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Full Length Research Paper

Isolation and bioactivity of endophytic filamentous actinobacteria from tropical medicinal plants

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Endophytic microorganisms are rich sources for drug discovery. Isolation of actinomycetes from the surface-sterilized tissues of 12 medicinal plants in Hainan, China, was carried out using different media of American Type Culture Collection (ATCC) 172 agar, Gauze's No. 2 agar, glucose-asparagine agar, humic acid-vitamin agar, and starch-casein-mineral salts agar. Of the 280 isolates recovered, 154 were from roots, 73 from stems and 53 from leaves. Bioactivity test of crude fermentation extracts were performed on all the isolates. About 41.1% of the extracts showed activity against liver cancer cell SMMC-7721, 19.3% against *Candida albicans* ATCC10231, and 10.0% against *Staphylococcus aureus* ATCC 51650. In addition, metabolites of nine isolates inhibited caspase 3, a protein related to neurodegenerative diseases, and three inhibited protein tyrosine phosphatase 1B (PTP1B), a protein related to diabetes. Based on their phenotypic and genotypic characteristics, endophytic actinomycetes were classified to actinobacterial genera including *Streptomyces, Micromonospora, Nocardia, Nonomuraea* and *Amycolatopsis* spp. The high bioactivity percentage, broad bioactivities and the novel taxa of the isolated endophytic actinomycetes presented their potential in pharmaceutical utilization.

Key words: *Filamentous actinomycetes, medicinal plants, antimicrobial activity, anti-tumor cell activity, protein tyrosine phosphatase 1B, caspase 3.*

INTRODUCTION

Endophytic microbes colonize the internal tissues of host plants and can form commensal, mutualistic symbiotic and trophobiotic relationships with them (Rosenblueth and Martínez, 2006; Ryan et al., 2008). These ecological roles have been applied on plant growth promotion (Compant et al., 2005; Nimnoi et al., 2010), induce disease resistance (Meguro et al., 2004) and drought tolerance (Hasegawa et al., 2004) in tissue-cultured seedlings, and act as biocontrol agents (Kunoh, 2002). Unique compounds discovered from endophytes secondary metabolites also show significant biological and ecological implications (Gunatilaka, 2006). The correlation of metabolites between endophytic actinomycetes and their host plant was clearly elucidated by the example of taxane producers. The capability of taxane production was not related to the geological source and taxonomic position of fungi or acinomycetes but connected to host plant (Caruso et al., 2000). The emergence of drug-resistant microorganisms and the increased threat of death caused by cancer are driving the search for a new generation of antimicrobial and antitumor agents for healthcare (Pervaiz, 2002; Talbot et al., 2006). Actinomycetes as promising pharmaceutical resources have been discovered from marine sediments (Bull and Stach, 2007), mangrove soil and plants (Hong et al., 2009), and desert soil (Okoro et al., 2009).

There is evidence that plant endophytic microbes

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represent an untapped sources of natural products (Tan and Zou, 2001; Strobel and Daisy, 2003; Strobel et al., 2004; Gunatilaka, 2006). Comparing with the highly explored soil source for actinomycetes discovery, less attention has been paid to actinobacteria present in the interstitial spaces of plant tissues (Stone et al., 2000), although it is known they are common in the tissues of healthy vascular plants (Hasegawa et al., 2006). Diverse actinomycetes have been uncovered from surface-sterilized plants, in addition to *Micromonospora*, *Microbispora*, *Nocardioides* and *Streptomyces* (Coombs and Franco, 2003), Verma et al. (2009) isolated *Sacchromonospora*, *Streptosporangium* and *Streptoverticillium*, and Qin et al. (2009) reported *Saccharopolyspora, Dietzia, Blastococcus, Dactylosporangium, Promicromonospora, Oerskovia, Actinocorallia* and *Jiangella*. There is evidence that such novel endophytic actinobacteria are a promising source of antimicrobial agents (Sasaki et al., 2001; Castillo et al., 2002, 2003, 2007; Ezra et al., 2004; Verma et al., 2009; Qin et al., 2009; Zhao et al., 2011) and antitumor agents (Bieber et al., 1998; Taechowisan et al., 2003, 2007; Igarashi et al., 2006, 2007). The present study was designed to isolate endophytic filamentous actinobacteria from 12 medicinal plants in a tropical reserved botanical garden in Hainan Province, and assess their biological activities for medicinal use.

