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Identification of *Streptomyces* sp. Tc022, an endophyte in *Alpinia galanga*, and the isolation of actinomycin D

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Abstract - Some endophytic actinomycetes (120) were isolated from the roots of *Alpinia galanga*. Identification of these endophytes was based on their morphology and amino acid composition of the whole-cell extract. Most isolates were classified as *Streptomyces* sp. (82), with the remainder belonging to *Nocardia* sp. (11), *Microbispora* sp. (3) and *Micromonospora* sp. (2). Eight isolates were unclassified and 14 were lost during subculture. The strain identified as endophytic *Streptomyces* sp. Tc022 strongly inhibited *Colletotrichum musae* and *Candida albicans*. This endophyte was cultured, the agar was extracted with organic solvent and the extract was purified on a column of silica gel to give a major component, which was identified to be actinomycin D on the basis of spectroscopic data. Actinomycin D showed antifungal activity against *Colletotrichum musae* and *Candida albicans* with the MIC of 10 and 20 mg ml^{-1}, respectively.

Key words: *Alpinia galanga*, antifungal activity, endophytic actinomycetes, actinomycin D.

INTRODUCTION

Most plants are host to one or more endophytic microorganisms. By definition, these organisms live between the living cells of their respective host and cause no overt tissue damage. Usually, fungi are the most commonly isolated endophytic microorganisms, but recently the endophytic actinomycetes were isolated from the tissues of healthy plants (Sardi *et al.,* 1992; Shimizu *et al.,* 2000). Some endophytes produced antimicrobial agents that may be involved in a symbiotic association with a host plant (Yang *et al.,* 1994). We have recently isolated endophytic actinomycetes from 36 plant species. The most prevalent group of isolates were the *Streptomycetes* sp. occurring in 6.4% of the tissue samples of *Zingiber officinale*. Some of the isolates showed strong antifungal activity (Taechowisan *et al*., 2003). In a separate study 59 endophytic actinomycetes were isolated from the roots of *Z. officinale* and *Alpinia galanga* and tested against some phytopathogenic fungi. The strain identified as *Streptomyces aureofaciens* CMUAc130 showed the most effective antifungal activity (Taechowisan and Lumyong, 2003). The major active ingredients from the culture filtrate were identified as 5,7-dimethoxy-4-*p*-methoxyphenylcoumarin and 5,7-dimethoxy-4-phenylcoumarin (Taechowisan *et al*., 2005). We report here the isolation from the roots of *Alpinia galanga* of another *Streptomyces* sp. Tc022, which was identified as being phylogenetically close to *Streptomyces parvulus*. Extraction of the culture medium of *Streptomyces* sp. Tc022 afforded actinomycin D, which displayed very strong antifungal activity.

MATERIALS AND METHODS

Isolation of endophytic actinomycetes. Five hundred samples of the root tissues of *Alpinia galanga* were collected from the environs of Nakorn Pathom, Thailand, during the period May 2004-February 2005. Most of them were healthy roots. The samples were washed in running tap water and cut into small pieces of ca. 4 x 4 mm^2. Tissue pieces were rinsed in 0.1% Tween 20 for 30 s, then in 1% sodium hypochlorite for 5 min, and then washed in sterile distilled water for 5 min. Next the tissue pieces were surface sterilized in 70% ethanol for 5 min and air-dried in a laminar flow chamber. Finally the pieces were transferred to dishes of humic acid-vitamin (HV) agar (Otoguro *et al.,* 2001) containing 100 mg ml^{-1} nystatin and cycloeximide, and incubated at 30 °C for 1 month. The colonies were inoculated onto International *Streptomyces* Project-2 (ISP-2) medium (Shirling and Gottlieb, 1966) for purification and stock cultures.

Morphological observations. The actinomycetes isolates were cultured on ISP-2 agar plates at 30 °C for 3 days, and then the cover slides were fixed down the actinomyces colony and incubated at 30 °C for further 5 days. The actinomycetes isolates grown on the cover slides were stained with crystal violet for 1 min. The actinomycetes morphology was observed under light microscope. For scanning electron microscopy (SEM, JEOL-JSM840A SEM, Tokyo, Japan)

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observation, the actinomycetes isolates grown on the cover slides were air-dried in a desiccator and mounted on stubs, splutter-coated with gold, and viewed by means of the SEM at an accelerating voltage of 20 KV. Photomicrographs were recorded on Kodak VP200 film (New York, USA).

