Characterisation of endophytic actinomycetes isolated from wattle trees (*Acacia auriculiformis* A. Cunn. ex Benth.) in Thailand

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ABSTRACT

Eleven strains of endophytic actinomycetes were isolated from healthy roots of *Acacia auriculiformis* A. Cunn. ex Benth. collected from Bangkok and Nakhonpathom, Thailand. No actinomycete could be isolated from leaf samples. Analysis of 16S rRNA sequencing of those strains revealed that they belong to members of genera *Streptomyces*, *Actinoallomurus*, *Amycolatopsis*, *Kribbella* and *Microbispora*. Within 11 endophytic actinomycetes, GMKU 944 showed strongest antibacterial activities against tested bacteria and fungi. In this work, the residing property of endophytic actinomycetes in the roots of wattle tree was verified by inoculation of GMKU 937 to germinated seeds. It was demonstrated that GMKU 937 could be re-isolated and was a true endophyte.

Keywords: endophytic actinomycetes, anti-microbial activity, 16S rRNA, wattle trees

INTRODUCTION

Endophytic microorganisms are microbes that colonized inside plant tissues with symptomless to their hosts. The most frequently isolated endophytes are fungi, however, both gram positive and negative bacteria can be found as endophytes (Bacon and White, 2000). One of the most interesting endophytic bacteria is the member of actinomycetes, soil dwelling Gram positive bacteria. The actinomycetes, especially *Streptomyces* spp. are valuable economical and biotechnological bacteria by providing over two third of antibiotics and bioactive compounds used these days (Baltz, 1998). Endophytic actinomycetes which associated with plants also play important role in protection their host from phytopathogenic invasions (Crawford *et al*., 1993). Recently, our laboratory has reported that several actinomycetes act as plant growth promoter by producing of phytohormone, indole-3-acetic acid (IAA) (Suttiviriya *et al*., 2008) or iron chelating molecules, siderophores *in vitro* (Indananda *et al*., 2009).

Previously, *Frankia* was recognized as the only endophytic actinomycete that resided in nodule of non-leguminous plants and fixed nitrogen (Benson and Silvester, 1993). Recently, reports of non-*Frankia* endophytic actinomycetes have been significantly increased. They were isolated from crop plants such as wheat (Conn and Franco, 2004), barley (Coombs *et al*., 2004) and rice (Tian *et al*, 2004). In Thailand, a variety of endophytic actinomycetes were reported from Thai crop plants (Indananda

et al, in press) as well as Thai medicinal plants (Taechowisan *et al*., 2003; Duangmal *et al*., 2008).

Wattle tree (*Acacia auriculiformis* A. Cunn. ex Benth.) is a fast-growing leguminous tree originated from Australia. *Acacia* spp. are well adapted to poor climatic and soil condition. For these reason, *Acacia* spp. have been introduced in limited environments such as Africa for soil fertility improvement and erosive inhibition. These abilities of *Acacia* spp. depend on root associated rhizobacteria which provide nitrogen to their host (Galiana *et al*., 1990). In addition, ecto- and endomycorrhiza also associated to *Acacia* spp. resulting in plant growth promotion and salt-stress improvement (Diouf *et al*., 2005).

In this study, we report endophytic actinomycetes isolated from wattle tree collected from Bangkok and Nakhonpathom, Thailand. The isolates were validated their genus position using 16S rRNA sequencing and phylogenetic tree analysis. Their antimicrobial activities were examined and the endophytic property was verified.

MATERIALS AND METHODS

Sample collections

Healthy leaves and roots of wattle tree (*Acacia auriculiformis* A. Cunn. ex Benth.) were collected from Bangkok at Kasetsart University, Bangken campus and Train garden, Chatuchak; and from Nakhonpathom at Kasetsart University, Kamphaengsaen campus and Mahidol University, Salaya campus. Plant samples were kept in plastic bags and stored at 4 °C until isolation.

