รายงานวิจัยฉบับสมบูรณ์

ชื่อโครงการวิจัย

ปฏิสัมพันธ์ระหว่างเอนโดไฟติกแอคติโนมัยสีทกับพืชโดยระบุตำแหน่งอาศัย และวิเคราะห์ยีนที่เกี่ยวข้องกับการสร้างสารส่งเสริมการเจริญของพืช

Interaction of endophytic actinomycetes and plants by localization of the endophytes and analysis of genes involved in plant growth promoting agents

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มีนาคม 2558

สัญญาเลขที่ **DBG5480002**

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สัญญาเลขที่ DBG5480002

โครงการ: ปฏิสัมพันธ์ระหว่างเอนโดไฟติกแอคติโนมัยสีทกับพืชโดยระบุตำแหน่งอาศัยและวิเคราะห์ยีนที่เกี่ยวข้องกับการสร้างสารส่งเสริมการเจริญของพืช
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บทคัดย่อ

เอนโดไฟติกแอคติโนมัยสีทจาก GMKU culture collection (TRF grant no. RMU4980033) และที่คัดแยกใหม่ (GKU culture collection) มีความหลากหลายทางชีวภาพสูง และมีคุณสมบัติเป็น plant growth promoting (PGP) bacteria โดยสามารถสร้างฮอร์โมนพืช IAA และ siderophore สามารถละลายหินฟอสเฟต สร้างเอนไซม์ ACC deaminase และ chitinase และยังสามารถยับยั้งเชื้อจุลินทรีย์ได้ทั้งแบคทีเรียและรา ในการศึกษานี้ได้ค้นพบแอคติโนมัยสีทสายพันธุ์ใหม่ คือ *Nonomuraea syzygii* sp. nov. และ *Actinomadura syzygii* sp. nov. และได้พิสูจน์ว่าเอนโดไฟติกแอคติโนมัยสีทอาศัยอยู่ในพืชจริง โดยการติดตาม *Streptomyces* sp. GMKU944 ด้วย eGFP เมื่อปลูกเชื้อเข้าไปในต้นกระถินณรงค์ พบว่าสามารถเห็นการเรื่องแสงของเชื้อโดยอาศัย laser scanning confocal microscope โดยพบเชื้อส่วนใหญ่อยู่ที่เซลล์ phoem parenchyma นอกจากนี้ยังศึกษาความสัมพันธ์ของเอนโดไฟติกแอคติโนมัยสีทกับพืช โดยการสร้างสายพันธุ์กลายที่ไม่สามารถสร้าง siderophore ใน *Streptomyces* sp. GMKU 3100 ด้วยการทำการกลายที่ยีน *desD*-like ซึ่งเกี่ยวข้องกับการสังเคราะห์ siderophore เมื่อนำเชื้อ *Streptomyces* sp. GMKU 3100 ที่สร้าง siderophore มาปลูกเข้าต้นข้าวและถั่วเขียวในระดับกระถาง พบว่าสามารถส่งเสริมการเจริญของพืช ทั้งความสูงและความยาวของต้นและราก รวมทั้งน้ำหนัก เมื่อเทียบกับพืชที่ไม่ปลูกเชื้อ หรือปลูกด้วยสายพันธุ์กลาย แสดงว่า PGP-trait นี้มีความสำคัญต่อการเจริญของพืชทั้งสอง นอกจากนี้ยังศึกษาผลของเอนไซม์ chitinase โดยเลือก *Streptomyces* sp. GMKU 301 and GMKU 322 ที่ผลิต chitinase และมียีน family 18 group A และ 19 chitinase มาปลูกลงในข้าวโพดก่อนที่จะปลูกเชื้อ *Fusarium moniliforme* ซึ่งก่อโรคเหี่ยวเน่า จากการทดลองในระดับกระถาง พบว่า สายพันธุ์ทั้งสองสามารถป้องกันข้าวโพดจากโรคเหี่ยวเน่าได้อย่างมีนัยสำคัญเมื่อเทียบกับพืชที่ไม่ปลูกเชื้อ แสดงว่าเอนโดไฟติกแอคติโนมัยสีทสามารถช่วยป้องกันโรคพืชได้ จากการศึกษานี้ยังค้นพบสารออกฤทธ์ทางชีวภาพชนิดใหม่ คือ linfuranone A จาก *Microbispora* sp. GMKU363 ซึ่งแยกจากพืชสมุนไพรลิ้นงูเห่า (*Clinacanthus siamensis* Bremek.) อีกด้วย

Abstract

Endophytic actinomycetes from previous GMKU culture collection (TRF grant no. RMU4980033) and newly isolates (GKU culture collection) were identified for their taxonomy at genus level and characterized for their plant growth promoting (PGP) properties. They were rich of diversity and carried PGP-traits including IAA and siderophore production, phosphate solubilisation, ACC deaminase and chitinase activities as well as anti-microbial activities. Two new species were validated as *Nonomuraea syzygii* sp. nov. and *Actinomadura syzygii* sp. nov. It was also proved that the endophytic actinomycetes actually resided in plant tissue by tagging *Streptomyces* sp. GMKU944 with *egfp*. eGFP-tagged strain in seedlings of wattle tree, were visualized under laser scanning confocal microscope mainly at phoem parenchyma cells. Futher study of the interaction between endophytic actinomycetes and plants, siderophore deficient endophytic *Streptomyces* sp. GMKU 3100 was generated by inactivation of a *desD*-like gene involved in siderophore biosynthesis. Pot culture experiments revealed that rice and mungbean plants inoculated with the wild type gave the best enhancement of plant growth and significantly increased root and shoot biomass and lengths compared with untreated controls and siderophore-deficient mutant treatments. The results indicated that this trait is important in enhancement of growth of the plants. Another PGP-trait, chitinase production, was studied by inoculated chitinase producing *Streptomyces* sp. GMKU 301 and GMKU 322, carried genes encoding family 18 group A and 19 chitinases, into maize plants before infected with *Fusarium moniliforme* causing foot rot and wilting diseases. Pot culture experiments revealed that chitinase producing strains significantly protected plants from foot rot and wilting diseases. In addition, a novel compound, linfuranone A, was discovered from an endophytic *Microbispora* sp. GMKU363 isolated from a root of Thai medicinal plant ‘Lin Ngu Hao’ (*Clinacanthus siamensis* Bremek.). Linfuranone A was active in an assay designed to screen antidiabetic and antiatherogenic activities.

Executive summary

Endophytic actinomycetes from previous GMKU culture collection (TRF grant no. RMU4980033) and newly isolates from Chamung (*Garcinia cowa* Roxb.), Jig (*Barringtonia acutangula* Gaertn.), Sumrong (*Scaphium macropodum* Beaum.), Waa (*Syzygium cumini* L.) and sugarcane (GKU culture collection) were characterized for their plant growth promoting (PGP) properties namely, indole-3-acetic acid (IAA) production, siderophore production, phosphate solubilisation, ACC deaminase and chitinase activities as well as anti-microbial activities. Most of them carried several PGP-traits. All isolates were identified their taxonomic relatedness by 16S rRNA sequencing and revealed that they belong to genera *Actinomadura*, *Dactylosporangium*; *Microbispora*, *Micromonospora*, *Nocardia*, *Nonomuraea*, *Streptomyces* and *Streptosporangium*. The most frequently isolated genus was *Streptomyces* following by *Microbispora*. For sugarcane plants, other bacterial endophytes were also isolated including *Acinetobacter*, *Bacillus*, *Enterobacter*, *Kluyvera*, *Lysinibacillus*, *Micrococcus*, *Paenibacillus*, *Pantoea*, *Pseudomonas* and *Staphylococcus*. *Bacillus* and *Enterobacter* were found as major genera. In the case of bacterial endophytes, they were additionally tested for nitrogen fixation and amplification of *nifH* gene, in which several of them carried the nitrogen fixation trait.

Amongst these new GKU strains, several putative novel species were postulated. Two isolates from the roots of jambolan plum tree (*Syzygium cumini* L. Skeels) collected at Khao Khitchakut National Park, *Nonomuraea syzygii* sp. nov. and *Actinomadura syzygii* sp. nov., were validated as new species based on morphological, chemotaxonomical, and biochemical data together with 16S rRNA sequences and DNA-DNA relatedness values.

In order to prove that these endophytic actinomycetes are really true endophytes, an endophytic *Streptomyces* sp. GMKU944 isolated from wattle tree was chose to study localization and visualization of the strain within plant tissues. *Streptomyces* sp. GMKU944 was tagged by introduction of plasmid pIJ10257*egfp* by intergeneric conjugation. After re-inoculation of the eGFP-tagged strain in seedlings of wattle tree, cross sections of the roots were visualized under laser scanning confocal microscope. The strain could be detected mainly at phoem parenchyma cells and some at xylem parenchyma cells. Furthermore, SEM micrographs revealed the presence of mycelia of eGFP-tagged *Streptomyces* sp. GMKU944 on the surface of nodule and root structure of wattle tree and also inside the crushed root tissue. The results strongly supported that the endophytic actinomycete is closely associated with plant.

To study the interaction between endophytic actinomycetes and plants, siderophore producing endophytic *Streptomyces* sp. GMKU 3100 isolated from roots of a Thai jasmine rice plant (*Oryza sativa* L. cv. KDML105) was selected to investigate the important of this trait. A

deficient strain of *Streptomyces* sp. GMKU 3100 was generated by inactivation of a *desD*-like gene encoding a key enzyme controlling the final step of siderophore biosynthesis. Pot culture experiments revealed that rice and mungbean plants inoculated with the wild type gave the best enhancement of plant growth and significantly increased root and shoot biomass and lengths compared with untreated controls and siderophore-deficient mutant treatments. Application of the wild type in the presence or absence of ferric citrate significantly promoted plant growth of both plants. This siderophore-deficient mutant clearly showed the effect of this important trait involved in plant–microbe interaction in enhancement of growth in rice and mungbean plants supplied with sequestered iron.

Another trait that was chose to study was chitinase. Two chitinase producing endophytic streptomycetes, *Streptomyces* sp. GMKU 301 and GMKU 322, carried genes encoding family 18 group A and 19 chitinases were selected to study the interaction with maize plants when infected with *Fusarium moniliforme* causing foot rot and wilting diseases. Pot culture experiments revealed that maize plants treated with the pathogen alone were severely damaged by the disease and completely dead, but plants that were inoculated with chitinase producing *Streptomyces* sp. GMKU 301 and GMKU 322 significantly protected plants from foot rot and wilting diseases. This chitinase producing endophytic actinomycetes clearly showed their biocontrol property to protect plants from disease.

Within this endophytic actinomycetes, a novel compound, linfuranone A, was discovered from an endophytic *Microbispora* sp. GMKU363 isolated from a root of Thai medicinal plant 'Lin Ngu Hao' (*Clinacanthus siamensis* Bremek.). Linfuranone A is a relatively rare 3-furanone derived from polyketide with a hemiketal at C-2 and an unsaturated alkyl chain at C-5. There are a few known structurally close metabolites isolated from *Streptomyces* spp., myxobacteria, fungi and marine molluscs. Biological screening of linfuranone A in diverse bioassays indicated that this compound was active in an assay designed to screen antidiabetic and antiatherogenic activities. Linfuranone A displayed no appreciable activities in antimicrobial and cytotoxic assays.