Antifungal activity of the actinomycetes isolates against phytopathogenic fungi and yeast. The fungal pathogen *Colletotrichum musae*, the causative agents of anthracnose of banana (the representative of hyphal fungi of plant pathogen), was used for screening antifungal activity. This fungal pathogen was obtained from Dr. Wipornpan Photita, Department of Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand. It was grown on potato dextrose agar (PDA). Mycelial disks of 8 mm diameter were cut from the pathogen colonies and transferred to the ISP-2 plates and positioned 6 cm away from each pre-grown actinomycete colony. For antagonistic action to *Candida albicans* ATCC 90028 (the representative of budding yeast of human pathogen), the yeast was cultured in ISP-2 broth at 30 ^oC for 24 h; the cells were diluted to 10^5 cells ml^{-1} in soft agar, and then were overlayed on pre-grown actinomycete colonies on ISP-2 plates. The plates were incubated at 30 ^oC for 5-7 days (for *C. musae*) and for 24 h (for *C. albicans*). The width of inhibition zones between the pathogen and the actinomycete isolates was measured.

Identification of the selected strain. Endophytic actinomycete Tc022 was selected for identification. For morphological characteristics, presence of aerial mycelium, spore mass colour, distinctive reverse colony colour, diffusible pigment, sporophore and spore chain morphology were recorded after 10 days incubation on ISP-2 medium. Diaminopimelic acid isomers and sugars from whole-cell extract were analysed for chemotaxonomic studies (Becker *et al.,* 1964; Boone and Pine, 1968).

16S rDNA sequencing. Genomic DNA was isolated from the endophytic actinomycete Tc022 by using the procedure of Hopwood *et al.* (1985). 16S rDNA was amplified by PCR using Taq DNA polymerase (Promega, USA) and primers A 7-26f (5′-CCGTCGACGAGCTCAGAGTTTGA TCCTGGCTCAG-3′) and primers B 1523-1504r (5′-CCCGGGTACCAAGCTTAAGGAG-GTGATCC AGCCGCA-3′). The conditions used for thermal cycling were as follows: denaturation at 95 ^oC for 5 min, followed by 35 cycles of denaturation at 95 ^oC for 1 min, annealing at 56 ^oC for 1 min and extension at 72 ^oC for 2 min. At the end of the cycles, the reaction mixture was kept at 72 ^oC for 10 min and then cooled to 4 ^oC. The 1.5 Kb amplified 16S rDNA fragment was separated by agarose gel electrophoresis and purified by using a QIAquick gel extraction kit (QIAGEN, Germany). The purified fragments were cloned into pGEM-T Easy vector (Promega). 16S rDNA nucleotide sequences were determined using the dideoxy chain termination method, with a Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The primers used for nucleotide sequencing were as follows: T7 promoter, SP6 promoter, C 704-685r (5′-TCTGCGCATTTCACCGCTAC-3′) and D 1115-1100r (5′-AGGGTTGC GCTCGTTG-3′). All of the obtained sequences were assembled and then compared with similar sequences from the reference organisms, with the BLAST database (a genome database of the National Center for Biotechnology Information).

Sequencing alignment and phylogenetic analysis. Reference strains were chosen from BLAST (Altschul *et al.,* 1997) search results. Multiple alignments of sequence determined in this study together with reference sequences obtained from databases and calculations of levels of sequence similarity were carried out using CLUSTAL W 1.74 (Higgins *et al.,* 1992). A phylogenetic tree was reconstructed by using treeing algorithms contained in the PHYLIP package (Felsenstein, 1995). The topology of the neighbour-joining phylogenetic tree was evaluated by using the bootstrap resampling method of Felsenstein (1995) with 1000 replicates.