MATERIALS AND METHODS

Sample collection site and plant material

12 healthy medicinal plants (Table 1) were selected from the Tropical Botanical Garden in Danzhou City (19° 31' N, 109° 35' E; elevation, 551ft/168 m), Hainan province, China. 36 samples of leaf, stem and root were collected from the plants in September, 2006. The plants were chosen by their medicinal function recorded (Dai and Mei, 2007, 2008).

Isolation of endophytic actinomycetes

The leaf, stem and root samples taken from the medicinal plants were washed in running tap water to remove soil particles then air-dried in a laminar flow hood. Five grams of each of the resultant preparations were weighed and treated with 70% ethanol for 5 min then with sodium hypochlorite (0.9% available chlorine) for 20 min, washed in sterile water three times to remove the surface sterilization agents and soaked in 10% $NaHCO_3$ solution to disrupt the plant tissues and to inhibit fungal growth. The treated plant materials were then cut into small fragments, macerated in 20 mL sterile phosphate-buffered saline (PBS) using a mortar and pestle. The resultant homogenates (0.2 mL) were plated into 5 selective isolation media: ATCC 172 agar (Hong et al., 2009): glucose 10 g/L, soluble starch 20 g/L, yeast extract 5 g/L, NZ-Amine A 5 g/L, calcium carbonate 1 g/L, agar 20 g/L, in distilled water pH 7.0; Gause's No. 2 agar (Ivantiskaya et al., 1978): glucose 10 g/L, peptone 5 g/L, tryptone 3 g/L, NaCl 5 g/L, agar 20 g/L, pH 7.2; glucose-asparagine agar (Takahashi et al., 1996): asparagine 1 g/L, glucose 2 g/L, K_2HPO_4 0.5 g/L, $MgSO_4.7H_2O$ 0.5 g/L, agar 20 g/L, pH 7.2; humic acid-vitamin agar (Hayakawa et al., 1987): humic acid 1.0 g/L, Na_2HPO_4 0.5 g/L, KCl 1.71 g/L, $MgSO_4{\cdot}7H_2O$ 0.05 g/L, $FeSO_4{\cdot}7H_2O$ 0.01 g/L, $CaCO_3$, 0.02 g/L, *p*-aminobenzoic acid 0.5 mg/L, biotin 0.25 mg/L, inositol 0.5 mg/L, niacin 0.5 mg/L, riboflavin 0.5 mg/L, pyridoxin-HCl 0.5 mg/L, Ca-pantothenate 0.5 mg/L, thiamine-HCl 0.5 mg/L, agar 20 g/L, pH 7.2; starch-casein-mineral salts agar(Küster and Williams, 1964): soluble starch 10 g/L, casein 0.3 g/L, KNO_3 2 g/L, NaCl 2 g/L, K_2HPO_4 2 g/L, $MgSO_4.7H_2O$ 0.05 g/L, $CaCO_3$, 0.02 g/L, $FeSO_4$ 0.01 g/L, agar 20 g/L, pH 7. 2.

All of the media were supplemented with the antifungal antibiotics actidione (50 µg/mL) and nystatin (50 µg/mL), and with 20 µg/mL of nalidixic acid to inhibit the growth of fast growing bacteria. Presumptive actinobacterial isolates grown on the isolated plates were plated into yeast extract - malt extract agar (ISP medium 2) (Shirling and Gottlieb, 1966) and incubated at 28°C for 7 days. The cultures were maintained on modified Bennett's agar (Jones, 1949) and long term preserved as mixtures of hyphal fragments and spores in glycerol (20%, v/v) at −20°C. The efficacy of the surface sterilization procedure was assessed by aseptically rolling surface-sterilized plant tissue into plates of each of the isolation media prior to incubation at 28°C.

Preparation of culture extracts

Each isolate was cultivated in 20 mL liquid medium (1.5% soybean, 2% starch, 0.2% peptone, 0.5% yeast extract, 0.4% $CaCO_3$ and pH 7.2) in a 100- mL Erlenmeyer flask at 28°C for 7 days at 200 rpm. Crude extracts were prepared by adding 60 mL of methanol to each of the cultures and the extraction was allowed to proceed for 2 weeks. Fractions of each 1 mL of the resultant extracts were transferred to wells in 96- deep well plates, vacuum dried at 60°C, dissolved in 200 µL DMSO and used in the biochemical screens (Hong et al., 2009).

