First Record of the Isolation, Identification and Biological Activity of a New Strain of *Spirillospora albida* from Thai Cave Soil

Nareeluk Nakaew¹, Wasu Pathom-aree² and Saisamorn Lumyong^{2*}

¹Department of Biotechnology, Graduate School, Chiang Mai University, Chiang Mai 50200, Thailand

²Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand

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An actinomycete, designated as CMU-PNK470, was isolated from the soil in Phanangkoi cave in northern Thailand and subjected to phenotypic and genotypic characterization. Morphological and chemical properties indicated that this isolate belonged to the genus *Spirillospora*. Phylogenetic analysis based on 16S rRNA gene sequencing confirmed its placement in the genus *Spirillospora* and it was most closely related to *Spirillospora albida* (98.86%). This is the first reported isolation of *Spirillospora albida* from a cave habitat. The crude extract of this strain showed antimicrobial activity against three Gram-positive bacteria, *Bacillus cereus* and Methicillin-resistant *Staphylococcus aureus* with a MIC value of 23.1 µg/ml and *Paenibacillus larvae* with a MIC value of 185 µg/ml. It also reduced the number of viable Human Small lung cancer cells (NCI-H187) to less than 50% at a concentration of 10.18 µg/ml.

INTRODUCTION

Rare actinomycetes (non-streptomycetes) are become an increasingly attractive source in the search for new antibiotics to fight drug resistance (Lazzarini *et al.*, 2000). Due to isolation difficulties from natural sources and cultivation and preservation complications, they have not been extensively studied. The genus *Spirillospora* was first proposed by Couch in 1963 for sporangia forming actinomycetes with motile spores and was placed in the family *Thermomonosporaceae* on the basis of 16S rRNA sequences and chemotaxonomic data (Zhang *et al.*, 1998). It comprises only two species, *Spirillospora albida* and *Spirillospora rubra*.

Caves are generally nutrient-poor habitats with low temperatures and high humidity (Schabereiter-Gurtner *et al.*, 2002). They are regarded as excellent sources for the discovery of novel strains of actinomycetes (Barton, 2006). Several new species have been isolated from cave soils including *Catelliglobospora koreensis* from a gold mine cave in Korea (Ara *et al.*, 2008), *Agromyces salestinus* and *Agromyces neolithicus* from the Grotta Dei Cervi cave in Italy (Jurado *et al.*, 2005a) and *Agromyces subbeticus* from a cave occupied by bats in Spain (Jurado *et al.*, 2005b). Recently, *Amycolatopsis jejuensis*, *Amycolatopsis halotolerans*, *Nocardia jejuensis* and *Nocardia spelunca* have been isolated from natural caves (Lee, 2006a, b; Soe *et al.*, 2007).

In the present study a rare actinomycete, strain CMU-PNK470, was isolated from a cave soil. Taxonomic characterization identified it as *Spirillospora albida* based on 16S rRNA gene sequence analysis in combination with

biochemical, chemotaxonomic and morphological data. Its ability to produce antimicrobial and anticancer compounds was also investigated.

MATERIALS AND METHODS

Selective isolation of actinomycetes from a cave soil

A soil sample was collected from Phanangkoi Cave in Phrae province in northern Thailand. The pH, and organic matter and total nitrogen contents of the soil sample were measured. It was sieved and air-dried for 7 days. One gram of soil sample was pretreated with dry heat in a hot air oven at 120 °C for 1 hour (Nonomura & Ohara, 1969). One gram of dried soil was suspended in 9 ml of 1.5% phenol solution for 30 minutes to kill fast-growing soil bacteria. A soil suspension (0.1 ml) was spread onto Humic acid-Vitamin (HV) agar (Hayakawa & Nonomura, 1987) containing nystatin and cycloheximide at final concentrations of 25 and 10 µg/ml, respectively. Plates were incubated at 28 °C for 20 days. Actinomycete isolates were maintained on Hickey-Tresner (HT) agar (Hickey & Tresner, 1952).