Isolation of endophytic actinomycetes

Plant materials were cut into small pieces (2 × 2 cm for leaf and 2 cm long for root). The samples were washed by running tap water for 1-2 min to remove soil particles and then surface sterilized by 70% ethanol for 10 min and 1% sodium hypochlorite for 15 min modified from the methods of Coombs and Franco (2003) and Cao *et al*. (2004). The plant materials were then rinsed with sterilized water for three times before soaking in 10% (w/v) $NaHCO_3$ solution for 5 min. At this point, the final washed solution was spread onto starch casein agar (SCA; Küster and Williams, 1964) containing 100 μg/ml ampicillin, 2.5 U/ml penicillin G, 50 μg/ml amphotericin B and 50 μg/ml cyclohexamide; to validate the surface sterilized protocol. Surface sterilized leaf and root samples were crushed in ¼ Ringer's solution. The solution and the crushed materials were spread onto SCA supplemented with above antibiotics. Endophytic actinomycetes were observed after incubation at 28 °C for 1-4 weeks.

Identification of actinomycetes

The isolates were picked from isolation plates and purified on mannitol soybean agar (MS; Hobbs *et al*., 1989) and ISP 2 and ISP 3 media (International *Streptomyces* Project; Shirling and Gottlieb, 1966). The isolates were identified according to morphological criteria, including characteristics of colonies on plate, morphology of substrate and aerial hyphae, morphology of spores and pigment production (Goodfellow and Cross, 1984). The isolates were preserved in 20% (v/v) glycerol and stored at -80 °C.

16S rRNA gene amplification and sequencing

The isolates were grown on MS medium for 5-7 days and genomic DNA was extracted according to Kieser *et al*. (2000). 50 mg of mycelium was harvested and resuspended in 500 μl lysis solution (0.3 M sucrose, 25 mM Tris-HCl pH 8.0, 25 mM EDTA pH 8.0) containing 10 μg/μl lysozyme and 50 μg/μl RNaseA and incubated at 37 °C for 1 hr. 250 μl of 2% sodium dodecyl sulfate

(SDS) was added and immediately mixed by inverting the tube. The mixture was extracted 2-3 times with 250 µl phenol:chloroform:isoamyl alcohol (25:24:1), vortexed and centrifuged at 12,000 rpm for 5 min. Genomic DNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 1 volume of isopropanol into the aqueous phase solution. The DNA pellet was washed twice with 70% ethanol and resuspended in 50 µl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and kept at -20 °C.

Genomic DNA of the isolate was used as a template to amplify 16S rRNA gene. The amplification was generated using STR1F (5′-TCACGGAGAGTTTGATCCTG-3′) and STR 1530R (5′-AAGGAGATCCAG CCGCA-3′) primers (Kataoka *et al*.,1997) by 35 cycle of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, proceeded by 1 min at 94°C, 1 min at 55°C and 4 min at 72°C. PCR products were purified by QIAquick PCR purification kit (QIAGEN, Germany) according to the manufacturer's protocol. PCR products were 5' partially sequenced using STR1F primer at Macrogen (Korea).

Phylogenetic analysis

16S rRNA sequence of each isolate was compared against the type strains in database using EzTaxon tool (Chun *et al*., 2007). Multiple alignments of 16S rRNA genes of the isolates and type strains which available on GenBank database were performed using the ClustalW version 1.8 (Thompson *et al*., 1994). Phylogenetic tree was inferred using neighhor-joining (NJ) analysis by MEGA software version 3.1 (Kumar *et al*., 2004) and was bootstrapped using 1,000 replicates for each sequence.

Determination of antimicrobial activities

Antibacterial activities of the isolates were tested against Gram-positive bacteria (*Bacillus cereus* ATCC 11778 and *Staphylococcus aureus* ATCC 25923), Gram-negative bacteria (*Escherichia coli* ATCC 8739) and phytopathogenic bacteria (*Xanthomonas campestris* pv. *glycine*, *Erwinia carotovora* pv. *carotovora* and *Ralstonia solanacearum*). The isolates were grown on MS agar for 5-7 days and were plugged using 0.6 mm cork borer and placed on nutrient agar (NA; Himedia, India) that had been flooded with 10^8 cfu/ml of test bacterial suspensions. 1 mg of ampicillin on a paper disk was used as positive control. All plates were incubated at 30°C overnight. Antibacterial activities of the isolates were observed by clear halo zone around each agar plugs.