The results obtained from this work strongly indicated that endophytic actinomycetes are extremely diverse. They are rich in finding new species and discovering new bioactive compounds. Furthermore, they carried PGP-traits that help enhance growth of plants and protect them from plant diseases. It is important to deeply study interaction between the endophytes and the plants at molecular level in order to understand their real function. The knowledge will be of value to be able to sustainably apply these endophytes as added values biocontrol/biofertilizers in crop plantation in the future.

เนื้อหางานวิจัย

วิธีดำเนินการวิจัย

1. Isolation and identification of endophytic actinomycetes

1.1 Endophytic actinomycetes and plant species

A total of 69 endophytic actinobacterial isolates were obtained from GMKU culture collection no. GMKU 3xxx (results from TRF grant no. RMU4980033). Newly endophytic bacterial isolates were from sugarcane varieties LK92-11 and KK3 obtained from Mitr Phol planting site, Chaiyaphum; and Chamung (*Garcinia cowa* Roxb.), Jig (*Barringtonia acutangula* Gaertn.), Sumrong (*Scaphium macropodum* Beaum.) and Waa (*Syzygium cumini* L.) collected at the foothills of Khao Khitchakut, Chantaburi.

1.2 Isolation of endophytic actinomycetes

The plant samples were surface-sterilized using the method described by Indananda *et al.* (2010). The plant materials were washed with tap water to remove soil particles and kept at 4 °C for a week to decrease surface bacteria. The roots, stems and leaves were cut into small pieces and then surface-sterilized using serial treatments of 95% EtOH for 10 minutes, 1% NaClO for 15 minutes, sterile DW for three times and then immersed in 10% (w/v) $NaHCO_3$ solution for 10 minutes. After air drying, the surface sterilized plant materials were ground in mortar containing Ringer's solution. Those of ground materials and their solution were placed and spread onto starch casein agar (SCA; Küster and Williams, 1964) and Water agar (WA) media supplemented with 2.5 U/ml penicillin G and 50 mg/ml cyclohexamide. The solution of the third washed DW was also spread on the medium to ensure that the surface plant materials were completely sterilized. Colonies of endophytic actinobacteria appeared on the media after incubation at 30 °C for 1-8 weeks. Each colony was isolated and purified on mannitol soya medium (MS; Hobbs *et al*., 1989). Each isolate was preserved by scrapping spores from agar plates and placed in a 1.5 sterilized tube containing 1 ml of 20% glycerol and maintained at -20 ºC or -80 ºC.

1.3 Characterisation and identification of endophytic actinomycetes

1.3.1 Morphological characterisation

Morphology of each isolates was determined on ISP 2, ISP 3, and ISP 4 media (Shirling and Gottlieb, 1966). The color of mycelium, spores and soluble pigment was determined by comparing its color with the color chips from Color Harmony Manual.

1.3.2 Determination of DAP isomers in cell wall peptidoglycan

The isomers of DAP in the cell wall were analyzed according to the method of Hasegawa et al. (1983). Dried cells were prepared by growing cell-mass of the isolate in appropriate media and the cell-mass were harvested by centrifugation at 3,000 g for 15 min. The resultant pellets were washed twice with sterile distilled water and then immersed in 70% EtOH at 4 °C for 16 h and then dried at 50 °C for 16 h. The dried cells were kept at -20 °C before using.

Dried cells (10 mg) were hydrolyzed with 1 ml 6N HCl in a small grass tube at 100 °C for 18 h. After cooling, the hydolysate was filtered using absorbent cotton to another clean small glass tube and 1 ml of sterile DW was added to the filter. The combined filtrate was concentrated to dryness by a rotary evaporator at 40 °C. The dried material was dissolved in 1 ml of water and dried again. After that, the residue was dissolved in 0.3 ml of sterile DW and 5 μl was applied on the base line of cellulose TLC plate (Merck no. 5716, 20×20 cm). A standard 0.01 M DL-DAP (Sigma, USA) for 3-5 μl was used. TLC was developed with the solvent system methanol: water: 6N HCl: pyridine (80: 26: 4: 10; v/v) (prepared fresh). Development was taken about 16 h. The TLC plate was then dried for about 30 min-1 h in fume hood and then development was repeated again about 4-5 h in the same solvent system. The spots were visualized by spraying with 0.2% ninhydrin solution followed by heating at 100 °C about 5-15 min in a dryer incubator.

1.3.3 Identification of endophytic actinomycetes by 16S rRNA gene sequencing

1.3.3.1 Genomic DNA preparation

Genomic DNA of endophytic actinomycetes was prepared by the method of Hopwood et al. (1985). Endophytic actinomycetes were grown on MS medium at 30 °C and two loopfulls of mycelia and spore were scraped from the plate and placed in a 1.5 ml tube containing 500 μl of lysozyme solution (10 μg/μl lysozyme, 50 μg/μl RNaseA, 0.3 M sucrose, 25 mM Tris-HCl, 25 mM EDTA, pH 8.0). The mixture was incubated at 37 °C for 1 h and inverted every 10 min during incubation. The lysate was cooled to room temperature and 250 μl of 2% SDS was added and the tube was immediately inverted for several times till the lysate was clear. 250 μl of phenol-chloroform (25 phenol: 24 chloroform: 1 isoamyl alcohol) was then added and the tube was vigorously mixed by vortex for 30 sec and centrifuged at 12,000 rpm for 10 min at RT. The supernatant was transferred to a fresh sterilized tube and 0.1 V of 3 M sodium acetate pH 4.8 and 1 V of isopropanol were then added and the tube was inverted before keeping at -20 °C for at least 5 min and centrifuged at 12,000 rpm for 2 min at 4 °C to precipitate DNA. The supernatant was discarded and the DNA pellet was washed twice with 200 μl of 70% EtOH and dried at RT with the tube lid open. The pellet was then suspended in 20-50 μl of 1X TE buffer. The total DNA was stored at -20 °C for a long period preservation.

1.3.3.2 Amplification of 16S rRNA gene

50 ng of the chromosomal DNA of each isolate was used to amplify 16S rRNA gene with specific primers, STR1F (5’-TCACGGAGAGTTTGATCCTG-3’) and STR1530R (5’-AAGGAGATCCAGCCGCA-3’) (Kataoka et al., 1997). The PCR was performed with the following reagents: 1X PCR buffer, 3 mM $MgCl_2$, 0.5 mM dNTP mixture, 0.125 µM each primer, 1.25 U *Taq* DNA polymerase, 10% DMSO in a total volume of 20 µl with sterilized ultra pure water. The PCR reactions were carried out following temperature cycling profile: 94 °C for 4 min, followed by 35 cycles at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and final cycle at 72 °C for 4 min. The expected size of 1.5 kb PCR product was determined by 0.8% agarose gel electrophoresis. The PCR products were purified using Gel/PCR DNA Fragments Extraction kit (Geneaid, Taiwan) following the manufacturer’s protocol. After that the purified PCR products were partially sequenced with STR1F primer to obtain the 5’-end sequences of 16S rRNA gene at Macrogen, Korea.

1.3.3.3 Sequence similarity analysis

To analyze the similarity values, the 16S rRNA gene sequences were preliminary compared with nucleotide sequences in the GenBank and the EzTaxon database at www.ncbi.nih.gov/BLAST and www.eztaxon-e.org, respectively.

1.4 Identification of new genus of endophytic actinomycetes

1.4.1 Full length 16S rRNA gene analysis and DNA-DNA hybridisation

PCR product of 16S rRNA gene was completely sequenced using primers STR1F (5'-TCACGGAGAGTTTGATCCTG-3') and STR1530R (5'-AAGGAGATCCAGCCGCA-3'; Kataoka *et al*., 1997), 926F (5'-AAACTCAAAGGAATTGACGG-3'; Tajima *et al.* (2001) and ATT026R (5'-TGGACTACCAGGGTATCTAATC-3'). The resulting 16S rRNA gene sequence was compared with corresponding sequences of the type strains available in the EzTaxon-e server (Kim *et al*., 2012). A multiple sequence alignment of new strain and other related taxa was performed using clustal X version 2.0 (Larkin *et al*., 2007). Phylogenetic trees were constructed using MEGA software version 6.0 (Tamura *et al*., 2013) by neighbour-joining (Saitou & Nei, 1987) and maximum likelihood (Felsenstein, 1981). Topology of the phylogenetic trees was evaluated with bootstrap analysis based on 1,000 resampling (Felsenstein, 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). The values of pairwise 16S rRNA gene sequence similarity among strain GKU 164^T and other related species were determined using the EzTaxon-e sever. DNA-DNA hybridization between GKU 164^T and its closely related type strains was fluorometrically determined using photobiotin-labelled DNA probes and microplate wells as described by Ezaki *et al.* (1989). The DNA-DNA relatedness values were examined from two independent determinations.

1.4.2 Morphological and physiological characterization

The morphological characteristics of new strain were observed by light and scanning electron microscopy (model JSM-5410; JEOL) using cultures grown on Czapek's agar at 28 °C for 30 days. Cultural characteristics of new strain were determined after incubation at 28 °C for 21 days on various ISP (International *Streptomyces* Project; Shirling & Gottlieb, 1966): yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6), tyrosine agar (ISP 7); modified Bennett's agar (MBA; Jones, 1949), nutrient agar (NA; Difco), yeast extract-starch agar (YS; JCM medium 61) and Czapek's agar (CZA). Colors were designated by comparison with color ships from the Color Harmony Manual (Jacobson *et al*., 1958). The range of temperature (5-50 °C), pH (4-11) and NaCl (1-7%) for growth tolerance was examined on NA for 14-21 days. Catalase and oxidase activities were observed with a 3% (v/v) hydrogen peroxide solution and 1% (v/v) tetramethyl-*ρ*-phenylenediamine solution, respectively. Reduction of nitrate was observed using ISP 8 medium by the method described by Shirling and Gottlieb (1966). Starch hydrolysis was examined on ISP 4 medium. Urease activity was determined based on a color change in Stuart's urea agar. Gelatin liquefaction was evaluated on glucose-peptone-gelatin agar. Coagulation and peptonization of milk were observed in 10% (w/v) skim milk broth (Difco). Hydrolysis of DNA was tested on DNase agar (Difco). Production of H_2S and melanin pigment were determined on ISP 6 and ISP 7 media, respectively. Citrate utilization was tested on Simmons citrate agar (Difco). Decomposition of casein, xanthine, hypoxanthine and L-tyrosine were evaluated by using the media of Gordon *et al.* (1974). Utilization of carbon source was examined on ISP 9 medium (Shirling & Gottlieb, 1966) supplemented with each filter-sterilization compound used at a final concentration of 1% (w/v) of the tested carbon sources. Enzyme activity profiles were tested by using the API ZYM system (bioMérieux) according to the manufacturer's instruction.