Phenotypic characteristics

Strain CMU-PNK470 was cultured on ISP-4 medium (Shirling & Gottlieb, 1966) and HT agar at 30 °C for 30 days. Morphological features of the substrate mycelium, aerial mycelium and spores were observed by light microscopy (BH-2, Olympus) and scanning electron microscopy (model JSM-5910, JEOL). The colors of the aerial mycelium, substrate mycelium and soluble pigment were observed on ISP-2, ISP-4 and HT agar using a NBS/IBCC color chart (Mundie, 1995). The temperature range for

*Corresponding author and address: Saisamorn Lumyong, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai 50200, Thailand., phone: +66-53-941947ext144, e-mail: scboi009@chiangmai.ac.th, fax: +66-53-892259.

growth was determined on ISP-2 agar. For examination of spore motility, the isolate was cultured on HV agar until spores were formed. The culture was flooded with distilled water or 0.01 M phosphate buffer (pH 7.0) containing 10% soil extract (Hamaki *et al.*, 2005) and incubated at 28 °C for 1 hour. Spore motility was observed under the light microscope. Carbohydrate utilization was determined by the method of Pridham & Gottlieb (1948). Gelatin liquefaction and starch hydrolysis were tested according to Gordon *et al.* (1974) and Goodfellow (1971), respectively.

Chemotaxonomic characteristics

Bacterial biomass for the chemotaxonomic studies and molecular identification was obtained after 14 days incubation with shaking at 28 °C in ISP-2 broth. The isomers of diaminopimelic acid and whole cell organism sugars were examined using standard procedures described by Hasegawa *et al.* (1983). Menaquinones were extracted and purified following Collins *et al.* (1977) and analyzed by High Performance Liquid Chromatography (HPLC) (Wu *et al.*, 1989).

Nucleotide sequencing of the 16S rRNA gene

Genomic DNA was isolated as described by Hopwood *et al.* (1985). The 16S rRNA gene was amplified by PCR using the universal primers 27F (5'-AGAGTTTGATC-MTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWT-CARCC-3') (Lane, 1991). The 50 µl PCR reaction mixture contained 5 µl of DNA template, 2 µl of 25 mM deoxyribonucleotide triphosphates (dNTPs), 2 µl of each 10 µM primer, 0.4 µl of Taq DNA polymerase, 5 µl of 10 × PCR buffer and 33.6 µl of sterilization distilled water. All reactions were preheated at 95 °C for 5 min and then 30 cycles of PCR amplification were performed using 95 °C 1 min denaturing, 50 °C 1 min annealing, and 72 °C 1 min extension. The reaction was kept at 72 °C for 10 min for final extension. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen) and sequenced by Macrogen Inc. (Seoul, Korea). The sequence was compared with all accessible sequences in a database using BLAST software. Multiple sequence alignment and computation of sequence similarity were conducted using ClustalW within the Bioedit program (Hall, 1999). A phylogenetic tree was constructed using the neighbor-joining method of Saitou & Nei (1987) in the Molecular Evolutionary Genetics Analysis (MEGA) program version 4 (Tamura *et al.*, 2007).

Biological activity

The ability of the CMU-PNK 470 strain to inhibit the growth of bacteria, yeast and filamentous fungi was determined using the agar well method (Barry & Thornsberry, 1985). The test microorganisms were *Bacillus cereus*, *Escherichia coli*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Paenibacillus larvae*, *Staphylococcus aureus* and yeast, *Candida*

albicans. Plant pathogenic filamentous fungi were *Colletotrichum*, *Didymella*, *Fusarium oxysporum* and *Sclerotium solani*. All of the test organisms were obtained from the Excellent on Sustainable Development of Biodiversity Resources Center, Chiang Mai University, Thailand. Strain CMU-PNK470 was grown in antibiotics production media for 20 days on shaking incubation at 30 °C. The culture broth was then extracted with ethyl acetate. The minimum inhibitory concentration of the crude extract was determined and defined as the lowest concentration of crude extract at which there was no visible growth of test microorganisms. The anti-cancer assay of crude extract against cancer cell lines was also determined by Sulphorhodamine B (SRB) assay (Skehan *et al.*, 1990). Doxorubicin and ellipticine were used as a positive control and DMSO as a negative control. Three cancer cell lines, Human breast cancer (MCF7), Human oral cavity cancer (KB), and Human small cell lung cancer (NCI-H187) were used in this study. The maximum concentration of the tested samples was 50 µg/ml.

RESULTS AND DISCUSSION

The soil sample had a pH of 8.29, with organic matter and total nitrogen contents of 15.26% and 0.841% respectively. A total of 78 actinomycetes were selectively isolated on Humic acid-Vitamin (HV) agar. One isolate, designated as CMU-PNK470, was selected for detailed studies as it showed distinct morphological and chemical characteristics and produced a diffusible blue pigment in HT agar.