Endophyte growth in tissue cultures of *Alpinia galanga*. *Alpinia galanga* Swartz used in this study was initiated from a surface-sterilized shoot tip derived from a sterilized soil-grown cutting, first with 70% ethanol for 5 min followed by 2% sodium hypochlorite with several drops of Tween 20 for 30 min. The culture was further propagated with subculturing at 1-3-month interval using 1-1.5 cm microcutting planted in screw-capped bottles (10 cm height x 5 cm diameter) as described elsewhere (Thomas, 1998). The culture medium was composed of MS (Murashige and Skoog, 1962) constituents, 3% sucrose, 1 mM indole 3-acetic acid and 0.25% phytagel, (Sigma Chemical Co, St Louis, MO, USA) on which microcuttings showed rooting and single shoot growth. Cultures were incubated at 26 ± 2 ^oC under 16 h photoperiod (30-40 mE m^{-2} s^{-1}) provided by cool white fluorescent tubes, 1.5-2 months after the previous subculturing. Healthy cultures that have been sanitized of covert bacteria through antibiotic treatment were used for elucidating endophytic association of *Streptomyces* sp. Tc022 and its effect on plant growth. After inoculation of *Streptomyces* sp. Tc022 on the thallus of *A. galanga* for 1 month, the root and stem materials from *A. galanga* were selected for microscopic observation by SEM. Specimens were washed several times using distilled water and fixed overnight in 2.5% glutaraldehyde at 4 ^oC. They were then dehydrated in a graded alcohol series (30-95%) followed by treatment in acetone and critical-point drying (Petrolini *et al*., 1986). The root and stem materials were isolated for *Streptomyces* sp. Tc022 on HV agar by surface-sterilization again.

Actinomycin D isolation procedures. Spores of *Streptomyces* sp. Tc022 were used to inoculate 100 plates of ISP-2 and incubated for 14 days at 28 ^oC. The culture medium was then cut into small pieces that were extracted with ethyl acetate (3 x 300 ml). This organic solvent was pooled and then taken to dryness under rotary evaporation to give a dark brown solid (530.5 mg). The solid was separated by column chromatography using silica gel 60 (Merck, 0.040-0.063 mm) and $CH_2Cl_2/MeOH/H_2O$ (20:3:1) as the eluent to give actinomycin D as a red solid (367.1 mg); m. p. 258-260 ^oC; TOFMS *m/z*: 1277.6182 (calcd for $C_{62}H_{86}N_{12}O_{16}Na$: 1277.6177). Its ^1H- and ^{13}C-NMR spectral data were identical with those of actinomycin D previously reported (Arison and Hoogsteen, 1970; Lackner, 1971; Hollstein *et al.,* 1974).

Disk bioassays on agar plates. *Candida albicans* ATCC 90028, used for this test was from the American Type Culture Collection. A single colony was cultured overnight in 10 ml Sabouraud broth (SB) at 37 ^oC; after 12 h incubation, 0.5 ml of the culture suspension was added to 4.5 ml pre-

warmed SB and the solution was incubated at 37 °C to obtain cultures in the exponential phase of growth. The inoculum for the disk diffusion assays was prepared as described in the protocols of the US National Committee for Clinical Laboratory Standards (NCCLS, 1997). The actinomycin D to be tested was dissolved in 2-3% methanol and applied to sterile (6-mm diameter) paper disks (Advantec, Toyo Roshi Kaisha, LTD., Japan), dried and then applied to the Sabouraud agar plate spreading with *Candida albicans* ATCC 90028. Incubation condition was 37 °C overnight. The inhibition zone was observed within 24 h.

The actinomycin D was also tested for antibiosis against *Colletotrichum musae*, using the paper disk method. Two pieces of 6 mm sterile paper disks were respectively soaked in the actinomycin D (0.25 mg per 50 ml). The air-dried disks were placed on a PDA. Each plate was then inoculated with an agar block (8 mm diameter) containing mycelial mats of the fungi in the center of the plate. The paper disks were 2.2 cm from the phytopathogen. The inhibition zone was observed within 3 days. Each treatment consisted of three replicates. The experiment was repeated twice.

Minimum inhibitory concentrations (MICs). Microbroth dilution assay of the yeast was performed as described in the NCCLS (1997) protocols. The assay was performed in sterile test tubes, and the total volume per tube was 1 ml. The yeast inoculum was prepared to give approximately 10^4-10^5 CFU per tube and the actinomycin D was tested at concentrations from 0.005 to 1.28 mg ml^{-1} in twofold step dilution. The actual number of CFU per tube was confirmed by plating onto Sabouraud agar. Two tubes were inoculated for a given concentration. The plates were incubated for 16-20 h at 37 °C. The MIC was defined as the minimum concentration of actinomycin D resulting in no visible growth of the test organism.