1.4.3 Chemotaxonomic characterisation

Biomass for chemotaxonomic studies was obtained from culture grown in yeast extract-glucose broth at 27 °C for 7 days and then cells were freeze-dried. Isomer of diaminopimelic acid in the cell wall was determined by TLC using whole-cell hydrolysates, according to the method of Hasegawa *et al.* (1983). Whole-cell sugars composition were analyzed by TLC using the method of Becker *et al.* (1965). The *N*-acyl type of muramic acid in peptidoglycan was examined by the method of Uchida & Aida (1984). The presence of mycolic acids were monitored by TLC following the procedure of Tomiyasu (1982). Isoprenoidquinones were extracted and purified by using the method of Collins *et al.* (1977) and analysed by LC/MS (JSM-T100LP, JEOL). Analyses of phospholipids and whole-cell fatty acids were carried out by the service at Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Thailand. Phospholipids in cells were extracted and identified by two-dimensional TLC as described by Minnikin *et al.* (1984). Cellular fatty acids were extracted, methylated and analysed by using the Microbial Identification System (MIDI) according to the method of Sesser (1990) and the manufacturer's instructions. Fatty acid methyl esters were then analysed by GLC and using the Microbial Identification software package (Sherlock version 6.1; MIDI database RTSBA6). The G+C content of genomic DNA of new strain, which was extracted according to the method of Marmur (1961), was determined by HPLC according to the procedure of Tamaoka & Komagata (1984).

2. Determination of plant growth promoting agents

2.1. Indole-3-acetic acid (IAA) production

Indole-3-acetic acid (IAA) was assayed based on the colorimetric method described by Glickmann and Dessaux (1995) with some modifications. Endophytic actinomycetes were inoculated into 5 ml TSB supplemented with tryptophan (500 µg/ml) in a rotary shaker at 200 rpm, 28°C in the dark for 7 days. The cultures were centrifuged at 12000 rpm for 5 min and 1 ml supernatant was mixed with 2 ml Salkowski's reagent (Gordon and Weber, 1951) and incubated for 30 min at room temperature. Development of a pink color indicated IAA production could be measured at OD_{530}.

2.2. Phosphate solubilization

Phosphate solubilization potential of endophytic actinomycetes was determined by growing actinomycetes in tryptic soy broth (TSB) in a rotary shaker at 200 rpm, 28°C for 5 days. 100 µl of cell culture was dropped onto Pikovskaya agar (Pikovskaya, 1948) containing tricalcium phosphate and incubated at 28°C for 5 days. The presence of clear zones around the microbial colonies was used as indicator for positive strains.

2.3. Siderophore production

Siderophore assay was done using the chrome azurol S (CAS) assay (Schwyn and Neilands, 1987). An agar plug of 5-day growth of endophytic actinomycetes on YM agar was placed onto a CAS agar plate and incubated at 28°C for 2 days. A positive strain was indicated by an orange halo around the agar plug.

2.4. ACC deaminase production

Isolates were grown in seed media and incubation at 28 ºC with shaking 250 rpm for 4-7 days. After good growing, the cells were inoculated in the 5 ml of minimum media supplemented with either 3.0 mM 1-aminocyclopropane-1-carboxylic acid (ACC) or 2 g $(NH_4)_2SO_4$ per liter as a sole nitrogen source (Tarabily, 2008) and incubation at 28 ºC on with shaking 250 rpm for 4 weeks. The cultures were harvested and the pellets washed twice with sterilized distill water. 50 µl of the washed cells were then re-inoculated and incubated in the same experimental condition. Newly colonies formed in the media with addition ACC were considerate positive for ACC deaminase activity.

2.5. Chitinase production

Isolates were grown in semi-solid starch casein agar and each 25 µl of cell suspension was dropped on a colloidal chitin agar (CCA) (Nawani et al., 2002) and incubated at 28ºC for 14 days. The size of clear zone around the microbial colonies was determined for chitinase production.

Isolates that were positive on CCA were subsequently grown on mycelial fragment agar (MFA) to determined fungal cell wall-degrading activities. The mycelium of *Fusarium moniliforme* DOAC 1224, plant pathogenic fungi, was prepared by method modified from Valois *et al*. (1996). MFA plates were incubated at 28ºC for 10 days. The size of clear zone around the microbial colonies was determined.

3. Localisation and visualization of endophytic actinomycetes in plant

3.1 Bacterial strains, plasmids and culture conditions for conjugation experiments

Methylation-deficient *E. coli* ET12567 carrying plasmid pUZ8002 (MacNeil *et al*. 1992) was used as the donor for intergeneric conjugation study. A pUZ8002 is a RK2 derivative which is not self-transmissible but supplies mobilization functions for *oriT* containing plasmids in trans. pIJ10257 (kindly provided from John Innes Centre, UK.) was used as a conjugative vector. *E. coli* ET12567(pUZ8002) derivative was grown in Luria-Bertani broth (LB) supplemented with hygromycin B (25 µg/ml), chloramphenical (25 µg/ml) and kanamycin (25 µg/ml). A representative endophytic actinomycete strain, *Streptomyces* sp. GMKU944 isolated from wattle tree (*Acacia auriculiformis* A. Cunn. ex Benth.), was used as a recipient and was grown in ISP2 broth (Shirling and Gottlieb, 1966) for mycelia preparation. MS, ISP4 (Shirling and Gottlieb, 1966) and tryptone soya agar (TSA; Oxoid CM0131, UK.) supplemented with 10 mmol/l $MgCl_2$ were used as conjugative media.

3.2 Intergeneric conjugation procedure

E. coli ET12567 (pUZ8002/pIJ10257) donor cells were grown on LB with antibiotics as previously described until an OD_{600} of 0.4-0.6. Cells were harvested by centrifugation and washed twice with LB to remove antibiotic residues. Cells were resuspended at a final volume of 500 µl of LB (10^8 cells). Conjugation using mycelia of recipients was done according to our previous report (Phornphisutthimas *et al*., 2010). *Streptomyces* sp. GMKU944 was grown in 50 ml ISP2 broth in an Erlenmeyer flask containing a stainless steel spring coil with shaking in orbital incubator at 250 rev/min and at 28°C, for 24, 36 and 48 hours and mycelia were harvested respectively. Equal volume (500 µl) of *E. coli* cell suspension and *Streptomyces* mycelia were mixed and spread on MS, ISP4 and TSA (Oxoid) supplemented with 10 mmol/l $MgCl_2$ and incubated at 28 °C for 16-20 hours. The plates were flooded with 1 ml of water containing nalidixic acid (500 ugml-1) to eliminate donor cells and hygromycin B (1000 ugml-1) to select ex-conjugants, and incubated further for 5-7 days.

3.3 Plasmid construction for egfp gene transfer

Plasmid carrying *egfp* gene was constructed in *E. coli* XL1-blue using standard protocol. The 1.4 kb *Nde*I-*Hind*III DNA fragment containing *egfp* gene from pIJ8655 (Sun *et al*., 1999) was ligated to pIJ12057 carrying a strong constitutive *ermE* promoter, a hygromycin B resistant marker, an *oriT*/RK2 region and phage chromosomal integration sequence (*int* ΦC31) to obtain a recombinant plasmid designated pIJ10257*egfp*.

3.4 Introduction of pIJ10257egfp into *Streptomyces* and verification of ex-conjugants

The pIJ10257*egfp* was transferred to *Streptomyces* sp. GMKU944 by intergeneric conjugation with the condition that gave highest efficiency (see results). The ex-conjugants were re-streaked on MS containing nalidixic acid (50 ug/ml) and hygromycin B (50 ug/ml).

Genomic DNA of ex-conjugants was isolated as described by Kieser *et al*. (2000) and amplified using PCR with primers flanking the multiple cloning site of pIJ10257: 10257seqF (5'-ACGTCCATGCGAGTGTCC-3') and 10257seqR (5'- CCAAACGGCATTGAGCGTC-3'). pIJ10257*egfp* and the wild type genomic DNA were used as positive and negative control, respectively.

3.5 Visualization of enhanced green fluorescent protein (EGFP)

Ex-conjugants harbouring pIJ10257*egfp* were examined for eGFP expression by growing in ISP2 broth supplemented with hygromycin B (25 µg/ml at 28°C, 250 rev/min for 3 days. The culture was examined under a fluorescence microscope (Olympus BX-51) using a mercury vapour lamp with blue excitation (BP450–480 nm, DM500 and barrier filter BA515).

3.6 Plant cultivation and inoculations

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 Dilworth, 1971). The germinated seeds were inoculated by endophytic actinomycete culture broth (in ISP 2 medium) alone or the mixture between endophytic actinomycete and *Rhizobium* sp. DASA36075 (obtained from Soil Microbiology Research Group, Department of Agriculture, Thailand). The geminated seeds without inoculation were used as control. The plastic growth pouches were placed in the growth chamber at 25°C with light for 12 hr/day. Two month after inoculation, seedlings were harvested and the endophytes were re-isolated from roots by surface-sterilized method (Indananda *et al.*, 2010).

3.7 Laser scaning confocal microscope

Two months old plantlets which inoculated with eGFP-tagged *Streptomyces* sp. GMKU944 and un-innoculated control plantlets were cut into 20-25 µm thick sections by using Cryo microtome (LEICA CM1800) with a freezing stage attachment. The sections were visualized under laser scanning confocal microscope (Olympus FV1000, Japan) with excitation wavelength 465-496 nm that give emission wavelength 515-555 nm for eGFP detection and with excitation wavelength 360-370 nm that give emission wavelength 420-460 nm. for plant autofluorescence detection.

4. Construction of siderophore-deficient mutant

A partial *desD*-like gene was amplified from genomic DNA of *Streptomyces* sp. GMKU 3100 using specific primers, ATT083F (5’-TGCTTCGTCGCCAACAACGG-3’) and ATT083R (5’-CTGGAGCCGGTTGAGGCAGGA-3’). The PCR reaction was carried out in a final volume of 20 µl containing 0.2 mM of each dNTP, 5% DMSO, 10 pmol of each primer, 2 µl of 10× *Taq* DNA polymerase buffer and 0.5 units of *Taq* DNA polymerase (Fermentas, USA). The PCR program was as follows: 3 min at 95°C for 1 cycle; 30 sec at 95°C, 30 sec at 68°C, 1 min and 30 sec at 72°C for 30 cycles and 4 min at 72°C for 1 cycle. The PCR fragment was confirmed by DNA sequencing and then cloned into an integrating vector, pIJ8671 (Sun *et al.*, 1999) to obtain pIJ8671/*desD* prior to transformation of *E. coli* ET12567(pUZ8002) (MacNeil *et al.*, 1992). Intergeneric conjugation was performed according to Phornphisutthimas *et al.* (2010). The mycelium of a 24-h culture of *Streptomyces* sp. GMKU 3100 was used as recipient to conjugate with donor *E. coli* ET12567(pUZ8002)/pIJ8671/*desD* on MS agar containing 10 mmol/l $MgCl_2$. The mutants were selected by thiostrepton resistance. The mutant was tested for siderophore production using the CAS assay described above (Schwyn and Neilands, 1987). The mutant was verified to result from integration of pIJ8671/*desD* into the chromosome by amplification of the thiostrepton resistance gene using specific primers, ATT012 and ATT013, and PCR condition as described by Phornphisutthimas *et al*. (2010). The absence of a 5.4-kb long PCR product was also determined comparing by comparison of the recombinant plasmid pIJ8671/*desD* using specific primers, ATT012 and Apr N-2 (5'-CCCCGGCGGTGTGCT -3'; Choi *et al*., 2004). The PCR reaction was carried out using 0.1 unit of Phusion High-Fidelity DNA polymerase (Finnzymes, Finland) with 10% DMSO as follows: 3 min at 98°C for 1 cycle; 30 sec at 98°C, 30 sec at 69.5°C, 3 min at 72°C for 30 cycles and 5 min at 72°C for 1 cycle.