Biological activity assays

Candida albicans ATCC 10231 and *Staphylococcus aureus* ATCC 51650 were cultured overnight at 30°C, 200 rpm in YPD medium (glucose 20 g/L, tryptone 20 g/L, yeast extract 1g/L, pH 5.0～5.5) and nutrient broth, respectively. The resultant cultures were diluted in the same media to $0.8\text{-}1.2{\times}10^6$ cfu/mL, and 100 µL aliquots of the inocula were transferred to individual wells in a 96-well plates. Culture extracts of each of the isolates were added to each of the wells. The two culture media were used as negative controls, and fluconazole and kanamycin as the positive controls for the anti- *C. albicans* and anti *S. aureus* assays, respectively. The 96-well plates were shaken at 200 rpm for 24 h at 30 °C then examined using a microplate spectrophotometer (Multiskan Mk3, Finland) at 570 nm. The absorbance readings from each well were used to record bioactivities as "+", "++", or "+++" for higher or equal to 4 to 6, 6 to 8 and 8 µg/mL of standard, respectively.

Serial dilutions of the positive control antibiotics at 128, 64, 32, 16, 14, 12, 10, 8, 6, 4, 2, and 1 µg/mL were used to generate standard curves. The construction, expression, purification, and enzymatic assays for PTP1B and caspase 3 were carried as follows. The recombinant enzyme proteins of PTP1B were expressed in an *Escherichia coli* system; for primary screening, 2 µL of the stock solution of each crude extract (1 mg/mL) in DMSO were transferred into individual wells of 96-well flat bottom plates to give a final concentration of 20 µg/mL of extract in 2% DMSO. After incubation with the enzymes for 15 min, 10 times concentrated substrates were added to initiate the enzymatic reaction, and the resultant enzymatic activity normalized against the control (2% DMSO) to obtain the inhibition rate of the compound. When the inhibition rate was more than 50% at 20 µg/mL, the dose-response inhibition assay of the compound was performed to determine the 50% inhibition concentrations (IC_{50}) (Hong et al., 2009).

Table 1. Number of filamentous actinomycetes isolated and their biological activities comparing with the recorded pharmacological activity of their host.

Medicinal plant	Number of isolate				Number of the bioactive strains						Pharmacological activity recorded
	Total	Leaves	Roots	Stems	Total	Anti *C. albicans*	Anti-*S. aureus*	Anti-tumor cell	Caspase 3 inhibition	PTP1B inhibition	
Antiaris toxicaria	5	0	5	0	2	0	1	1	0	0	Cardiotonic effect (Dai and Mei, 2007)
Aphanamixis grandifolia	24	12	10	2	16	5	4	10	1	0	Treating rheumatoid arthritis (Wu, 1988)
Areca catechu	17	4	3	10	4	2	1	1	0	0	Antimcrobial activity (Dai and Mei, 2007)
Cephalotaxus hainanensis	1	0	1	0	0	0	0	0	0	0	Anticancer activity (Dai and Mei, 2007)
Cerbera manghas	50	0	45	5	25	13	1	20	1	1	Anticancer activity (Dai and Mei, 2007)
Dracaena cambodiana	24	1	21	2	19	10	7	8	1	0	Anti-inflammatory, analgesic action, anti- fungal activities and anticancer activity (Dai and Mei, 2008)
Evodia lepta	36	4	29	3	27	5	7	22	0	1	Detumescence and analgesic action, releasing heat and resolving toxins (Dai and Mei, 2007)
Hydnocarpus hainanensis	52	12	36	4	34	13	4	19	3	1	Anti-*Mycobacterium leprae,* anticancer activity (Dai and Mei, 2007)
Maytenus confertiflorus	7	0	2	5	5	2	0	2	1	0	Anticancer activity (Wang et al.,1981)
Plumeria obtuse	47	11	0	36	29	3	2	26	1	0	Releasing heat and resolving toxins, moistenning lung and relieving cough*
Strychnos hainanensis	13	7	2	4	9	1	1	6	1	0	Detumescence and analgesic action (Hu et al., 2008)
Thevetia peruriana	4	2	0	2	0	0	0	0	0	0	Cardiotonic effect (Gao and Zeng, 1983)
Total	280	53	154	73	169	54	28	115	9	3	

*From State Administration of Traditional Chinese Medicine "Chinese Materia Medica" Editorial Board (2000).

Cell growth inhibition assays were then carried out. Fractions (100 µL) of each cell suspension (40,000 cells/mL) of the adherent cell line SMMC-7721 were dispensed into individual wells in 96-well plates which were incubated in Dulbecco's Modified Medium (Beijing Xinjingke Biotechnology Company Limited) at 37°C in an incubator containing 5% CO_2 for 24 h to allow surface attachment of the cells. Preparations in DMSO (1 µL) were added and the 96-well plates incubated under the same conditions for 72 h when 50 µL 3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) (5 mg/ml) was added to each of the wells and the plates incubated for 3 h under the same conditions. The media were removed using a pipette and 100 µL of specimen in DMSO were added to each of the wells of the plates. Readings were taken using a Multiskan apparatus (Leibo, Shanghai, China) at an absorbance of 570 nm, and a reference wavelength at 690 nm, mitmycin C was used as the positive control. The IC_{50} values of each of the inhibitors were calculated in GraphPad Prism (GraphPad Software, San Diego, CA) using non-linear regression analysis.