Antifungal activities of the isolates were tested against fungi and yeast (*Aspergillus niger* and *Candida albicans*) and phytopathogenic fungi (*Collectotrichum* sp., *Fusarium* sp., *Fusarium proliferatum* DOAC 0842, and *Rhizoctonia solani* DOAC 1406). The isolates were inoculated at the margin of NA plates and incubated at 30°C for 5-7 days, and then the test fungi (except *C. albicans*) were inoculated at the center of the NA plates and incubated further for 3-5 days. Antifungal activities of each isolates were observed by inhibition zone. Determination of antifungal activity against *C. albicans* was done as described for bacteria.

Validation of endophytic property

The inoculation of a representative endophytic actinomycete to germinated wattle tree seeds was performed using the protocol modified from Somasegaran and Hoben (1994). Seeds of wattle tree were washed with tap water and treated with conc. H_2SO_4 for 30 min. The treated seeds were rinsed with running tap water for 15 min and germinated on moist sterilized cotton sheets. The 2 days germinated seeds were transferred to plastic

growth pouches (Somasegaran and Hoben, 1994) containing N-free nutrient (Broughton and Dillworth, 1971). The germinated seeds were inoculated by endophytic actinomycete culture broth (in ISP 2 medium). The plastic growth pouches were placed in the growth chamber at 25°C with light for 12 hr/ day. One month after inoculation, seedlings were harvested and the endophyte was re-isolated from roots as describe earlier.

RESULTS

Isolation and identification endophytic actinomycetes from wattle trees

Several colonies of endophytic actinomycetes could be morphologically observed on SCA medium spreading with crushed root solution after 1-4 weeks of incubation. Hyphal growth of endophytic actinomycetes was also detected on the surface of root materials. No endophytes were obtained from leaf samples. In addition, the last wash from all surface sterilized root and leaf samples showed no microbial growth which indicated that the surface sterilized protocol was exceptionally efficient. After purification of the endophytes, 11 isolates were identified (Table 1). The results indicated various types of endophytic actinomycetes obtained from four different places of plant samples. The isolates were labeled as GMKU (Genetics Microbiology Kasetsart University) culture collection numbers as shown in Table 1.

All 11 isolates were subsequently identified using partial 16S rRNA sequencing and phylogenetic analysis. The results showed that the isolates were actinomycetes (Table 1). Three isolates from roots collected from Kasetsart University, Bangken campus belonged to genera *Actinoallomurus*, *Amycolatopsis* and *Microbispora*. Both isolates from Kasetsart University, Kamphangsan campus belonged to genus *Streptomyces*. Two isolates from Train Garden, Bangkok belong to genera *Kribbella* and *Streptomyces*. Four isolates from Mahidol University, Salaya campus belonged

Table 1 Identification of endophytic actinomycetes isolated from wattle tree roots collected from Bangkok and Nakhonpathom, Thailand based on 5’ partial 16S rRNA sequences.

Location	Strain no.	Species	% Identity
KU, BK[1]	GMKU 931	*Actinoallomurus caesius* A3015^T (AB364589)	99
	GMKU 932	*Amycolatopsis tolypomycina* DSM 44544^T (AJ508241)	99
	GMKU 936	*Microbispora corallina* DF-32^T (AB018046)	97
KU, KPS[2]	GMKU 937	*Streptomyces coelicoflavus* NBRC 15399^T (AB184650)	99
	GMKU 940	*Streptomyces coelicoflavus* NBRC 15399^T (AB184650)	99
TG, BKK[3]	GMKU 938	*Kribbella jejuensis* HD9^T (AY253866)	99
	GMKU 939	*Streptomyces regensis* NRRL B-11479^T (DQ026649)	96
MU, NKP[4]	GMKU 941	*Microbispora mesophila* JCM 3151^T (AF002266)	98
	GMKU 942	*Microbispora mesophila* JCM 3151^T (AF002266)	98
	GKMU 943	*Actinoallomurus coprocola* TT04-09^T (AB364579)	99
	GMKU 944	*Streptomyces sioyaensis* NRRL B-5408^T (DQ026654)	100

[1] Kasetsart University, Bangken campus, Bangkok

[2] Kasetsart University, Kamphaengsaen campus, Nakhonpathom

[3] Train Garden, Chatuchak, Bangkok

[4] Mahidol University, Salaya campus, Nakhonpathom

to genera *Actinoallomurus*, *Microbispora* and *Streptomyces*. Most of the isolates shared 96-100% identity to those of closely related actinomycete type strains (Table 1). Phylogenetic tree analysis of 11 endophytic strains with some closet members clearly indicated that they were dissimilar and belonged to different genus/species (Fig. 1).