The fungal pathogen *Colletotrichum musae* was tested for its response to the actinomycin D using a potato dextrose agar (PDA) dilution technique. The actinomycin D (2 mg) was dissolved in methanol (200 ml), diluted serially in the same solvent and added to PDA at 48 °C. The medium (5 ml) was added to a 5 cm diameter Petri dish. The final concentrations were 0, 1, 5, 10, 30, 60, 90, 120, 150 and 180 m g ml^{-1}. An 8 mm diameter plug of the fungi, removed from the margin of a 4-day-old colony on PDA, was placed 1.5 cm from the edge of the plate. Linear growth of the fungi at 30°C was recorded 2 days after treatment. Each treatment consisted of three replicates. The experiment was repeated twice.

RESULTS AND DISCUSSION

Five hundred samples of the root tissues of *Alpinia galanga* yielded at least 120 endophytic microorganisms. In total 120 isolates were recovered, the majority of which were *Streptomyces* sp. (82), with the remainder identified as *Nocardia* sp. (11), *Microbispora* sp. (3), *Micromonospora* sp. (2) and unidentified isolates (8). Fourteen isolates did not develop sporing structures, although meso-diaminopimelic acid was detected in whole cell extracts, confirming an actinomycete status. However, an endophyte designated actinomycete Tc022 was of great interest, because of its potent antifungal activity. Morphological observation of 3-day-old cultures of Tc022 grown on ISP-2 medium revealed that sporophores to be monopodially branched and flexuous, producing open spirals of oval-shaped spores (1 x 1.5 mm) with smooth surfaces. The substrate mycelium was extensively branched with non-fragmenting hyphae. The aerial mycelium was white changing to ash-grey with yellow soluble pigment occasionally discernible. Based on results in morphological observation (light microscopy and SEM) as well as on the presence of LL-diaminopimelic acid in the whole-cell extract, endophytic actinomycete Tc022 was identified as belonging to the genus *Streptomyces*. Almost the complete 16S rDNA sequence was determined for the endophytic *Streptomyces* sp. Tc022 (>95% of the *Escherichia coli* sequence) from position 8 to position 1523 (*E. coli* numbering system; Brosius *et al.*, 1978). BLAST search results for strain Tc022 came from the non-redundant GenBank + EMBL + DDBJ; when reference sequences were chosen, unidentified and unpublished sequences were excluded. The 16S rDNA analysis and BLAST search results, and the phylogenetic tree (Fig. 1) generated from representative strains of the related species showed that strain Tc022 had high levels of sequence similarity (98%) to *S. parvulus* (accession number: AB122767), confirming a close phylogenetic relation of the 2 species. The nucleotide sequence data reported in this paper appeared in the GenBank, EMBL and DDBJ databases with the accession number AB218615.

Results of the dual cultures showed that *Streptomyces* sp. Tc022 was inhibitory to the growth of *Colletotrichum musae* and *Candida albicans*. The inhibition zone of these tested fungi was over 20 mm when they were cultured dually with *Streptomyces* sp. Tc022 for 7 days (for *Colletotrichum*

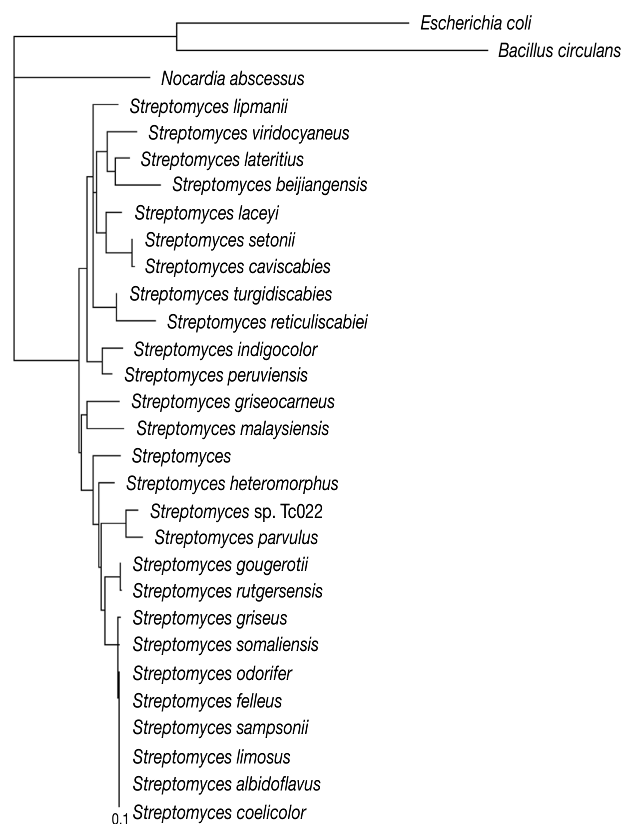

FIG. 1 – Phylogenetic tree showing the relationships of endophytic *Streptomyces* sp. Tc022, related species of the same genus and other taxa based on 16S rDNA genes sequences.