4.1 Siderophore producing strain interaction with plants

4.1.1 Pot culture growth conditions

Healthy rice seeds (*Oryza sativa* L. cv. KDML105) were immersed in water for 24 h before surface-sterilization. Rice and mungbean seeds (*Vigna radiata* (L.) Wilczek cv. CN72) were then surface-disinfected with 70% ethyl alcohol for 1 min followed by a 1% solution of sodium hypochlorite for 5 min. The seeds were washed six times for 1 min each with sterile distilled water. The seeds were then transferred onto sterilized moistened tissue paper in a tissue culture flask and incubated at room temperature in the dark for 2 days for seed germination. The pruned-root dip method (Musson *et al*., 1995) was used to inoculate the seedlings. The roots were trimmed using a sterilized scalpel and the seedlings were then immediately placed in sterile glass beakers containing a 10^8 CFU/ml spore suspension of *Streptomyces* sp. GMKU 3100 and its mutant and soaked for 4 h. Ten seedlings were used for each experiment and replicated twice. The seedlings were then transferred to a pot containing autoclaved vermiculite overnight at room temperature. Hoagland's solution (Hoagland and Arnon, 1950) supplemented with and without a less complex iron source, 10 µM ferric citrate, was added to the pot in order to assess the role of bacterial siderophores (Sharma *et al*., 2003). The pots were placed in the greenhouse and supplied with water once a day for 2-4 weeks.

4.1.2 Evaluation of shoot and root length and biomass

After 14- and 28-day growth of rice and mungbean plants, respectively, in the greenhouse, they were surface-sterilized and examined for endophytes according to the protocol previously described (Indananda *et al*., 2010). Root and shoot lengths, root and shoot fresh weights, and root and shoot dry weights were observed. All measurements of root and shoot parameters were conducted on ten seedlings for each condition. The one-way ANOVA and Tukeys multiple range tests (TMRT) were calculated using SPSS (version 11.5) at p=0.05 to determine the efficacy of wild type, mutant and untreated control in promotion of root and shoot development with and without 10 µM ferric citrate.

5. Chitinase producing endophytic streptomycetes control fungal disease

5.1 Screening of chitinase production

Chitinase production potential of the endophytic actinomycestes isolates were screened by growing actinomycetes into 6 ml semi-solid starch-casein (Küster and Williams1964) with the addition of 0.1 % agar and incubated on a rotary shaker (200 rpm) at 28 °C for 7 days. Aliquot of 25 µl of culture suspension was dropped onto colloidal chitin agar (CCA) plate containing the basal medium (Nawani *et al*. 2002) supplemented 0.5 % colloidal chitin. The colloidal chitin was prepared from shrimp shell chitin powder (Sigma) according to the method of Ahmadi et al. (2008) with some modifications. Five grams of chitin powder was added slowly into 90 ml of concentrated HCl under vigorous stirring for 2 hours. The mixture was added to 500 ml of ice-cold 95 % ethanol with rapid stirring and kept overnight at 4 °C. The precipitate was collected by centrifugation at 6000 rpm for 10 mins at 4 °C and washed with 0.1 M sodium phosphate buffer (pH 7.0) until the colloidal chitin become natural. There were triplicate plated for each isolates. Development of clear halos around the colonies were recorded after 14 days of incubation at 28 °C in the dark.

5.2 Determination of cell-wall degrading activity

Cell-wall degrading ability of the endophytic actinomycetes strains were determined on mycelial fragments agar (MFA) by modified the method of Valois et al. (1996). The phytopathogenic fungus *F. moniliforme* DOAC 1224 was grown in 30 mL of potato dextrose broth (PDB; Difco) and incubated on a rotary shaker (110 rpm) at 25 °C for 7 days. The mycelial mats were collected by filtration through Whatman no. 1 filter paper, washed with sterile deionized water, resuspended in 30 mL of sterile deionized water, and then homogenized in a Waring blender. The suspended mycelial fragments were centrifuged in a refrigerator at 7,500 rpm for 20 min at 4 °C and washed with sterile deionized water. The mycelial fragments were then resuspended in 30 mL of sterile deionized water and subjected to ultrasonic disintegration in an ice bath for 20 min of 30 s power on and 15 s power off in ultrasonicator. The mycelial pellets were centrifuged, washed three times with sterile deionized water and then added three volumes of sterile deionized water. The suspended mycelial pellets were added to the basal medium (Nawani et al. 2002). The cell culture of 7-days growth of the endophytic actinomycetes in semi-solid starch-casein agar were dropped onto MFA plate and incubated at 28 °C for 10 days in the dark. There were triplicate plated for each isolates.

5.3 Molecular detection of chitinase genes

The endophytic actinomycetes isolates were detected the partial of genes encoding family 18 group A and 19 chitinases using degenerate primers by PCR. Genomic DNA was extracted by the method of Kieser (2000). The family 18 group A chitinase gene was amplified using primer ATT107F (5'-GACACCTGGGACCAGCCGCTG-3') and ATT107R (5'-TAGAAGCCGAYGCCGAKSAGCA-3'). The family 19 chitinase gene was amplified using primer ATT092F (5'-CAGTTCRACCARATGTTCCCG-3') and ATT092R (5'-CGTTGATSGASCGGATSGTCT-3'). The PCR reaction was performed in a total volume of 20 µl containing 14 µl ultrapure water, 0.4 µl of 0.2 mMdNTPs, 0.4 µl of 10 pmol of each primes, 2 µl DMSO, X µl 0f 3mM $MgCl_2$, 2 µl of 50 ng DNA, 10 µl of 10X Taq polymerase buffer and 1 µl of 1UTaq polymerase enzymes (Fermentas, USA). The PCR program of family 18 group A chitinase gene was as follows: initial denaturation at 94 °C for 4 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 1 min 30 s and extension at 72 °C for 1 min. with a final extension step at 72 °C for 4 min. For family 19 chitinase gene was used the PCR program same family 18 group A chitinase gene, but annealing temperature was changed to 60 °C. The PCR product was purified using a Gel/PCR DNA Fragment Extraction kit (Geneaid) and then sent to Macrogen (Korea) for confirmed by DNA sequencing. The nucleotide sequences were manually assessed for similarities against the NCBI non-redundant protein database using BLASTX algorithm.

5.4 *In vitro* evaluation of fungal antagonism

The endophytic actinomycetes strains were evaluated for in *vitro* antagonism again the phytopathogenic fungus *F. moniliforme* DOAC 1224 using the dual culture technique as modified the method of Tahtamouni et al. (2006). Each endophytic actinomycetes strain was streak-inoculated on one side of the SCA plates and incubated at 28 °C for 7 days. A 6-mm mycelial plug of *F. moniliforme* DOAC 1224 from a fresh growing colony was placed at the opposite side of the actinomycetes cultures at a 4-cm distance. The fungal plug was also placed on un-inoculated agar plates separately as control treatment. There were triplicate plated for each actinomycetes strain. The diameter of the inhibition zone was measured after 8 days of incubation at 25 °C.

5.5 Microscopic observations of fungal cell wall lysis

The selected endophytic actinomycetes strains were employed to observe the effect on the *F. moniliforme* DOAC 1224 cell walls using a slide culture technique. An agar block (4x2 cm.) taken from a CCA plate and that transferred to the center of slide. The cell culture of 7-days growth of the endophytic actinomycetes in semi-solid starch-casein agar were dropped on one side of the agar block and incubated at 28 °C for 5 days in the dark. The mycelial of *F. moniliforme* DOAC 1224 from a fresh growing colony was placed at the opposite side of the actinomycetes cultures at a 2-cm distance. The inoculated slides were incubated at 25 °C for 3 days. The fungal hyphae from the interaction zone was observed using a light microscope. The subsequent changes in the morphology of the fungal hyphae were collected and sent to the sample preparation by the service at Scientific and Technological Research Equipment Centre, Thailand. The samples were fixed with osmium tetroxide for 1-2 hours at room temperature. The fixed samples were then dehydrated in a graded ethanol series (30, 50, 70, 95 and 100%), each grade for 10 min and 3 times for 100% ethanol. Fully dehydrated samples were dried in a critical point dryer (Balzersmodel CPD 020), mounted on stubs, and then coated with gold in a sputter coater (Balzers model SCD 040). The coated specimens were examined with a scanning electron microscope (SEM; model JSM-5410).

5.6 Evaluation of bicontrol efficacy of chitinase-producing strain

The individual chitinolytic *Streptomyces* GMKU 301 and GMKU 322 strain were evaluated the biocontrol efficacy against *F. moniliforme* DOAC 1224in maize plants (*Zea Mays* L. cv. *Nakhonsawan*3). Maize seeds were surface sterilized by soaking in 1% sodium hypochlorite for 1 min and subsequently immersing in 70% ethanol for 5 min. Surface-sterilized seeds were then washed in sterile water three times. The seeds were pre-germinated on moist paper towels in a plastic box at room temperature in the dark for 3-4 days. The seeds germinated were inoculated with each of *Streptomyces* using the pruned-root dip method (Musson et al. 1995) and soaked in the bacterial suspension (10^8cfu/ml) for 4 hours on a rotary shaker at 110 rpm. The seed germinated were soaked in distilled water served as control. Excess bacterial suspensions were drained off and seed germinated were then planted into mini-pots containing autoclaved soil. The mini-pot were placed in the greenhouse and supplied with water once a day. Thirteen-day-old seedlings were transplanted into fresh pot with five plants per pot with three replicates for each treatment. One day after transplanting of seedlings, each seedling was inoculated with two mycelial plugs (6 mm in diameter) of *F. moniliforme* DOAC 1224 on the collar region of maize plants and covered with parafilm to avoid dryness. The parafilm was removed from the maize seedling 3 days after incubation. For un-inoculated control was treated with agar plug without mycelial of fungi. The experiment was included six treatments: 1-control a (no pathogen); 2-treated with *Streptomyces* GMKU 301; 3-treated with *Streptomyces* GMKU 322; 4-control b (inoculated with pathogen); 5-treated with *Streptomyces*

GMKU 301 and inoculated with pathogen; 6-treated with *Streptomyces* GMKU 322 and inoculated with pathogen. Fourteen-day after inoculation pathogen, maize plants were recorded the disease index and calculating biocontrol efficacy of endophytic *Streptomyces* against *F. moniliforme* DOAC 1224 causing foot rots and wilting diseases of maize plants. The disease index was evaluated as previously described, with some modification (Pal *et al*. 2001) based on the score 0-5; 0-no symptoms; 1-appearance of lesion at the collar region; 2-large lesions; 3-moderate rotting of the collar region, loss of turgor at the top with slight groping; 4 extensive rotting at the collar region, wilting and drooping of the shoot; 5-plant completely wilted, dead and dry. Disease scores were converted to disease incidence (Xue *et al*. 2009) by using the following formula; disease incidence = [Σ(the number of plants in this index x disease index)/(total number of plants investigated x highest disease index)] x 100%. The one-way ANOVA and Tukeys multiple range tests (TMRT) were calculated using SPSS (version 11.5) at p = 0.05 to determine the disease incidence. Biocontrol efficiency was calculated as described by Xue *et al.* (2009) using the following formula; biocontrol efficiency = [(disease incidence of control-disease incidence of bacteria treated plants) / disease incidence of control] x 100%. The stem of maize plants were cut into longitudinal-half sections and observed under a stereomicroscope.