Strain CMU-PNK470 was grown on several media to enable cultural characterization. The strain grew very slowly on all tested media. It formed very small colonies and rarely formed aerial mycelium. The colony was deep violet purple on ISP-2 but white on ISP-4 and HT agars. Diffusible pigments were purplish blue on ISP-4 and HT agar plates and deep violet purple on ISP-2 agar (Table 1). Spherical sporangia with diameters of 5 µm (Fig. 1) were formed on aerial mycelium after cultivation on ISP-4 agar for 30 days.

Strain CMU-PNK470 was examined for key phenotypic markers in order to determine whether it had a chemotaxonomic profile consistent with its classification in the genus *Spirillospora*. Strain CMU-PNK470 has chemical properties consistent with its assignment to the genus *Spirillospora* (Couch 1963). It contains *meso*-diaminopimelic acid in cell wall hydrolysates as the characteristic cell wall diamino acid with madurose, galactose and arabinose as diagnostic sugars. The major menaquinones were MK-9 (H₆), MK-9 (H₄) and MK-9 (H₈). The motility of its sporangiospores were also shown. These phenotypic properties were consistent with the description of the genus *Spirillospora*.

For phylogenetic analysis, the 16S rRNA gene sequence of strain CMU-PNK470 was submitted to GenBank under

Table 1. Phenotypic characteristics of strain CMU-PNK470 and related species

Characteristics	CMU-PNK470	<i>Spirillospora albida</i> NBRC 12248 ^{T*}	<i>Spirillospora rubra</i> JCM 6875 ^{T**}
Growth on ISP2	AM: –; SM: purple; SP: purple	AM: white; SM: beige; SP: –	ND
Growth on ISP4	AM: white; SM: pale purple; SP: purple	AM: white; SM: beige; SP: –	ND
Motile spore formation	+	+	+
Production of sporangia	+	+	+
Production of Citrate lyase	+	ND	ND
Degradation of:			
Tyrosine	+	+	–
Gelatin	–	–	+
Starch	–	ND	ND
Casein	+	+	+
(+)-D-Cellobiose	+	–	ND
Myo-inositol	+	–	ND
(–)-D-Lactose	–	ND	ND
(–)-D-Mannitol	–	+	ND
(+)-L-Rhamnose	–	–	ND
(+)-D-Xylose	–	–	ND
D-Fructose	+	ND	ND
D-Mannose	+	ND	ND
D-Raffinose	+	ND	ND
L-Arabinose	–	ND	ND
Sucrose	+	ND	ND

AM = aerial mycelium, SM = substrate mycelium, SP = soluble pigment, ND = Not determined, *Data taken from *Rose & Meyers 2007, **Goodfellow 1989

Fig. 1. Scanning electron micrograph of CMU-PNK470 cultured on ISP-4 medium for 30 days at 30°C.

the accession number EU399546. It is evident from Figure 2 that this strain belongs to the genus *Spirillospora* and is closely related to *Spirillospora albida* NBRC12248^T. This relationship is supported by a high bootstrap value of 94%. Strain CMU-PNK470 shared 98.86% 16S rRNA sequence similarity to *Spirillospora albida* NBRC12248^T, a value that corresponds to 16 nucleotide differences at the 1408 locations available for alignment (Table 2). The high level of 16S rDNA sequence similarity suggests that strain

CMU-PNK470 should be identified as *Spirillospora albida*. A 16S rRNA sequence similarity of 97.21% was observed between strain CMU-PNK470 and *Spirillospora rubra* JCM6875^T. Also, they were located on a different clade although they belong to the same genus. A similar observation was reported by Zhang *et al.* (2001) who suggested that *S. rubra* should be separated as an independent genus. Due to *S. albida* being located on the same clade of *Actinomadura* and being more closely related than *S. rubra*, Zhang *et al.* (2001) suggested that the lateral transfer of a distant gene cluster source produced a unique morphology in an ancestor of *Spirillospora*. Consequently, they suggested combining them. Further studies are needed to clarify the relationship between *S. albida* and *S. rubra*.

Phenotypic comparisons between strain CMU-PNK470 and related species are shown in Table 1. Unfortunately there is little phenotypic information relating to this genus available for comparison. However, strain CMU-PNK470 may represent a new species within the genus *Spirillospora* because a higher 16S rDNA similarity was found between representatives of validly described members of the family *Thermomonosporaceae* such as *Actinomadura citrea* and *Actinomadura coerulea* which shared 99.4% similarity. The clarification of the taxonomic status of strain CMU-PNK470 requires further investigation.