Phenotypic identification of isolated filamentous actinomycetes

Cultural and physiological features of the isolates were acquired using media and methods described by Shirling and Gottlieb (1966). The isolates were grown on yeast extract-malt extract agar (ISP medium 2) and peptone- yeast extract-iron agar (ISP medium 6) plates at 28°C for 10 days. The incubated plates were then examined visually to determine aerial spore mass color, substrate mycelial pigmentation and the color of any diffusible pigment. The peptone-yeast extract-iron agar plates were examined to see whether any of the isolates produced melanin pigments. Spore chain morphology was determined by light microscopy of 10 day-old cultures grown on the ISP medium 2 plates. Biomass samples for the chemotaxonomic studies were prepared from cultures grown either in yeast-glucose or trypticase soy broths on a rotary shaker at 28°C prior to harvesting and preparation of freeze-dried cells. Standard TLC procedures were used to detect the isomers of diaminopimelic acid (Becker et al., 1964) and whole-organism sugars (Boone and Pine, 1968) using appropriate controls.

Extraction of DNA from representative strains

Seventeen strains (Table 4) represented for isolates of most of the plants and bioactivities were selected for identification. A loopful of hyphal fragments and spores of each strain was scraped from colonies grown on ISP medium 2 and suspended in 500 µL of 1×TE buffer (0.01 M Tris-HCl, 0.01 M EDTA [pH 8.0]) and mixed by vortexing. The resultant preparations were treated with lysozyme (2 mg/ml), incubated at 37°C for 60 min, proteinase K (5 µL of 20 mg/mL) and 20% sodium dodecyl sulfate (SDS) (50 µL) were then added and the preparations incubated at 55°C for 60 min. Following incubation, a further 10 µL of 25% SDS was added to each of the preparations which were then incubated at 55°C for 30 min. The resultant lysates were centrifuged (15,000×*g*, 5 min) to pellet the cell debris before sequential extractions with phenol –chloroform–isoamyl alcohol (25:24:1, v/v). The resultant aqueous phases were precipitated with 2 volumes of isopropanol, washed with 70% (v/v) ethanol, and dried under vacuum. The resultant DNA samples were redissolved in 50 µL of TE buffer (pH 8.0) prior to use (Coombs and Franco, 2003).

16S rRNA genes analysis of the selected actinomycetes

Oligonucleotide primers with specificity for eubacterial 16S rRNA genes (forward primer 8–27: 5'-AGACTTTGATCCTGGCTCAG-3', reverse primer 1492: 5'-CGGCTACCTTGTTACGACTTC-3') were used to amplify 16S rRNA genes of representative isolates. PCR was achieved using a DNA thermal cycler (PE 2400) with the following amplification profile: initial denaturation at 94°C (5 min), 30 cycles of 94°C (1 min), 55°C (50 s), and 72°C (2 min), and a final extension step of 10 min at 72°C. The PCR products were purified and sequenced by Yingjun Biotechnology Limited (Shanghai, China). The resultant 16S rRNA gene sequences were compared with those deposited in the public databases and the EzTaxon server (http://www.Eztaxon.org; Chun et al., 2007) which was also used to calculate pairwise sequences similarities. The representative sequences of related type strains were retrieved from the GenBank/EMBL/DDBJ databases. The highest 16S rRNA gene sequence similarities to the type strains were obtain using BioEdit software (Hall, 1999). A neighbor joining (Saitou and Nei, 1987) phylogenetic tree was generated using MEGA version 4.0 software (Tamura et al., 2007), was evaluated in a bootstrap analysis (Felsenstein, 1985) of 1000 replicates; a distance matrix was generated using Kimura's 2-parameter model (Kimura, 1980).

RESULTS

Isolation of endophytic filamentous actinomycetes

A total of 280 actinomycetes were isolated from 36 plant samples collected from the twelve medicinal plants; 154 were from roots, 73 from stems and 53 from leaf samples. The average number of isolates per sample was 7.7. Between 1 and 52 isolates were obtained from the 12 plants, most from the root material. Most of the isolates were obtained from materials of *Hydnocarpus hainanensis* (18.6%), *Cerbera manghas* (17.9%) and *Plumeria obtuse* (16.8%) with relatively few from the remaining plants and only a single strain from *Cephalotaxus hainanensis* (Table 1).