6. Identification of new bioactive compound from endophytic actinomycetes

6.1 Fermentation

The selected strain of endophytic actinomycetes was cultured on Bn-2 slant agar medium and then was inoculated into 500-mL K-1 flasks each containing 100 ml of the V-22 seed medium (pH 7.0). The cultures were cultivated on a rotary shaker (200 rpm) at 30 °C for 4 days. The seed culture (3 ml) was transferred into 500 ml K-1 flasks each containing 100 ml of the A-11M production medium (pH 7.0) The inoculated flasks were cultured on a rotary shaker (200 rpm) at 30 °C for 6 days.

6.2 Extraction and isolation

At the end of the fermentation period, 100 ml of 1-butanol were added to each flask, and they were allowed to shake for 1 additional hour. The mixture was centrifuged at 6,000 rpm for 10 min and the organic layer was separated from the aqueous layer containing the mycelium. Evaporation of the solvent gave a dried crude extract from 1.5 l of culture. The crude extract was subjected to silica gel column chromatography with a step gradient of $CHCl_3$-MeOH (1: 0, 20: 1, 10: 1, 4: 1, 2: 1, 1: 1 and 0: 1 v/v). The potential fraction was collected and concentrated and further purified by preparative C-18 HPLC using a gradient of MeCN/0.1% HCO_2H (MeCN concentration: 15 to 40% over 60 min). Finally, the compound was analysed by NMR.

ผลงานวิจัย

1. Isolation and identification of endophytic actinomycetes

Sixty endophytic actinomycetes were isolated from local plants namely, Chamung (*Garcinia cowa* Roxb.), Jig (*Barringtonia acutangula* Gaertn.), Sumrong (*Scaphium macropodum* Beaum.) and Waa (*Syzygium cumini* L.) designated GKU1xx (Table 1). Eight-three endophytic actinomycetes designated GKU8xx (Table 2) and 52 strains of bacterial endophytes designated EN-x (Table 3) were isolated from sugarcane plants variety LK92-11 and KK3.

GKU and EN collections were identified their taxonomic relatedness by 16S rRNA sequencing. For GKU1xx (Table 1), 42 strains belong to genus *Streptomyces*, while 18 strains belong to rare actinomycetes. For GKU8xx, 29 strains belong to genus *Microbispora*, 47 strains belong to genus *Streptomyces*, 5 strains belong to *Actinomadura* and 2 strains belong to *Micromonospora* (Table 2).

For bacterial endophytes, *Acinetobacter*, *Bacillus*, *Enterobacter*, *Kluyvera*, *Lysinibacillus*, *Micrococcus*, *Paenibacillus*, *Pantoea*, *Pseudomonas* and *Staphylococcus* were found in sugarcane plants. *Bacillus* and *Enterobacter* were found as major genera (Table 3).

Table 1 Endophytic actinomycetes GKU1xx isolated from local plants at Khao Khitchakut, Chantaburi.

Plants	A	D	N	No	S	St
Thai name: sumrong Common name: malva nut Scientific name: *Scaphium macropodium* Beaum					14	
Thai name: chamung Common name: cowa Scientific name: *Garcinia cowa* Roxb.	3				8	
Thai name: jig Common name: indian oak Scientific name: *Barringtonia acutangula* (L.) Gaertn.	7	1	2	1	20	
Thai name: waa Common name: jambolan plum Scientific name: *Syzygium cumini* (L.) Skeels	2			1		1
Total	12	1	2	2	42	1

Note: A, *Actinomadura*; D, *Dactylosporangium*; N, *Nocardia*; No, *Nonomuraea*; S, *Streptomyces*; St, *Streptosporangium*

2. Characterisation of plant growth promotion (PGP) of endophytes

Sixty-nine endophytic actinomycetes from GMKU culture collection (results from TRF grant no. RMU4980033) (Table 4) and 83 newly isolates GKU culture collection isolated from sugarcane plants (Table 2), and 52 strains of bacterial endophytes (EN collection, Table 3) were tested for plant growth promoting (PGP) traits namely, indole-3-acetic acid (IAA) production, siderophore production, phosphate solubilisation, ACC deaminase and chitinase activities. All activities were summarized in Table 2, 3 and 4.

Table 2 Plant growth promoting and antimicrobial activities of the representative isolates of endophytic actinomycetes

Strains	**Genus**	**PGP-traits**			**Antifungal**			**Antibacterial**			
		IAA	SP	PS	AN	FM	CF	BC	SA	EC	PA
GKU800	*Microbispora* sp.	++	+	+	-	+	-	-	-	-	-
GKU801	*Microbispora* sp.	+	-	++	-	+	-	-	-	-	-
GKU802	*Microbispora* sp.	+++	+	-	-	+	+	-	-	-	-
GKU803	*Microbispora* sp.	-	+	-	-	+	-	-	-	-	-
GKU804	*Microbispora* sp.	+	-	-	-	+	-	-	-	-	-
GKU805	*Microbispora* sp.	-	-	-	-	+	-	-	-	-	-
GKU806	*Microbispora* sp.	+	+	-	-	+	-	-	-	-	-
GKU807	*Microbispora* sp.	-	++	+	-	+	-	-	-	-	-
GKU810	*Streptomyces* sp.	+++	+	++	+	-	-	-	-	-	-
GKU813	*Microbispora* sp.	+	++	+	-	-	-	-	-	-	-
GKU816	*Streptomyces* sp.	+++	+++	-	-	+	++	-	-	-	-
GKU818	*Microbispora* sp.	-	-	+	-	+	-	-	-	-	-
GKU819	*Microbispora* sp.	+	-	+	-	-	-	-	-	-	-
GKU820	*Microbispora* sp.	++	+	++	-	+	-	-	-	-	-
GKU821	*Microbispora* sp.	++	-	++	-	+	-	-	-	-	-
GKU822	*Actinomadura* sp.	-	++	-	-	++	-	-	-	-	-
GKU823	*Microbispora* sp.	+	+	++	+	-	-	-	-	-	-
GKU824	*Microbispora* sp.	-	+	+++	+	+	-	-	-	-	-
GKU825	*Microbispora* sp.	-	+	+	-	+	-	-	-	-	-
GKU826	*Streptomyces* sp.	-	++	-	++	-	+++	-	-	-	-
GKU827	*Microbispora* sp.	+++	+	++	-	+	-	-	-	-	-
GKU830	*Streptomyces* sp.	++	++	-	-	-	-	-	-	-	-
GKU831	*Streptomyces* sp.	+++	+++	++	+++	-	+++	-	-	-	-
GKU832	*Microbispora* sp.	-	+	+	-	-	++	-	-	-	-
GKU833	*Streptomyces* sp.	++	+++	+	+++	+++	+++	++	-	-	-

Strains	**Genus**	**PGP-traits**			**Antifungal**			**Antibacterial**			
		IAA	SP	PS	AN	FM	CF	BC	SA	EC	PA
GKU835	*Streptomyces* sp.	-	-	+	-	-	-	-	-	-	-
GKU840	*Streptomyces* sp.	-	++	-	+	-	-	-	-	-	-
GKU841	*Microbispora* sp.	++	+	+	-	+	-	-	-	-	-
GKU842	*Microbispora* sp.	-	++	+	-	-	-	-	-	-	-
GKU843	*Microbispora* sp.	+	++	-	+	-	-	-	-	-	-
GKU846	*Microbispora* sp.	-	+	-	-	-	-	-	-	-	-
GKU847	*Microbispora* sp.	-	+	++	-	-	-	-	-	-	-
GKU848	*Microbispora* sp.	-	+++	+	+	+	-	-	-	-	-
GKU852	*Streptomyces* sp.	+++	+++	++	-	-	+	-	-	-	-
GKU855	*Streptomyces* sp.	+++	+++	+	-	+	-	-	-	-	-
GKU856	*Streptomyces* sp.	+++	-	-	-	+	+	-	-	-	-
GKU858	*Streptomyces* sp.	+	-	+	+	+	+	-	-	-	-
GKU859	*Streptomyces* sp.	+	-	-	-	-	-	-	-	-	-
GKU860	*Streptomyces* sp.	-	-	-	-	-	-	-	-	-	-
GKU861	*Microbispora* sp.	-	-	+	-	-	-	-	-	-	-
GKU862	*Streptomyces* sp.	+	-	+++	+	-	-	-	-	-	-
GKU863	*Microbispora* sp.	++	+	-	-	-	-	-	-	-	-
GKU865	*Streptomyces* sp.	++	+	++	+	-	++	-	-	-	-
GKU866	*Streptomyces* sp.	+	-	+	-	-		+++	-	-	-
GKU867	*Streptomyces* sp.	-	+++	-	++	+	+	+++	+++	-	-
GKU868	*Microbispora* sp.	-	++	-	-	-	-	+++	-	-	-
GKU869	*Streptomyces* sp.	-	+	-	+	+	+	+++	-	-	-
GKU870	*Actinomadura* sp.	+	+	-	+++	++	+++	-	-	-	-
GKU872	*Streptomyces* sp.	+	+	+	-	+	+	-	-	-	-
GKU874	*Streptomyces* sp.	+	+	-	++	+	-	+	-	-	-
GKU876	*Streptomyces* sp.	-	+++	-	-	+	-	++	-	-	-
GKU877	*Streptomyces* sp.	+	+++	-	+	+	-	-	-	+	-
GKU878	*Streptomyces* sp.	-	+	-	++	++	+++	-	-	-	-
GKU879	*Streptomyces* sp.	+	+	+	-	-	++	-	-	-	-
GKU880	*Streptomyces* sp.	+++	++	-	-	+	+	+	-	-	-
GKU883	*Streptomyces* sp.	+	+	+	-	+	+	-	-	-	-
GKU885	*Micromonospora* sp.	-	+	-	++	+	++	-	-	-	-
GKU886	*Actinomadura* sp.	-	+	-	-	-	-	-	-	-	-
GKU887	*Streptomyces* sp.	-	++	-	-	-	-	-	-	-	-
GKU889	*Streptomyces* sp.	+	++	+	+	+	-	+++	+++	-	-
GKU891	*Streptomyces* sp.	+	++	-	+	++	+++	-	-	-	-