Antimicrobial activities of endophytic actinomycetes

Within 11 endophytic actinomycetes, there

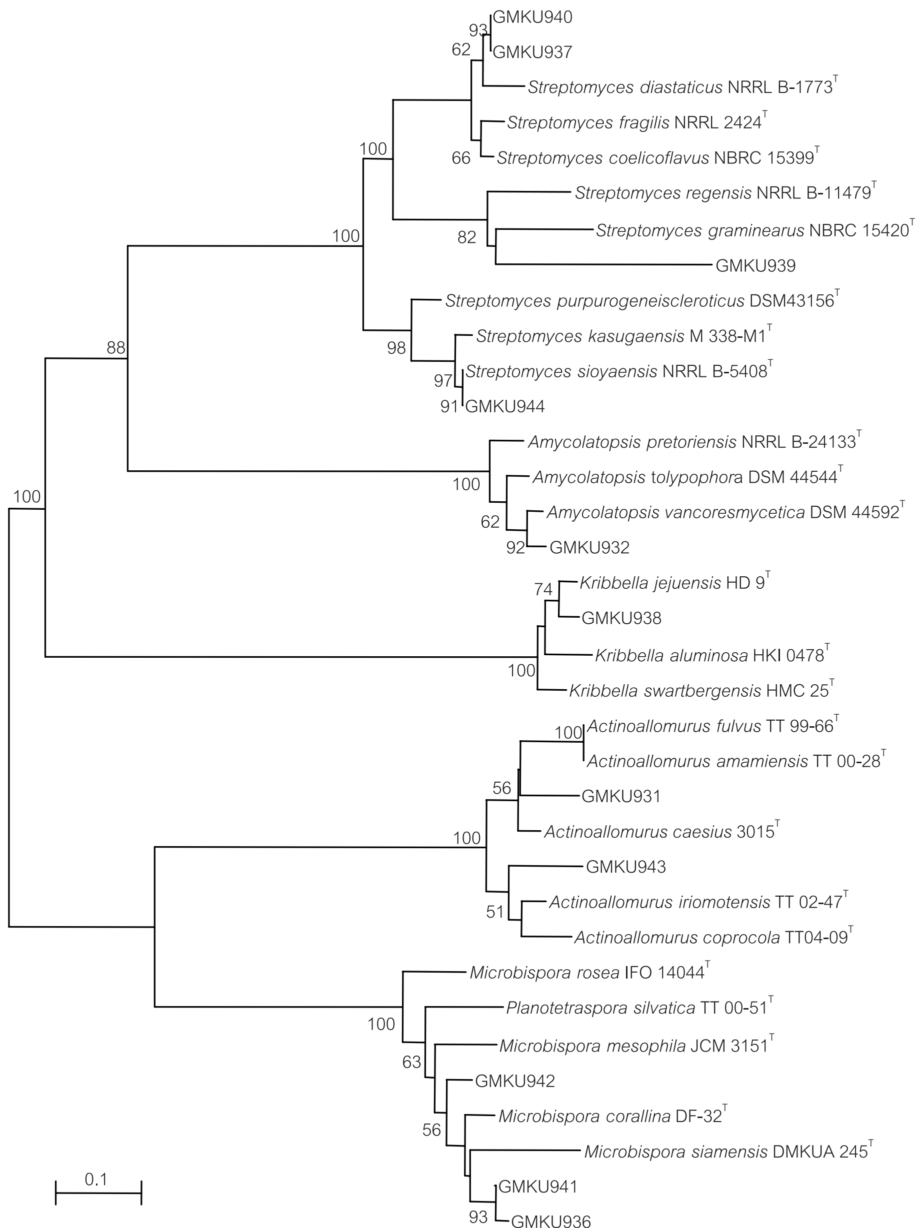

Figure 2 Phylogenetic tree based on 16S rRNA sequences of 11 endophytic actinomycetes and related species. The numbers at nodes represent the percentage of 1000 bootstrap re-samplings. Bar, the number of mutations per sequence position.