Bioactivity of the endophytic filamentous actinomycetes

Culture extracts of 115 strains showed activity against the tumor cell line (41.1%), 54 against *C. albicans* (19.3%), 28 against *S. aureus* (10.0%), and 9 against caspase 3 (3.2%); only 3 inhibited PTP1B (Table 1). Extracts of isolates from *Cerbera manghas* and *Hydnocarpus hainanensis* showed activity against all of the screen models, including four isolates which inhibited caspase 3 and 2 that inhibited PTP1B (Table 1). The highest incidence of bioactivity was observed from the extracts of cultures isolated from *Dracaena cambodiana*. The small number of strains isolated from *C. hainanensis* and *Thevetia perurian* did not show any bioactivity. High number of antimicrobial isolates was found from *D. cambodiana*, and *H. hainanensis*, which were recorded for antimicrobial activities. Anti-tumor cell strains with high percentage were isolated from *C. manghas* (40.0%), *D. cambodiana* (33.3%), and *H. hainanensis* (36.5%), which are in accordance with their anticancer activity in the record (Dai and Mei, 2007, 2008). Higher percentage of anti-tumor cell isolates were noticed from

Table 2. Distribution of isolated filamentous actinomycetes in twelve medicinal plants.

Medicinal plant	Number of isolates in different taxonomic groups of actinomycetes				
	Streptomyces	***Micromonospora***	***Nocardia***	***Nonomuraea***	***Amycolatopsis***
Antiaris toxicaria	4	1	0	0	0
Aphanamixis grandifolia	18	5	1	0	0
Areca catechu	15	2	0	0	0
Cephalotaxus hainanensis	1	0	0	0	0
Cerbera manghas	48	2	0	0	0
Dracaena cambodiana	23	0	0	0	1
Evodia lepta	36	0	0	0	0
Hydnocarpus hainanensis	34	12	0	6	0
Maytenus confertiflorus	6	0	0	0	1
Plumeria obtuse	47	0	0	0	0
Strychnos hainanensis	13	0	0	0	0
Thevetia peruriana	4	0	0	0	0
Total	249	22	1	6	2

Table 3. Distribution of bioactive endophytic actinomycetes isolates in different genus.

Genera	Number of tested isolates	Number of the active strains				
		Anti-*C. albicans*	**Anti-*S. aureus***	**Anti- tumor cell**	**Caspase 3 inhibition**	**PTP1B-inhibition**
Amycolatopsis	2	1	0	1		
Micromonospora	22	4	0	3	1	
Nocardia	1	0	0	1		
Nonomuraea	6	3	0	0	1	
Strepomyces	249	46	28	110	7	3

Aphanamixis grandifolia (41.6%), *Evodia lepta* (61.1%) and *Plumeria obtuse* (55.3%), which have no recorded anticancer activity.

Identification of isolates

249 out of the 280 isolates (89%) were presumptively assigned to the genus *Streptomyces* as they produced an abundant aerial spore mass on yeast extract-malt extract agar. Chemotaxonomic characteristics such as LL-diaminopimelic acid (LL-A_2pm) of the isolates whole-cell hydrolysates analysis were in consistent with their assignment to this genus (Shirling and Gottlieb, 1972). *Streptomyces* strains were isolated from all of the investigated plants (Table 2), notably from *C. manghas* and *P. obtuse.* Similarly, 22 of the isolates (7.9%) were presumptively assigned to the genus *Micromonospora* as they contained *meso*-A_2pm, arabinose and xylose, in the whole organism hydrolysate, and formed substrate mycelium that carried single spores mostly at the tips of the hyphae, properties typical of *Micromonospora* (Kawamoto, 1989). The largest number of bioactive isolates was assigned to *Streptomyces.* However, it is worthy to note that the *Micromonospora* and *Nonomuraea* isolates showed caspase 3 inhibition activities (Table 3). Seventeen isolates were selected for 16S rRNA gene sequencing based on their bioactive, chemotaxonomic and morphological properties (Table 4).