Spirillospora albida is very difficult to cultivate due to its slow growth. Hence, there has been little published information describing its distribution, isolation and bio-

Fig. 2. Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the position of *Spirillospora albida* CMU-PNK 470 amongst its related neighbors. The number of branches indicated the percentage bootstrap values of 1,000 replicates (greater than 50%).

Bar, 0.01 nucleotide substitutions per position.

active compound production since the identification of *Spirillospora* as a separate genus. Spirillomycin is the only known specific antibiotic produced by this genus (Domnas, 1968). Hacène *et al.*, (1994) and Hacène & Lefebvre (1995, 1996) reported that *Spirillospora* strain 719 from a soil sample in the Algerian Sahara produced several unspecified antibiotics including a new deep red pigment-like anti-bacterial HP17, which could inhibit the growth of coccoidal

Gram-positive bacteria, a new polyene antifungal HM17 and a broad spectrum antibiotic, AH17. There have been no reports of anticancer compounds produced from members of the genus *Spirillospora*. Thus, the antibiotic activity of strain CMU-PNK470 was investigated. It showed an ability to produce an anti-Gram-positive bacteria substance which was active against *Bacillus cereus*, a clinical isolate of Methicillin-resistant *Staphylococcus aureus* (MRSA) and

Table 2. 16S rRNA sequence percentage similarity between *Spirillospora albida* CMU-PNK470 and related taxa.

Strain: 1, *Actinomadurula rudentiformis*; 2, *Actinomadurula hibisca*; 3, *Actinomadurula umbrina*; 4, *Actinomadurula echinospira*; 5, *Actinomadurula catellatospora*; 6, *Actinomadurula livida*; 7, *Actinomadurula yumaensis*; 8, *Actinomadurula rugatobispora*; 9, *Actinomadurula vinacea*; 10, *Actinomadurula viridis*; 11, *Actinomadurula latina*; 12, *Actinomadurula meyerii*; 13, *Actinomadurula formosensis*; 14, *Actinomadurula cremea* subsp. *cremea*; 15, *Actinomadurula pelleteri*; 16, *Actinomadurula fibrosa*; 17, *Actinomadurula fulvescens*; 18, *Actinomadurula napierensis*; 19, *Actinomadurula namibiensis*; 20, *Spirillospora albida*; 21, *Spirillospora rubra*; 22, *Excelsospora viridilutea*; 23, *Thermomonospora curvata*; 24, *Actinocorallia glomerata*; 25, *Actinocorallia cavernae*; 26, *Actinocorallia libanitica*; 27, *Actinocorallia herbida*; 28, *Nocardiopsis dassonvillei*; 29, strain CMU-PNK470. (GenBank accession numbers of each strain were similar to Figure 2)