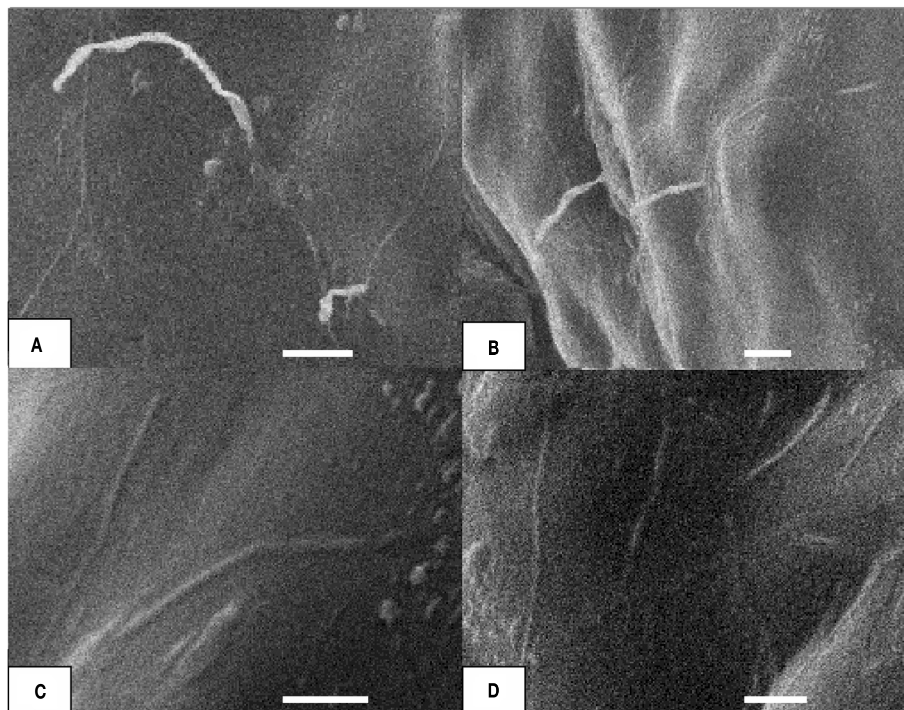

FIG. 2 – Scanning electron micrograph of the hyphae of *Streptomyces* sp. Tc022 colonized in root tissues A) and B), and stem tissues C) and D) of *Alpinia galanga* after 1 month inoculation. Bar = 2 mm.

musae) and 24 h (for *Candida albicans*) at 30 ºC. The different concentration of the crude extract showed efficacy in suppressing *Colletotrichum musae* and *Candida albicans*. The minimum concentrations of actinomycin D for inhibition of *Colletotrichum musae* and *Candida albicans* were 10 and 20 mg ml^{-1}, respectively.

With SEM observation for the association of endophyte in the tissue culture of *A. galanga*, hyphae of *Streptomyces* sp. Tc022 could colonize the stem and root tissues (Fig. 2). The tissue-associated *Streptomyces* could be re-isolated after surface-sterilization and culturing on HV agar for 3 weeks. The thallus of *A. galanga*, which was colonized by this endophyte, could grow healthily. Thus, the *Streptomyces* sp. Tc022 is considered an endophytic rather than ectophytic microbe. There are a several reports about the isolation of antibiotic producing endophytic *Streptomyces*, *Streptomyces* sp. NRRL 30566, an endophyte of *Grevillea pteridifolia*, which produced kakadumycins (Castillo *et al.,* 2003), *Streptomyces* sp. NRRL 30562, an endophyte of *Kennedia nigriscans*, which produced munumbicins (Castillo *et al.,* 2002) and *Streptomyces aureofaciens* CMUAc130, an endophyte of *Zingiber officinale*, which produced antifungal 4-arylcoumarins (Taechowisan *et al.,* 2005). The present study is the first report of the isolation and identification of an endophytic *Streptomyces* producing actinomycin D (from healthy root tissue of *A. galanga*).

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