium 2 using a temperature gradient incubator (Tokyo Kagaku Sangyo, Tokyo, Japan) with low and high temperatures between 5 and 50 °C. Enzyme activity profiles were carried out using the API ZYM (bioMèrieux, Lyon, France) test kits. Melanin pigment was examined on ISP medium 7.[13] Urease activity was determined by a color change on urea agar.[15] The production of hydrogen sulfide was detected using lead acetate strips. Hydrolysis of casein, gelatin and nitrate reduction was examined following the methods of Gordon and Mihm.[16] *Micobispora mesophila* JCM 3151^T was used in this study for comparison.

Isolate NN276^T was examined for key chemical markers. The freeze-dried cells used for chemotaxonomic analysis were obtained from cultures grown in shake flasks of ISP medium 2 broth for 10 days at 27 °C. The isomers of diaminopimelic acid (A_2pm) in the cell wall peptidoglycan and whole-cell sugar composition were determined by thin-layer chromatography as described by Staneck and Roberts.[17] The acyl type of the cell wall was analyzed according to the method of Uchida and Aida.[18] Polar lipids were examined using two-dimensional thin-layer chromatography and identified by the method of Minnikin *et al.*[19] Menaquinones were extracted and purified by the method of Collins *et al.*,[20] and isoprene units were subsequently analyzed by LC/MS (JMS-T100LP, JEOL) with PEGASIL ODS column (2 ø × 50 mm; Senshu, Tokyo, Japan) using methanol/2-propanol (7:3). Mycolic acids were detected by thin-layer chromatography according to the method of Tomiyasu.[21] Analysis of the fatty acids was performed according to the procedures of the Sherlock Microbial Identification System (MIDI, version 4.5; MIDI Inc., Newark, DE, USA) using a gas chromatograph (model HP6890; Hewlett Packard, Palo Alto, CA, USA) and identified with the ACTIN1 database.

Genomic DNA of the isolate NN276^T was extracted and purified from biomass according to the method of Kieser *et al.*[22] The G + C content (mol%) of the DNA was determined by HPLC according to the method of Tamaoka and Komagata.[23] DNA–DNA relatedness was measured fluorometrically using the microplate hybridization method.[24] The phylogenetic position of the isolate was determined based on 16S rRNA gene sequence. The 16S rDNA was amplified as described by Duangmal *et al.*[25] and the PCR products were sequenced (First Base, Seri Kembangan, Malaysia) using universal primers.[26] The resultant almost-complete 16S rRNA gene sequence, consisting of 1479 nucleotides, was aligned with the corresponding sequences of representatives of the genus *Microbispora* and of related genera in the family *Streptosporangiaceae* (retrieved from the GenBank databases) using the CLUSTAL X[27] and PHYDIT programs (http://plaza.snu.ac.kr/~jchun/phydit/). Phylogenetic trees were inferred by the least-squares,[28] maximum-parsimony[29] and neighbor-joining[30] tree-making algorithms from the PHYLIP suite of programs[31] and TREECON software.[32] The resultant phylogenetic trees were viewed by TREEVIEW program.[33]

RESULTS AND DISCUSSION

Strain NN276^T was aerobic, Gram-positive, non-motile actinomycete, and produced branched and non-fragmented substrate mycelia. Single spherical spores with smooth surfaces were born on short sporophores (Figure 1), similar to those of *M. mesophila*.[2] The results from chemical analysis indicated that strain NN276^T contained *meso*-diaminopimelic acid in the peptidoglycan. The major menaquinones were MK-9(H_4), MK-9(H_2) and MK-9. Glucose, madurose and ribose were detected as the components of sugars in the whole-cell hydrolysates. Polar lipid analysis showed that the organism