Strains	Genus	PGP-traits			Antifungal			Antibacterial			
		IAA	SP	PS	AN	FM	CF	BC	SA	EC	PA
GKU893	*Streptomyces* sp.	+	+++	-	+	++	-	-	-	-	-
GKU895	*Streptomyces* sp.	+++	++	+	-	++	+	+	-	-	-
GKU897	*Streptomyces* sp.	-	++	-	++	+	++	-	+++	-	-
GKU898	*Microbispora* sp.	-	-	+	-	+	-	-	-	-	-
GKU8001	*Actinomadura* sp.	+	++	-	+	+	+	-	-	-	-
GKU8002	*Micromonospora* sp.	-	++	-	+	+	-	-	-	-	-
GKU8003	*Actinomadura* sp.	+	+	-	-	-	+	-	-	-	-
GKU8004	*Streptomyces* sp.	-	+	-	-	-	-	-	-	-	-
GKU8005	*Streptomyces* sp.	-	++	+	+	+	-	-	-	-	-
GKU8006	*Streptomyces* sp.	-	-	-	-	-	-	-	-	-	-
GKU8008	*Streptomyces* sp.	+	++	-	+	+++	++	-	-	-	-
GKU8009	*Streptomyces* sp.	+	+	+	+	-	++	-	-	-	-
GKU8010	*Streptomyces* sp.	-	+++	-	+	++	+++	-	-	-	-
GKU8019	*Streptomyces* sp.	-	+	-	-	-	++	+++	-	-	-
GKU8020	*Streptomyces* sp.	-	-	-	-	-	-	-	-	-	-
GKU8021	*Streptomyces* sp.	-	++	+	-	+	-	-	-	-	-
GKU8022	*Streptomyces* sp.	-	++	-	+	-	-	++	-	-	-
GKU8024	*Microbispora* sp.	-	-	-	-	-	-	-	-	-	-
GKU8025	*Streptomyces* sp.	-	++	+	-	-	-	++	+	-	-
GKU8026	*Streptomyces* sp.	-	-	-	-	-	-	-	-	-	-
GKU8028	*Streptomyces* sp.	-	+	-	-	-	-	-	-	-	-
GKU8031	*Streptomyces* sp.	+	-	-	-	-	-	-	-	-	-

Notes: IAA (indole-3-acetic production); SP (siderophore production); PS (phosphate solubilization); AN (*Aspergillus niger* ATCC 6275); FM (*Fusarium moniliforme* DOAC 1224); CF (*Colletotrichum falcatum* DOAC 1655); BC (*Bacillus cereus* ATCC 11778); EC (*Escherichia coli* ATCC 8739); PA (*Pseudomonas aeruginosa* ATCC 15442); SA (*Staphylococcus aureus* ATCC 25923)

Table 3 Plant growth promoting antimicrobial activities of the representative isolates of endophytic bacteria

Strains	Genus	PGP-traits					Antifungal			Antibacterial			
		IAA	SP	PS	ARA	*nifH*	AN	FM	CF	BC	SA	EC	PA
EN-1	*Enterobacter* sp.	-	-	-	-	-	-	-	-	-	-	-	-
EN-2	*Enterobacter sp.*	-	-	-	-	-	-	-	-	-	-	-	-
EN-3	*Enterobacter sp.*	-	-	-	-	-	-	-	-	-	-	-	-
EN-4	*Pantoea sp.*	+	-	-	-	-	-	-	-	-	-	-	-
EN-5	*Acinetobacter sp.*	-	-	-	-	+	-	-	-	-	-	-	-
EN-6	*Bacillus sp.*	-	+	-	-	+	-	++	++	+	-	++	-

Strains	Genus	PGP-traits					Antifungal			Antibacterial			
		IAA	SP	PS	ARA	*nifH*	AN	FM	CF	BC	SA	EC	PA
EN-7	*Bacillus sp.*	-	+	-	-	+	-	++	++	-	-	+	-
EN-8	*Bacillus sp.*	-	++	-	-	-	+	++	++	-	-	+	-
EN-9	*Lysinibacillus sp.*	-	-	-	+	+	-	-	-	-	-	-	-
EN-10	*Enterobacter sp.*	+	-	-	-	+	-	-	-	-	-	-	-
EN-11	*Bacillus sp.*	-	-	-	-	-	-	-	-	-	-	-	-
EN-12	*Enterobacter sp.*	+++	-	-	-	-	-	-	-	-	-	-	-
EN-13	*Bacillus sp.*	-	+	-	-	-	-	-	+	+	-	++	-
EN-14	*Bacillus sp.*	-	-	-	-	-	-	-	-	+	-	++	-
EN-15	*Bacillus sp.*	+	++	-	-	-	+	++	+++	++	-	-	-
EN-16	*Enterobacter sp.*	+++	++	-	-	-	-	-	-	-	-	-	-
EN-17	*Enterobacter sp.*	-	-	-	-	-	-	-	-	-	-	-	-
EN-18	*Bacillus sp.*	+	+++	-	-	-	-	++	+++	-	+	+	-
EN-19	*Enterobacter sp.*	+++	+	-	-	-	-	-	-	-	-	-	-
EN-20	*Enterobacter sp.*	++	-	-	-	+	-	-	-	-	-	-	-
EN-21	*Enterobacter sp.*	+++	+	-	+	+	-	-	-	-	-	-	-
EN-22	*Enterobacter sp.*	+++	+	-	-	-	-	-	-	-	-	-	-
EN-23	*Enterobacter sp.*	-	-	-	-	-	-	-	-	-	-	-	-
EN-24	*Bacillus sp.*	-	++	-	+	-	++	+++	+++	++	-	-	-
EN-25	*Bacillus sp.*	-	-	++	-	-	-	-	-	-	-	-	-
EN-26	*Paenibacillus sp.*	+	++	-	-	-	-	+	++	+	-	-	-
EN-27	*Bacillus sp.*	+	+	-	+	+	-	-	-	-	-	-	-
EN-28	*Staphylococcus sp.*	-	-	-	-	-	-	-	-	-	-	-	-
EN-29	*Pantoea sp.*	-	-	+++	-	+	-	+	-	-	-	-	-
EN-30	*Enterobacter sp.*	+++	-	-	+	+	-	-	-	-	-	-	-
EN-31	*Micrococcus sp.*	++	-	-	-	-	-	-	-	-	-	-	-
EN-32	*Enterobacter sp.*	++	-	-	-	-	-	-	-	-	-	-	-
EN-33	*Enterobacter sp.*	-	-	-	-	-	-	-	-	-	-	-	-
EN-34	*Pseudomonas sp.*	+++	-	-	-	-	-	-	-	-	-	-	-
EN-35	*Enterobacter sp.*	+	-	+	-	-	-	-	-	-	-	-	-
EN-36	*Enterobacter sp.*	-	-	-	-	-	-	-	-	-	-	-	-
EN-37	*Kluyvera sp.*	+++	-	+	-	-	-	+	-	-	-	-	-
EN-38	*Enterobacter sp.*	++	-	-	-	-	-	-	-	-	-	-	-
EN-39	*Pantoea sp.*	-	-	+++	+	+	-	+	-	-	-	-	-
EN-40	*Enterobacter sp.*	+++	-	-	-	-	-	-	-	-	-	-	-
EN-41	*Acinetobacter sp.*	-	-	-	-	-	-	-	-	-	-	-	-
EN-42	*Enterobacter sp.*	-	+++	-	-	-	-	+	++	-	-	-	-

Strains	Genus	PGP-traits					Antifungal			Antibacterial			
		IAA	SP	PS	ARA	*nifH*	AN	FM	CF	BC	SA	EC	PA
EN-43	*Enterobacter sp.*	-	+++	-	-	-	-	++	++	-	-	-	-
EN-44	*Enterobacter sp.*	-	+	-	-	-	-	++	++	-	-	-	-
EN-45	*Enterobacter sp.*	-	+	-	-	-	-	+	++	-	-	-	-
EN-46	*Enterobacter sp.*	-	-	-	-	-	-	-	-	-	-	-	-
EN-47	*Enterobacter sp.*	-	+	+	-	-	-	+	-	-	-	-	-
EN-48	*Bacillus sp.*	-	++	+	-	-	+	+++	-	-	-	-	-
EN-49	*Bacillus sp.*	-	-	+	-	-	-	+	+++	-	-	-	-
EN-50	*Paenibacillus sp.*	-	+++	+	-	-	-	+	+++	-	-	-	-
EN-51	*Bacillus sp.*	-	+++	-	-	-	-	-	-	-	-	-	-
EN-52	*Staphylococcus sp.*	-	-	-	-	-	-	+	++	-	-	-	-

Notes: IAA (indole-3-acetic production); SP (siderophore production); PS (phosphate solubilization); ARA (acetylene reduction assay); AN (*Aspergillus niger* ATCC 6275); FM (*Fusarium moniliforme* DOAC 1224); CF (*Colletotrichum falcatum* DOAC 1655); BC (*Bacillus cereus* ATCC 11778); EC (*Escherichia coli* ATCC 8739); PA (*Pseudomonas aeruginosa* ATCC 15442); SA (*Staphylococcus aureus* ATCC 25923)

Table 4 Plant growth promoting (PGP) traits of endophytic actinomycetes GMKU 3xx (TRF grant no. RMU4980033): indole-3-acetic acid (IAA) production, siderophore production, phosphate solubilisation, ACC deaminase and chitinase activities from GMKU culture collection. + = positive; - = negative; ND = not yet determined.

Strains	IAA	Siderophore	Phosphate	ACC deaminase	Chitinase
GMKU 301	-	-	+	-	+
GMKU 302	-	-	-	-	+
GMKU 303	-	-	-	-	+
GMKU 305	-	-	-	-	+
GMKU 306	+	+	+	-	+
GMKU 307	+	+	+	-	+
GMKU 308	+	+	+	+	+
GMKU 309	-	-	+	-	+
GMKU 311	+	-	-	-	+
GMKU 312	-	-	+	-	+
GMKU 313	-	+	-	+	+
GMKU 314	-	+	+	+	-
GMKU 315	-	+	-	+	+
GMKU 316	-	+	+	+	+
GMKU 317	-	-	-	-	+
GMKU 318	-	-	+	-	+

GMKU 319	-	-	+	+	+

Strains	IAA	Siderophore	Phosphate	ACC deaminase	Chitinase
GMKU 320	-	-	-	+	+
GMKU 322	-	-	-	-	+
GMKU 323	-	-	-	-	+
GMKU 324	-	-	+	-	+
GMKU 325	-	-	-	+	-
GMKU 326	-	-	-	-	+
GMKU 327	-	-	+	-	-
GMKU 328	-	+	-	-	+
GMKU 329	-	+	-	-	+
GMKU 330	-	+	-	-	+
GMKU 331	-	+	-	-	+
GMKU 333	-	+	-	-	+
GMKU 334	+	+	-	-	+
GMKU 335	+	+	-	+	+
GMKU 336	-	+	+	+	+
GMKU 337	+	-	+	-	+
GMKU 340	-	-	-	-	+
GMKU 341	-	-	-	-	+
GMKU 342		-	-	-	+
GMKU 343	+	-	-	-	+
GMKU 344	+	+	+	+	+
GMKU 345	-	+	+	+	+
GMKU 346	-	+	-	-	-
GMKU 347	-	-	-	-	+
GMKU 348		-	-	-	+
GMKU 350	-	-	-	-	+
GMKU 351	-	+	-	-	+
GMKU 352	-	+	-	-	+
GMKU 353	-	-	-	-	+
GMKU 354	+	+	+	-	+
GMKU 355	+	+	+	-	+
GMKU 356	-	-	-	-	-
GMKU 357	-	-	-	-	+
GMKU 358	-	+	-	-	+
GMKU 359	-	+	+	-	+
GMKU 360	-	+	-	-	-
GMKU 361	-	+	-	-	+