It is evident from Figure 1 that 8 of the isolates belong to the genus *Streptomyces*, 4 to the genus *Nonomuraea*, 2 to the genus *Micromonospora*, 2 to the genus *Amycolatopsis* and 1 to the genus *Nocardia*. The *Streptomyces* strains were isolated from roots and stems of most of the collected medicinal plants. In contrast, all of the *Nonomuraea* strains were isolated on Gause's No. 2 agar that had been inoculated with suspensions only from *H. hainanensis* root (Table 4). It is indicated that three of the isolates 102113, 110160 and 310132 which belong to the rare actinomycetes genera were new species, as their highest 16S rRNA sequence similarity to the known type strains were below 98.7% (Table 4) according to Stackebrandt and Ebers (2006), and formed distinct phyletic lines from the known species' (Figure 1). The streptomycete isolate 103105 also could be new species as it is distinctly separated from the type strain neighbors on the phylogenetic tree, and by sequence similarities of 98.105% (Table 4).

Table 4. Characteristics of the seventeen representative endophytic actinomycetes isolates.

Genera	Isolates	Colony characteristics on ISP medium 2 after 10 days			Diagnostic amino acids	Diagnostic sugar	Plant source[a]	Bioactivity	Highest 16S rRNA gene sequence match	
		AM	SM	PG					Type strains	Similarities (%)
Amycolatopsis	102113	–	Brown	–	*meso*-A_2pm	Arabinose galactose	R- *D.cambodiana*	Anti- tumor cell	*A. tolypomycina* DSM 44544^T	98.548
	308201	–	Red	–	*meso*-A_2pm	Arabinose galactose	S- *Maytenus confertiflorus*	Anti-*C. albicans*	*A. pretoriensis* NRRL B-24133^T	98.767
Micromonospora	310118	–	Black	–	*meso*-A_2pm	Arabinose xylose	R- *H.hainanensis*	Anti-*C. albicans*	*M. tulbaghiae* TVU1^T	99.781
	110160	–	Yellow	–	*meso*-A_2pm	Arabinose xylose	R- *H.hainanensis*	Anti-*C. albicans*	*M. endolithica* DSM 44398^T	98.422
Nocardia	305107	–	Brown	–	*meso*-A_2pm	Arabinose galactose	R- *Aphanamixis grandifolia*	Anti- tumor cell	*N. niigatensis* IFM 0330^T	99.280
Nonomuraea	310132	White	Orange	–	*meso*-A_2pm	Madurose	R- *H.hainanensis*	Anti-*C. albicans*	*N. candida* HMC10^T	98.542
	310168	White	Yellow	–	*meso*-A_2pm	Madurose	R- *H.hainanensis*	Anti-*C. albicans*	*N. helvata* IFO 14681^T	98.700
	310103	Trace	Cream	–	*meso*-A_2pm	Madurose	R- *H. hainanensis*	Anti-Caspase 3	*N. candida* HMC10^T	99.057
	310116	Trace	Pink	–	*meso*-A_2pm	Madurose	R- *H. hainanensis*	Anti-*C. albicans*	*N. candida* HMC10^T	99.273
Strepomyces	103105	Gray	Cream	–	LL-A_2pm	–	R- *Areca catechu*	Anti-*C. albicans*	*S. indonesiensis* DSM 41759^T	98.105
	402109	Gray	Orange	–	LL-A_2pm	–	R- *D. cambodiana*	Anti-*C. albicans*	*S. rhizosphaericus* NBRC 100778^T	98.849
	111215	White	Yellow	–	LL-A_2pm	–	S- *P.obtuse*	Anti-*C. albicans*	*S. rapamycinicus* NRRL B-5491^T	99.125
	301106	Gray	Pale brown	Green	LL-A_2pm	–	R- *Antiaris toxicaria*	Anti-*C. albicans* Anti-*S. aureus*	*S. youssoufiensis* X4^T	99.149
	110136	White	Red	–	LL-A_2pm	–	R- *H. hainanensis*	Anti-*C. albicans*	*S. lilacinus* NBRC 3944^T	99.502
	411208	Gray	Violet	–	LL-A_2pm	–	S- *P. obtuse*	Anti-*C. albicans* Anti- tumor cell	*S. puniceus* NBRC 12811^T	99.857
	102111	Gray	Brown	–	LL-A_2pm	–	R- *D. cambodiana*	Anti-*S. aureus*	*S. pactum* NBRC 13433^T	99.716
	303207	Gray	Yellow	–	LL-A_2pm	–	S- *Areca catechu*	Anti-*S. aureus*	*S. pactum* NBRC 13433^T	100.000

[a]: plant source: "R" for root, "S" for stem, AM: color of aerial mycelium; SM: color of substrate mycelium; PG: diffusible pigments; –: none.