	Percentage similarity																												Different /Total nucleotides
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1	—																												25/1395
2	97.73	—																											32/1407
3	95.70	95.62	—																										60/1395
4	96.28	95.77	96.43	—																									49/1381
5	95.28	95.42	95.31	95.01	—																								60/1402
6	96.95	96.84	96.95	96.35	98.11	—																							44/1406
7	97.22	97.24	96.74	96.20	96.61	98.48	—																						37/1403
8	97.10	97.36	95.92	96.34	95.97	97.44	97.22	—																					44/1387
9	97.24	97.82	96.97	96.49	96.49	98.04	98.36	—																					43/1402
10	97.30	97.56	96.64	96.63	96.76	98.13	97.92	97.94	99.37	—																			43/1402
11	96.37	95.85	95.97	95.39	95.10	96.28	96.34	96.49	96.65	96.60	—																		49/1386
12	97.43	97.02	96.52	96.41	96.10	97.23	97.58	97.64	97.54	97.49	97.30	—																	35/1400
13	97.09	96.41	95.84	96.28	95.92	96.83	96.69	97.29	97.84	97.85	95.38	96.55	—																38/1404
14	96.01	96.22	96.41	95.34	95.54	96.92	97.06	96.65	96.85	97.05	96.50	97.26	97.69	—															49/1403
15	96.65	96.47	96.11	96.13	95.69	97.24	97.17	97.57	97.48	97.43	96.48	97.23	97.52	97.41	—														46/1402
16	97.86	98.27	95.82	96.26	95.96	97.44	97.51	97.07	97.54	97.98	96.40	97.43	97.65	97.26	97.44	—													25/1406
17	98.87	98.14	95.74	96.42	95.86	97.37	97.29	97.43	97.62	97.57	96.61	97.43	97.44	96.01	96.88	98.19	—												27/1409
18	97.02	96.87	96.62	95.79	95.97	97.24	97.61	97.53	97.39	97.24	98.21	98.36	97.54	97.32	96.80	97.46	97.47	—											33/1336
19	96.97	98.37	95.23	95.73	94.09	95.83	96.17	96.83	96.76	96.32	96.02	96.87	96.18	96.09	96.10	97.45	96.97	96.78	—										35/1402
20	97.67	97.24	95.47	96.04	95.36	96.89	96.88	96.63	96.93	97.01	95.86	96.83	96.30	96.27	96.32	97.93	97.78	97.00	96.62	—									16/1408
21	97.56	96.88	96.25	96.28	94.74	96.26	96.19	96.28	96.41	96.30	95.75	96.59	95.84	95.56	96.60	96.87	97.55	96.55	96.16	96.86	—								39/1396
22	95.87	96.18	94.51	95.55	93.60	95.37	95.35	95.71	95.59	95.47	94.81	95.58	94.52	95.21	94.86	95.55	95.68	95.73	96.19	95.68	95.48	—							58/1395
23	94.74	94.35	95.82	95.93	93.73	95.41	95.68	95.21	96.15	95.82	94.72	95.38	94.86	94.94	95.19	94.55	94.58	95.13	94.44	94.02	94.91	95.22	—						81/1399
24	96.55	96.89	95.48	95.43	94.16	95.86	95.42	95.34	95.80	95.53	94.72	95.90	94.10	94.92	94.86	96.38	96.81	95.80	96.43	95.75	96.53	94.51	93.90	—					52/1397
25	95.57	95.79	93.91	94.68	93.43	94.73	94.56	94.61	94.48	94.48	94.16	94.68	94.35	93.98	94.05	95.62	95.58	95.05	95.57	95.05	95.34	94.04	93.28	97.44	—				60/1384
26	95.20	95.71	94.48	94.25	93.21	94.44	94.27	94.68	94.34	94.19	94.30	95.04	94.41	94.26	94.12	95.56	95.21	95.18	96.33	95.20	95.13	94.11	92.92	97.50	97.31	—			56/1388
27	95.72	96.38	94.83	94.67	93.42	95.07	94.70	94.53	94.94	94.89	94.63	95.49	94.76	94.55	94.27	96.17	96.02	95.71	96.34	95.95	95.61	94.43	93.32	98.02	97.21	98.00	—		51/1395
28	90.63	91.26	91.45	92.04	89.31	90.96	90.99	90.70	91.26	90.88	90.11	90.97	89.93	90.71	90.36	91.17	91.10	91.48	91.75	90.68	91.31	91.82	91.00	91.58	91.33	91.03	91.21	—	127/1398
29	98.21	97.73	95.70	96.45	95.72	96.87	97.36	96.83	96.93	96.93	96.46	97.50	97.29	96.51	96.72	98.22	98.08	97.53	97.50	98.86	97.21	95.84	94.21	96.28	95.66	95.97	96.34	90.92	—

Paenibacillus larvae, a causative agent of American Foulbrood in honey bees. The MIC value of the antimicrobial extracted from strain CMU-PNK470 against *Bacillus cereus* and Methicillin-resistant *Staphylococcus aureus* was 23.1 µg/ml and against *Paenibacillus larvae* was 185 µg/ml. Also, a crude extract of strain CMU-PNK470 showed anticancer activity against human small lung cancer cells (NCI-H187) with an IC₅₀ value of 10.18 µg/ml. This result encourages us to investigate this compound in the future.

We conclude from the genotypic and phenotypic data that strain CMU-PNK470 represents a new strain within the species *Spirillospora albida*. This is the first reported isolation of *Spirillospora albida* from a cave soil. The strain showed biological activity against selected pathogenic Gram-positive bacteria and cancer cell lines. The results obtained show the potential value of this strain as a source of novel bioactive compounds. Hence, it merits further studies to purify and characterize the active components. This study also supports the hypothesis that rare microorganism are a promising source of new bioactive compounds.

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