GMKU 362	-	+	-	+	+

Strains	IAA	Siderophore	Phosphate	ACC deaminase	Chitinase
GMKU 363	-	+	-	+	+
GMKU 364	-	+	-	+	+
GMKU 365	+	-	-	-	+
GMKU 366	-	+	-	-	+
GMKU 367	-	+	-	+	ND
GMKU 368	-	-	-	-	+
GMKU 369	-	-	-	-	+
GMKU 370	-	+	-	-	+
GMKU 372	-	-	-	-	+
GMKU 377	+	+	+	-	+
GMKU 378	-	-	-	-	+
GMKU 3100	-	+	-	ND	ND
GMKU 3101	-	-	-	ND	ND
GMKU 3102	-	+	-	ND	ND

3. Identification of new species from endophytic actinomycetes

3.1 *Nonomuraea syzygii* sp. nov. isolated from jambolan plum tree (*Syzygium cumini* L. Skeels)

3.1.1 16S rRNA gene analysis

Comparison of the almost complete 16S rRNA gene sequence (1,448 nt) of strain GKU 164^{T} with those related valid type strains indicated that strain GKU 164^{T} was a member of the genus *Nonomuraea*. The most closely related strains were *N. monospora* PT708^{T} (98.77%; 17 nt difference at 1380), *N. thailandensis* KC-061^{T} (98.73%; 18 nt difference at 1,422), *Nonomuraea solani* NEAU-Z6^{T} (98.68%, 19 nt difference at 1442), *Nonomuraea rhizophila* YIM 67092^{T} (98.34%, 24 nt difference at 1444), *N. shaanxiensis* NEAU-st1^{T} (98.20%, 26 nt difference at 1442) and *Nonomuraea rosea* GW 12687^{T} (98.04%, 27 nt difference at 1375). Levels of 16S rRNA gene sequence similarity values to the type strains of other species of the genus *Nonomuraea* were lower than 98%. The neighbour-joining tree was in agreement with the maximum likelihood tree in which strain GKU 164^{T} always formed a closely phyletic line with *N. monospora* PT708^{T} and *N. thailandensis* KC-061^{T} (Fig. 1 and Fig. 2).

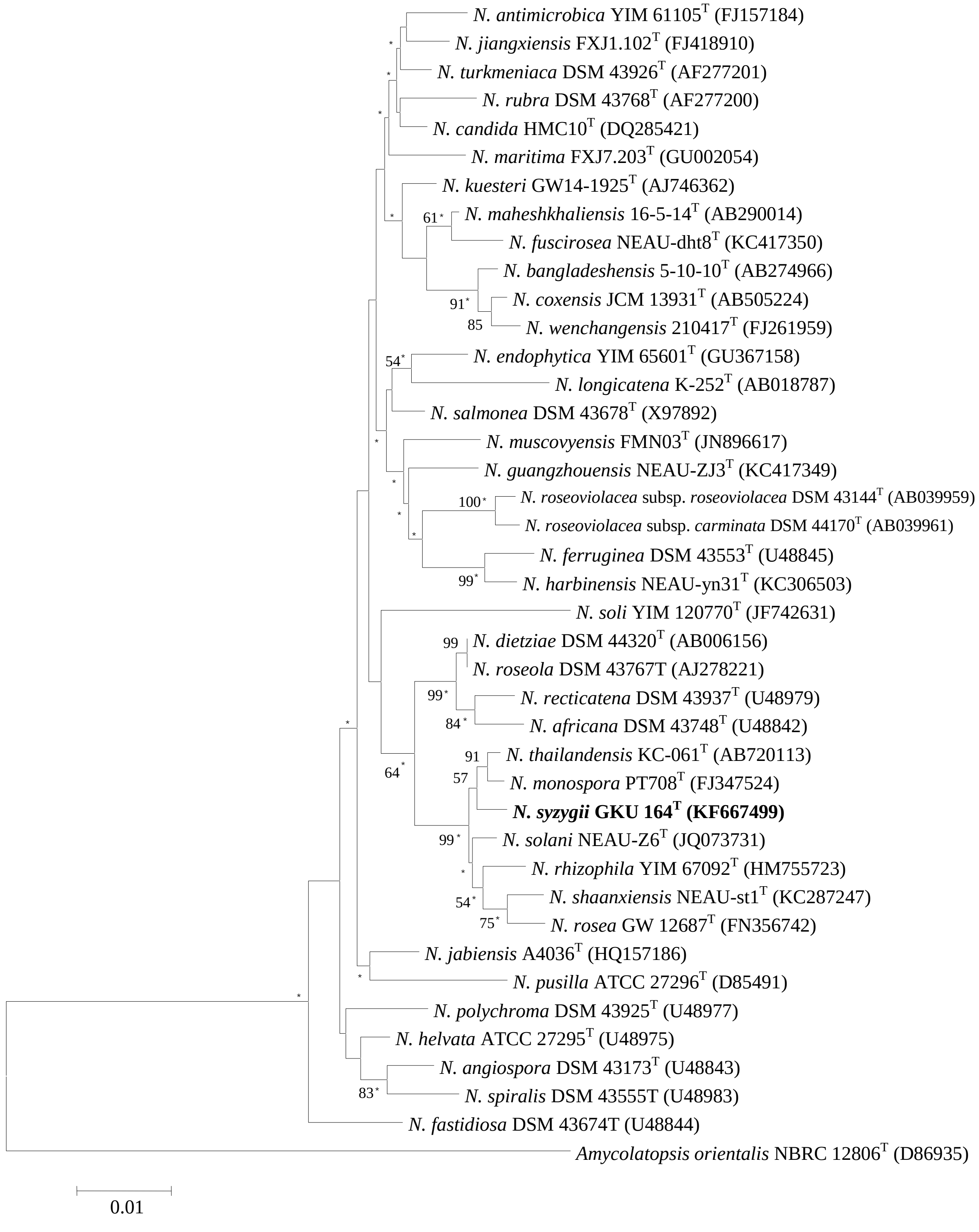


Figure 1 Neighbour-joining tree based on almost complete 16S rRNA gene sequences showing the relationship between strain GKU 164^T and all recognized species of the genus *Nonomuraea*. *Amycolatopsis orientalis* NBRC 12806^T was used as an outgroup. Asterisks denote branches that were also recovered from maximum-likelihood tree. Numbers at branch points indicate bootstrap percentage (based on 1,000 replications); only values above 50% are showed. Bar, 0.01 substitutions per nucleotide position.

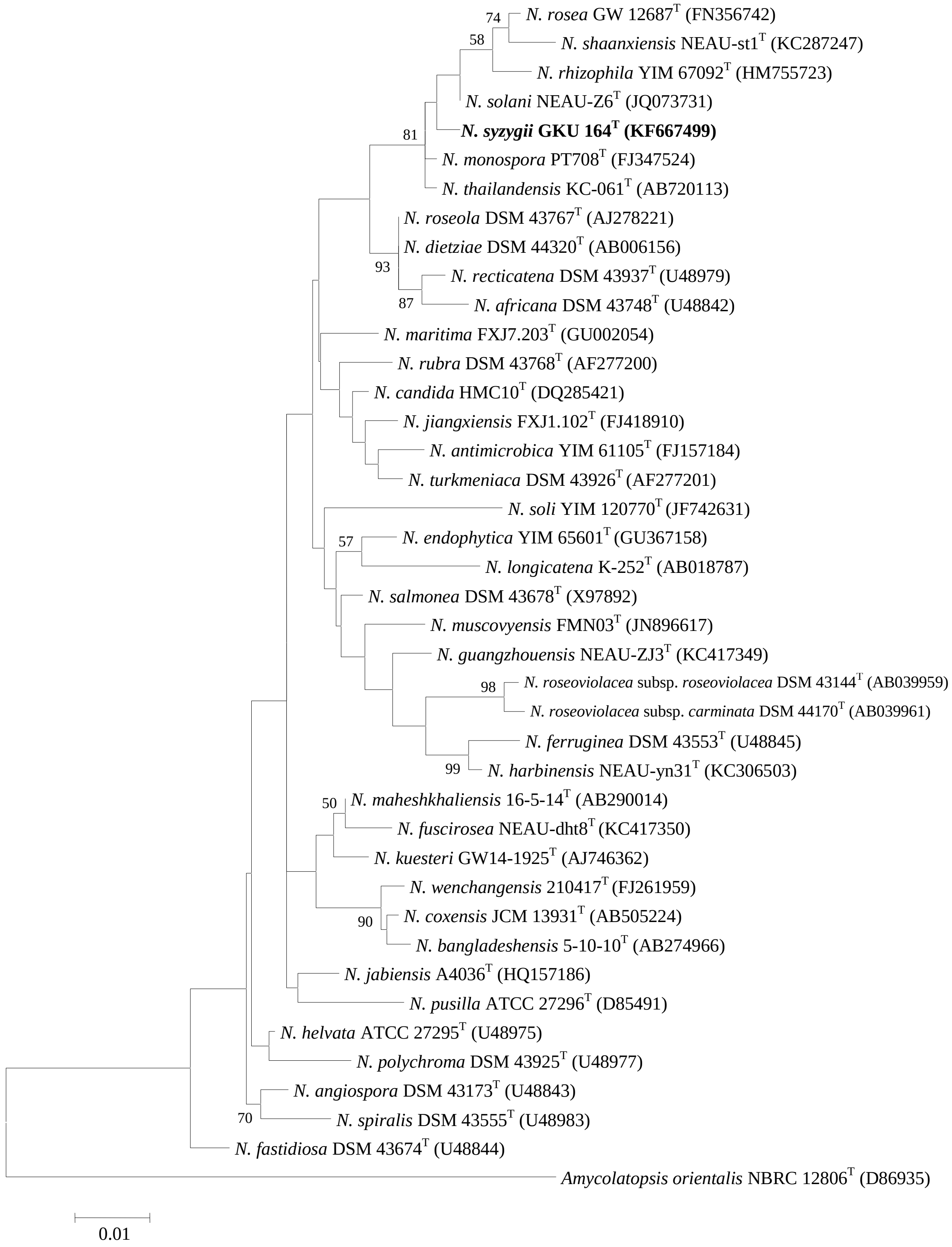


Figure 2 Maximum-likelihood tree based on almost complete 16S rRNA gene sequences showing the relationship between strain GKU 164^{T} and all recognized species of the genus *Nonomuraea*. *Amycolatopsis orientalis* NBRC 12806^{T} was used as an outgroup. Numbers at branch points indicate bootstrap percentage (based on 1,000 replications); only values above 50% are showed. Bar, 0.01 substitutions per nucleotide position.

3.1.2 Morphological and physiological characteristics

The morphological characteristics of strain GKU 164^T were consistent with those of members of the genus *Nonomuraea*. Aerial and substrate mycelia of strain GKU 164^T were developed without fragmentation. Sporangia were not found. Strain GKU 164^T showed good growth on ISP 2, ISP 3, ISP 4, MBA, NA and YS agar, but no growth on ISP 5. Substrate mycelia were abundantly produced on most of the media tested and the colors were variable depending on the growth medium (Table 5). No soluble pigment was detected on any of the media tested. Aerial mycelium was absent on most of the media except for Czapek's agar that poor growth was observed after incubation at 28 °C for 21 days. Non-motile spores were borne singly and the spore surface was rough (Fig. 3). The temperature and pH range for growth of strain GKU 164^T were observed at 10–36 °C and pH 5–9, with optimum growth at 18–32 °C and pH 7–8. Other physiological and biochemical characteristics of strain GKU 164^T were compared to closely related type species and revealed the differences from *N. monospora* PT708^T, *N. thailandensis* KC-061^T, *N. solani* NEAU-Z6^T, *N. rhizophila* YIM 67092^T and *N. shaanxiensis* NEAU-st1^T (Table 6). Strain GKU 164^T possesses single spore similar to those of *N. monospora* PT708^T, *N. solani* NEAU-Z6^T and *N. shaanxiensis* NEAU-st1^T but spore surface is rough while others are smooth.