were 3 isolates active against test bacteria namely, GMKU 932 (against *B. cereus*), GMKU 940 (against *B. cereus* and *E. coli*) and GMKU 944 (against *B. cereus*, *S. aureus* and *R. solanacearum*). In addition, the clear halo zone generated by GMKU 944 was greater than the positive control indicated that GMKU 944 had strong antibacterial activities. Unfortunately, all of the isolates showed no activity against test phytopathogenic fungi. Only 4 isolates, GMKU 937, GMKU 938, GMKU 940 and GMKU 944 showed strong activity against *A. nigers*.

Validation of endophytic property

Strain GMKU 937 was selected as a representative of endophytic actinomycetes to study the residing property in the wattle tree. One month after inoculation of strain GMKU 937 to germinated seeds of wattle tree, abundant colonies of GMKU 937 could be obtained from the crunched roots using the surface sterilized protocol described earlier. On the contrary, there was no actinomycete obtained from the non-inoculated seedlings. However, there was no significant phenotypic difference between inoculated and non-inoculated seedlings of wattle tree.

DISCUSSION

In this study, 11 strains of endophytic actinomycetes were isolated from wattle tree. The result of effectiveness of the surface sterilization procedures suggested that the epiphytic microbes were completely removed by this procedure. Ethanol and sodium hypochlorite are often used in plant surface sterilization procedures which made it possible to isolate and characterize endophytic actinomycetes associated with plant roots (Conn and Franco, 2004; Cao *et al*., 2004; Tian *et al*., 2004). There was no actinomycete grown on surface sterilized leaves and crushed leaves isolation plates. The result was in agreement with the report of Taechowisan *et al*. (2003) that roots represented a good habitat for endophytic actinomycetes. This may be related to the fact that actinomycetes reside abundantly in rhizosphere (Sardi *et al*., 1992) and could easily move into the plant roots.

Endophytic actinomycetes in wattle tree roots obtained from four collection sites in Thailand were taxonomically different. It was previously suggested that differences in chemical composition of soil could influence the endophytic microbial communities (Conn and Franco, 2004; Tian *et al*., 2004). In addition, many biological and environment factor also effected on the endophytic populations in plants, such as plant cultivar, plant age, tissue type and time of sampling (Araújo *et al*., 2001; Adams and Klopper, 2002; Zinniel *et al*., 2002)

Identification of endophytic actinomycetes based on 16S rRNA sequencing showed that these isolates belong to five different genera. Variety of genera of actinomycetes was also found in Thai medicinal plants (Indananda *et al*., 2009). These suggested the great diversity of endophytic actinomycetes from tropical plants of Thailand. At the same time of this report, novel species of endophytic actinomycetes, *Pseudonocardia acaciae* sp. nov. (Duangmal *et al*., in press) and *Actinoallomurus acaciae* sp. nov. (Thamchaipenet *et al*., in press) were isolated from wattle trees. These supported the idea that plant-associated actinomycetes are great diversity and are the good resources for novel species discovery.

Determination of antimicrobial activities revealed that most of the isolates which were active against test bacteria and fungi, belonged to genus *Streptomyces*. These supported well-known knowledge that the actinomycetes, especially *Streptomyces* often produce bioactive compounds (Baltz, 1998). Amongst those positive strains,

GMKU 944 showed strongest activities against test bacteria and fungi. The results suggested that endophytic actinomycetes are good resources for bioactive compounds. In the term of plant-microbe interaction, the plant hosts are protected from phytopathogen by bioactive metabolites produced by the endophytic actinomycetes, while the endophytes gain some benefits from plants by getting nutrient supply (Hasegawa *et al*., 2006).

Strain GMKU937 was re-inoculated to germinated wattle tree seeds. No significant phenotypic difference between inoculated and non-inoculated plants was detected which indicated that GMKU 937 was not pathogenic bacteria. GMKU 937 could be re-isolated from the roots of wattle tree suggested that endophytic actinomycetes obtained in this study were indeed true endophytes. The absence of actinomycete in non-inoculated seedlings indicated that these seeds did not contain endophytes and the endophytic actinomycetes were exogenous and obtained directly from soil.

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