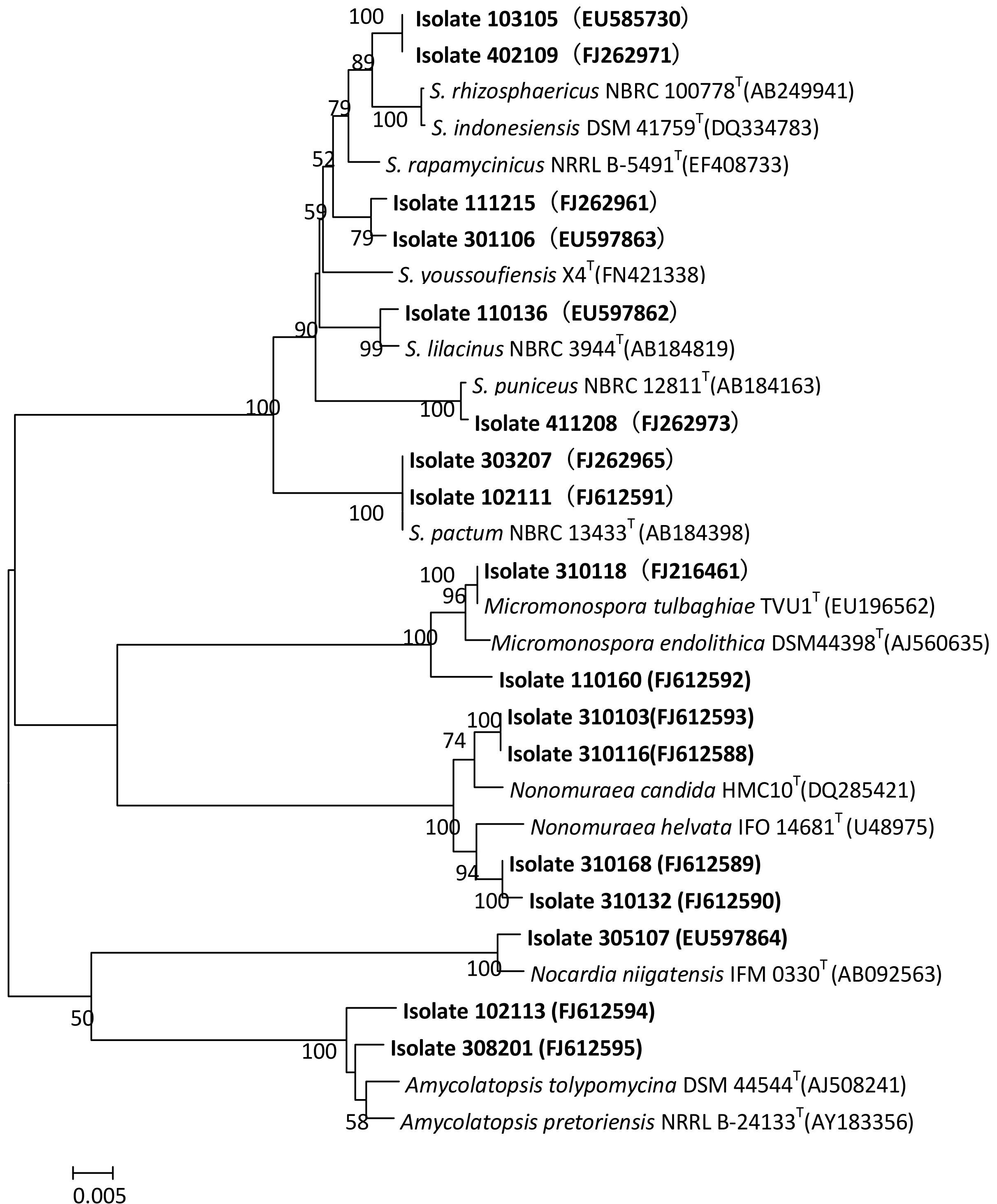

Figure 1. Neighbor-joining 16S rRNA gene tree showing relationships between the representative isolates, and the strains of highest 16S rRNA gene sequence similarity type species. The numbers at the nodes indicate bootstrap values based on 1000 replicates; only values above 50% are shown. Bar 0.5% sequence divergence. GenBank accession numbers are given in bracket.