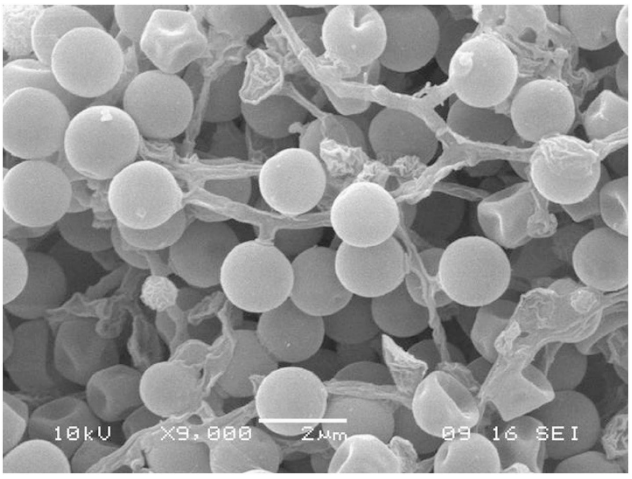

Figure 1 Scanning electron micrograph of spherical spores with smooth surfaces of strain NN276^T grown on ISP medium 3 for 15 days at 30 °C. Bar, 2 μm.

contained diphosphatidylglycerol, hydroxy-phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylinositol mannoside and an unknown phosphoglycolipid. The cellular fatty acid profile was represented by the predominant of iso-$C_{16:0}$ (58.0%). Fatty acids found in smaller proportions included 10 methyl-$C_{17:0}$ (11.2%), unknown 16.048 (7.0%), iso H-$C_{16:1}$ (4.4%), iso-$C_{14:0}$ (3.7%), iso 2OH-$C_{16:0}$ (2.8%), *cis* 9-$C_{17:1}$ (2.4%), $C_{15:0}$ (2.4%), 10 methyl-$C_{16:0}$ (2.2%), $C_{18:0}$ (1.8%), iso-$C_{15:0}$ (1.2%), $C_{17:0}$ (0.8%), iso-$C_{18:0}$ (0.6%), $C_{16:0}$ (0.4%), *cis* 9-$C_{16:1}$ (0.4%), iso F-$C_{16:1}$ (0.4%), iso-$C_{12:0}$ (0.2%) and $C_{14:0}$ (0.2%). The G + C content was 70 mol%.

The almost-complete 16S rRNA gene sequence (1479 nt) of strain NN276^T was compared with sequences against the GenBank database. The results indicated that the isolate belongs to the genus *Microbispora*. The strain NN276^T showed the highest level of 16S rRNA gene similarity with the type strain of *M. mesophila* (97.9%, a value corresponded to 31 nucleotide differences of 1456 locations). It is evident from the phylogenetic tree (Figure 2) that the strain NN276^T and the members of the genus *Microbispora* formed a coherent cluster that was supported by high bootstrap values and the strain NN276^T was clustered to *M. mesophila* in a well-separated branch with a high bootstrap value of 99% by neighbor-joining analysis. Similar tree topologies were obtained when the least-squares and maximum-parsimony methods were applied. Determination of DNA–DNA relatedness studies were carried out between strain NN276^T and *M. mesophila* JCM 3151^T by reciprocal hybridizations. The DNA–DNA reassociation value similarities were in the range of 45–49%, confirming that the strain NN276^T represents a separate genomic species based on whole-genomic DNA relatedness of less than 70% cut-off point.[34]

From biochemical and physiological properties (Table 1), strain NN276^T was readily differentiated from its closest relatives, *M. mesophila* JCM 3151^T; in particular, the utilization of arabinose, D(+)mannose, D(−) ribose, L(−)fucose and L(−)rhamnose was negative in NN276^T, properties that were positive in *M. mesophila*. The degradation of arbutin, casein, starch and xylan hydrolysis was positive in *M. mesophila* but negative in strain NN276^T. Moreover, the strain NN276^T did not produce spore on ISP medium 2 and GYE agar, properties that were detected in *M. mesophila* JCM 3151^T. The temperature range for growth of the strain NN276^T was 16–40 °C, where as *M. mesophila* JCM 3151^{T}was at 20–45 °C.

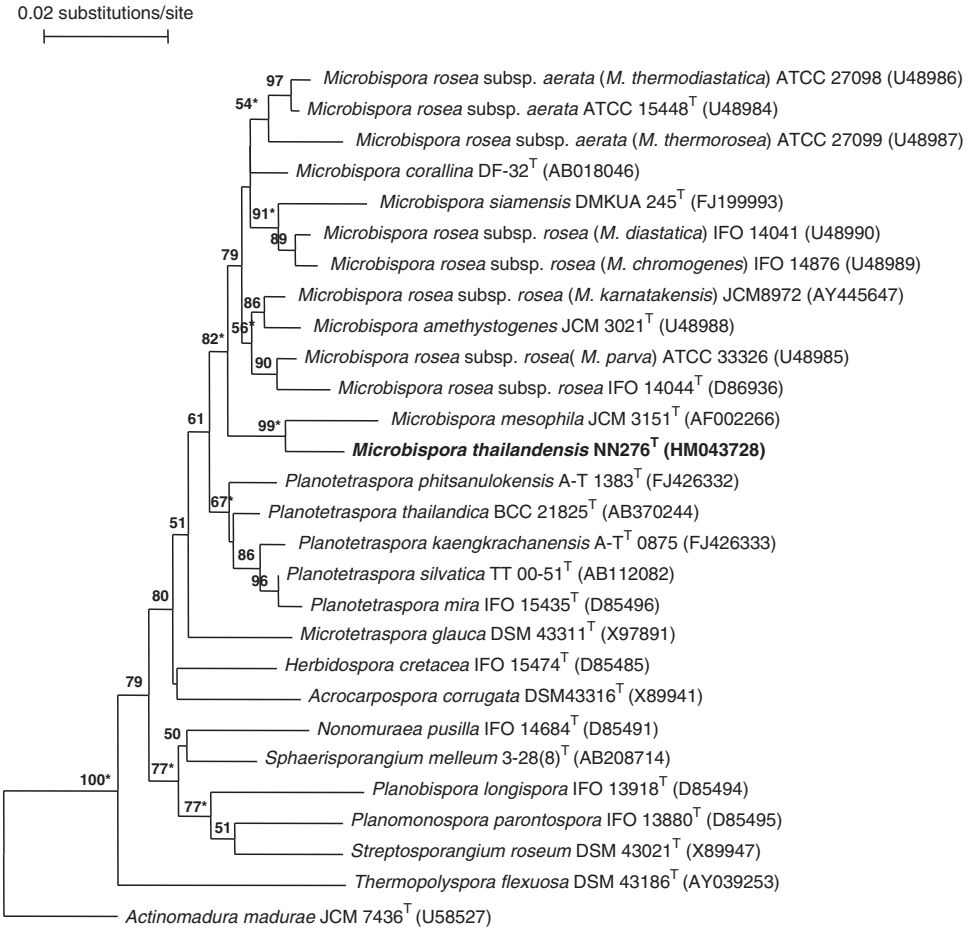

Figure 2 Neighbor-joining[30] phylogenetic tree based on nearly complete 16S rRNA gene sequences showing the relation ship between the strain NN276^T, species of the genus *Microbispora* and selected members of the family *Streptosporangiaceae*. *Actinomadura madurae* JCM 7436^T (U58527) was used as an outgroup. Asterisks indicate branches that were also found using the least-squares[28] and maximum-parsimony[29] tree-making algorithms. Numbers at the nodes indicate the percentage bootstrap support from an analysis of 1000 resampled data sets (only values >50% are indicated). Bar, 0.02 substitutions/site.

It is interesting to note that both *M. mesophila*[2] and isolate NN276^T are the only two species in the genus *Microbispora* that shared the same characteristic of single spherical spore formed on short sporophores. This unique single spore prompted a question whether these two species may represent a new genus. However, at present, we think it is premature to assume that they are a new genus based on only this observation. More isolates that shared this unique single spore are needed to help clarify its novel genus status.