DISCUSSION

Endophytic filamentous actinomycetes were successfully isolated from all of the 12 selected medicinal plants in one tropical botanic garden, Hainan. The highest number of endophytic actinomycetes was obtained from an

endemic plant *H. hainanensis*. But only one strain was isolated from *C. hainanensis,* another endemic plant which was recorded for anticancer activity. It was suggested that not the origin or location of the plant but the plant itself affect the distribution of endophytic actinomycetes, which is similar to the report of Caruso et al. (2000) that the capability of producing taxanes was not related to the geological source and taxonomic position of acinomycetes but connected to host plant. Many of the strains isolated in the present study showed bioactivity against one or more of the screening models, thereby underpinning and extending results from previous studies which showed endophytic actinobacteria to be a source of natural bioactive compounds (Tan and Zou, 2001; Strobel et al., 2004; Gunatilaka, 2006). Thus, culture extracts of 115 isolates showed activity against the tumor cell line (41.1%), 54 against *C. albicans* (19.3%), 28 against *S. aureus* (10.0%).

Though it is lower inhibition frequencies of biochemical assays on molecular level comparing to growth inhibition assays on cell level, as 9 culture extracts active against caspase 3 (3.2%) and 3 isolates inhibited PTP1B, it is promising for the potential of exploring pharmaceuticals of treating degenerative disease and diabetes from the endophytic actinomycetes of medicinal plants. Endophytes may be a viable alternative pharmaceutical resource instead to the medicinal plants, especially those facing extinction, as some endophytes have been reported to produce the same or similar bioactive natural products generated by host plants, especially medicinal plants (Strobel and Daisy, 2003). A high percentage of strains (10% of fungi, 14% of actinomycetes) isolated from one species of *Taxus baccata* tissues collected from different localities in Italy gave positive results of taxane production (Caruso et al., 2000). The distribution of bioactive actinomycetes in our results showed some correlation to the host plant biochemistry itself.

The highest percentage of isolates showing anti- *C. albicans* and anti- *S. aureus* activity, for example, were from the *D. cambodiana* samples whereas the plant itself was reported to produce 4′-hydroxy-3 5-dimethoxystillben and resveratrol which are known to have antimicrobial activity (Dai and Mei, 2008). Isolates from this plant were particularly effective in the anti-microbial assays. Similarly, higher numbers of anti-cancer cell strains were obtained from the samples of anti-cancer plants (that is, *C. mangha*). It is exciting that *E. lepta* and *P. obtuse* were a particularly rich source of anti-cancer active strains although so far anti-cancer activity has not been recorded in these two plants, but both have been recorded characteristics of releasing heat and getting rid of toxin. These results are not only interesting but will also guide the future bioprospecting strategies. Filamentous actionbacteria, especially *Streptomyces*, have a unique capacity to produce novel bioactive compounds, especially antibiotics (Watve et al., 2001; Bérdy, 2005). Most of the reported natural products produced by endophytic actinomycetes were from streptomycetes (Bieber et al., 1998; Sasaki et al., 2001; Castillo et al., 2003; Ezra et al., 2004; Igarashi et al., 2006; Taechowisan et al., 2007).

It is not surprising that the most active strains in this study belonged to the genera *Streptomyces*, as revealed by previous studies (Coombs and Franco, 2003; Taechowisan et al., 2003; Verma et al., 2009). Indeed, only members of this taxon showed activity against protein tyrosine phosphatase 1B (Table 3). Although streptomycetes now encompass more than 500 species as the largest genus among actinomycetes, much evidence demonstrates that the taxonomic and metabolic diversity of streptomycetes is enormous (Kumar and Goodfellow, 2008; Hong et al., 2009). It is evident from the 16S rRNA gene sequence data of this study that the streptomycetes which showed activity in the anti-*Candida* assay can be assigned to new species as they are separated from the phylogenetic type strains neighbors by sequence similarities below the recommended 98.7% level (Stackebrandt and Ebers, 2006).

Isolates assigned to other taxa, such as the genera *Amycolatopsis, Micromonospora*, *Nonomuraea* and *Nocardia*, also demonstrated promising activities in this study. Two *Amycolatopsis* isolates and one *Nocardia* isolate all showed bioactivities. Four out of six isolates of *Nonomuraea* showed bioactivities and *Micromonospora* showed broader bioactivities just less than streptomycetes, and caspase 3 inhibition activities was detected from these two latter genera. It also can be found that three of the isolates belonging to these rare actinomycetes genera were new species from the phylogenetic tree as their highest 16S rRNA sequence similarity to the known type strains were below 98.7% according to Stackebrandt and Ebers (2006). Endophytic actinomycetes belong to these taxa have been isolated from other plants (Caruso et al., 2000; Coombs and Franco 2003; Verma et al., 2009; Qin et al., 2009; Zhao et al., 2011), this indicated that habitats in the medicinal plants are likely to be a good pharmaceutical source of rare actinomycetes.

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