It is apparent from this polyphasic study that the strain NN276^T can be distinguished from representatives of members of the genus *Microbispora*. We proposed that strain NN276^T represents a novel species of *Microbispora*, because of which the name *M. thailandensis* sp. nov. is proposed.

Description of *M. thailandensis* sp. nov.

M. thailandensis (thai.lan.den'sis.) N.L. fem. adj. thailandensis pertains to Thailand, where the strain was originally isolated.

It is an aerobic, Gram-positive, non-motile actinomycete. Abundant pale-yellow substrate mycelia are well-developed on ISP medium 2 and GYE agar with light brown diffusible pigment. Poor growth with scantly whitish spores, without soluble pigments, is found on ISP media 3, 4 and 5. Melanin pigment is not observed. Single spherical spores with smooth surfaces are borne on short sporophores alternately branched from aerial mycelia. Good growth occurs between 26–38 °C. The pH range for growth is 5–9 and cells can grow in the presence of 0–3% NaCl (w/v). Catalase, oxidase and nitrate reduction are positive. Urease and H_2S production are negative. Utilization of D(+)cellobiose, D(−)fructose, D(+) galactose, glucose, lactose, maltose, D(−)mannitol, raffinose, D(−) sorbitol and D(+)xylose is positive. Utilization of adonitol, arabinose, L(−)fucose, D(+)mannose, melibiose, L(−)rhamnose, D(−)ribose, sucrose and xylitol is negative. Gelatin, hypoxanthine, L-tyrosine and tween 40 are degraded. Aesculin, allantoin, arbutin, casein, cellulose, guanine, starch, xanthine and xylan are not degraded. Acid phosphatase, cystine aminopeptidase, esterase C4, α-glucosidase, β-glucosidase, leucine, aminopeptidase, lipase C8, N-acetyl-β-glucosaminidase, phosphoamidase and valine aminopeptidase are detected with the API ZYM enzyme assay; alkaline phosphatase, chymotrypsin, α-fucosidase, α-galactosidase, β-galactosidase, β-glucuronidase, lipase C14, α-mannosidase and trypsin are negative. The diagnostic diamino acid of the peptidoglycan is *meso*-diaminopimelic acid. Madurose is detected in whole-cell hydrolysates. The glycan moiety of the murein is acetylated. Major phospholipids were diphosphatidylglycerol, hydroxy-phosphatidylethanolamine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylinositol mannoside. The major menaquinone is MK-9(H_4), MK-9(H_2) and MK-9. Mycolic acids are not detected.

Table 1 Differential characteristics of strain NN276^T and *Microbispoa mesophila* JCM 3151^T

Characteristics	*NN276^T*	*M. mesophila^T*
On ISP medium 2		
Growth	Good	Good
Sporulation	None	Good; light gray
On ISP medium 3		
Growth	Poor	Moderate
Sporulation	Poor; white	Moderate; light gray
Utilization of		
Arabinose	−	+
Maltose	+	−
D(+)Mannose	−	+
D(−)Ribose	−	+
L(−)Fucose	−	+
L(−)Rhamnose	−	+
Degradation of		
Arbutin	−	+
Casein	−	+
Hypoxanthine	+	−
L-Tyrosine	+	−
Starch	−	+
Xylan	−	+
NaCl tolerance	0–3%	0–2%
Growth at pH 10	−	+
Temperature range	16–40 °C	20–45 °C

+, positive; −, negative.

The major fatty acid in cellular fatty acids profile is iso-$C_{16:0}$. The G + C content of the type strain DNA is 70 mol%.

The type strain, strain NN276^T (= BCC 41490^T = NRRL B-24806^T = NBRC 107569^T), was isolated from cave soil, Nakhon Sawan province, Thailand.

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Accession number: The GenBank accession number for the 16S rRNA gene sequence of strain NN276^T is HM043728.

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