Table 5 Growth and culture characteristics of strain GKU 164^T at 28 °C for 21 days.

Medium	Growth	Aerial mycelium	Substrate mycelium
ISP 2	Good	None	Oak brown
ISP 3	Good	None	Grayish red
ISP 4	Good	None	Olive brown
ISP 5	None	-	-
ISP 6	Moderate	None	Clay
ISP 7	Poor	None	Olive brown
MBA	Good	None	Light yellow
NA	Good	None	Clay
YS	Good	None	Topaz
CZA	Moderate	Yellow white	Grayish yellow

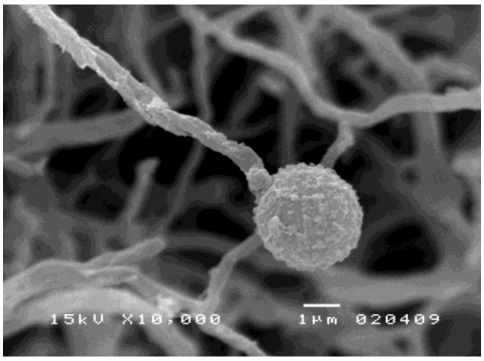


Figure 3 Scanning electron micrograph of the single spore on aerial mycelia of strain GKU 164^T grown on Czapek's agar at 28 °C for 30 days. Bar indicates 1 µm.

Table 6 Comparison of the phenotypic properties of strain GKU 164^T and closely related type species of the genus *Nonomuraea* with validly published names. Species: 1. GKU 164^T; 2. *N. monospora* PT708^T; 3. *N. thailandensis* KC-061^T; 4. *N. solani* NEAU-Z6^T; 5. *N. rhizophila* YIM 67092^T; 6. *N. shaanxiensis* NEAU-st1^T. Data for strains 1-3 are taken from this study. Data for strains 4-6 are from Wang *et al.* (2013), Zhao *et al.* (2011) and Zhang *et al.* (2014b), respectively. ND, no data available; +, positive; -, negative; w, weakly positive.

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**
Spore morphology						
Arrangement	Single	Single	Spiral	Single	Spiral	Single
Ornamentation	Rough	Smooth	Smooth	Smooth	Rough	Smooth
Number of spore	1	1	>10	1	7-10	1
Growth on ISP 3						
Aerial mycelium	None	Grayish brown	Reddish grey	White	White	None
Substrate mycelium	Grayish red	Cuba	Violet brown	White	Brown-yellow	Yellowish gray
Soluble pigment	None	Reddish brown	Brownish grey	None	None	None
Temperature growth (°C)	10-36	ND	14-38	20-39	10-37	18-28
pH range	5-9	5-9	5-9	7-9	6-8	6-9
NaCl tolerance (%)	3	3	3	2	7	2
Nitrate reduction	-	+	+	-	-	+
Starch hydrolysis	+	+	+	+	-	-
Gelatin liquefaction	+	w	-	+	-	+
Urease activity	-	-	-	-	+	-
Oxidase activity	+	+	+	-	-	-
Coagulation of milk	-	-	-	ND	+	+
Peptonization of milk	-	w	w	ND	+	ND
Decomposition of:						
Hypoxanthine	w	w	w	ND	+	ND
L-tyrosine	+	+	+	+	+	-
Utilization of:						
L-arabinose	-	+	+	+	-	-

Characteristic	**1**	**2**	**3**	**4**	**5**	**6**
D-cellobiose	-	+	+	ND	+	ND
Dulcitol	-	+	w	ND	ND	ND
D-fructose	-	+	w	+	+	-
Myo-inositol	-	-	w	+	+	+
Maltose	-	-	-	+	+	+
D-raffinose	-	+	w	+	+	-
D-sorbitol	-	+	-	+	-	+
Sucrose	-	+	+	+	-	+
D-xylose	-	+	-	+	-	-

3.1.3 Chemotaxonomic characteristics

Chemotaxonomic properties of strain GKU 164^T revealed typical characteristics of members of the genus *Nonomuraea*. The strain contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. Madurose, galactose, mannose, ribose, rhamnose and glucose were detected as the diagnostic sugars in the whole-cell hydrolysates. The presence of *meso*-diaminopimelic acid and madurose indicated that cell wall and whole cell sugar of strain GKU 164^T was of type III and type B, respectively (Lechevalier & Lechevalier, 1970). The *N*-acyl type of muramic acid in peptidoglycan was acetyl. Mycolic acids were not detected. The predominant menaquinone was MK-9(H_4) (76%), while the minor amount of MK-9(H_6) (10%), MK-9(H_2) (9%) and MK-9(H_0) (5%) were also detected. The diagnostic phospholipids were diphosphatidylglycerol (DPG), phosphatidyethanolamine (PE), hydroxy-phosphatidylethanolamine (OH-PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylinositolmannosides (PIMs), phosphatidylmonomethylethanolamine (PME), hydroxy-phosphatidylmonomethylethanolamine (OH-PME), an unidentified aminophosphoglycolipid (APGL) and four unknown phospholipids (PLs) (Fig. 4). Phospholipid pattern of strain GKU 164^T corresponded to type IV (Lechevalier *et al.*, 1977). The major cellular fatty acids were iso-$C_{16:0}$ (31.8%) and 10-methyl $C_{17:0}$ (25.0%) which were generally similar to those of recognized members of the genus *Nonomuraea* (Qin *et al.*, 2009; Li *et al.*, 2011; Zhao *et al.*, 2011; Nakaew *et al.*, 2012; Sripreechasak *et al.*, 2013). The genomic DNA G+C content of strain GKU 164^T was 70.4 mol%.

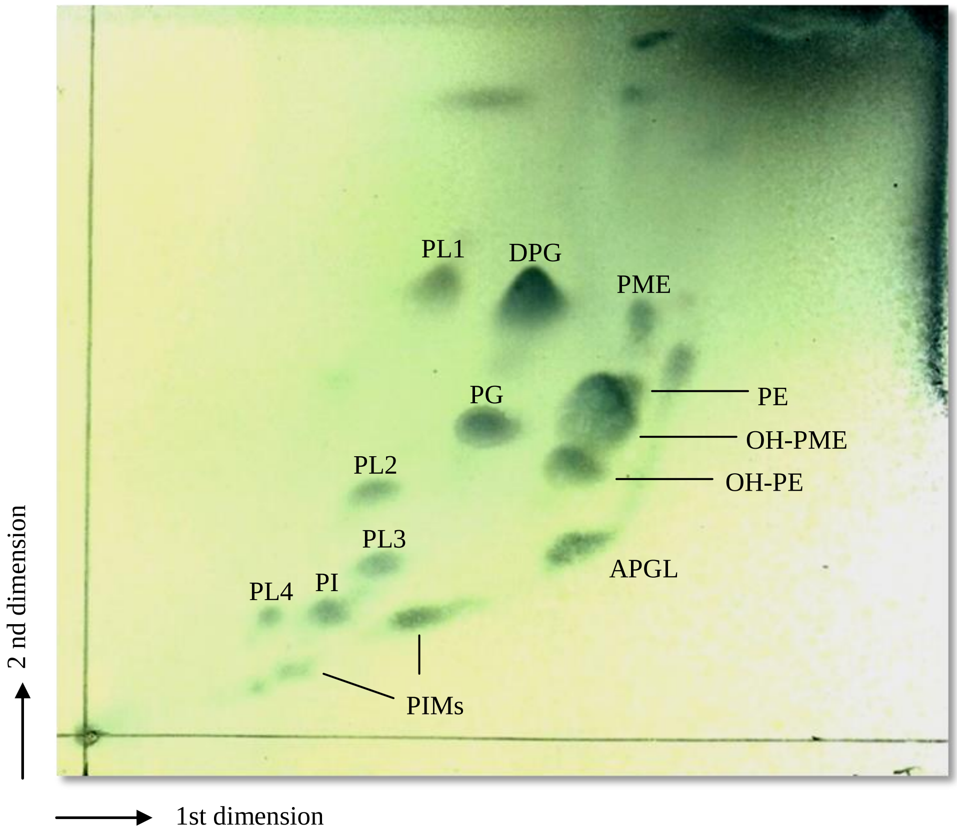


Figure 4 Phospholipid profile by two-dimensional TLC detected with phosphomolybdic acid of strain GKU 164^{T}. APGL, aminophosphoglycolipid that possible contains *N*-acetylglucosamine; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; OH-PE, hydroxy-phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIMs, phosphatidylinositolmannosides; PME, phosphatidylmonomethylethanolamine; OH-PME, hydroxy-phosphatidylmonomethylethanolamine and PLs (PL1, PL2, PL3 and PL4), unknown phospholipids.

3.1.4 DNA-DNA hybridisation

According to phenotypic and genotypic results, strain GKU 164^{T} was evidently different from the close related type species. Therefore, DNA-DNA hybridization between GKU 164^{T} and the closest strains, *N. monospora* PT708^{T} and *N. thailandensis* KC-061^{T} (Fig. 1) was fluorometrically determined using photobiotin-labelled DNA probes and microplate wells as described by Ezaki *et al.* (1989). The DNA-DNA relatedness values were examined from two independent determinations and revealed the values of 40.11±2.33% and 37.61±0.23%, respectively. The results clearly indicated that strain GKU 164^{T} was distinguished from *N. monospora* PT708^{T} and *N. thailandensis* KC-061^{T} because the values were below the recommended threshold value of 70 % for the definition of bacterial species (Wayne *et al*., 1987).

On the basis of the polyphasic data, strain GKU 164^T differs from the closely related type species of the genus *Nonomuraea*; therefore, strain GKU 164^T represents a novel species, for which the name *Nonomuraea syzygii* sp. nov. is proposed.

3.1.5 Description of *Nonomuraea syzygii* sp. nov.

Nonomuraea syzygii (sy.zy'gi.i. N.L. gen n. of *Syzygium cumini* L. Skeels, the jambolan plum tree from which the type strain was isolated)

Aerobic, Gram-stain-positive, non-acid-fast actinomycete that form branched, non-fragmenting substrate and aerial hyphae. Non-motile spores are borne singly on the aerial mycelia and the spore surface is rough. Sporangia are not detected. No soluble pigment is observed in any of the media tested. The optimal temperature for good growth occurs at 18–32 °C and pH at 7–8. The NaCl tolerance range for growth is up to 3% (w/v). Catalase, oxidase, DNase, gelatin liquefaction and starch hydrolysis are positive, but negative for urease, milk peptonization and coagulation, nitrate reduction, H_2S and melanin production. Casein, L-tyrosine and xanthine are degraded, while hypoxanthine is weakly, but citrate is not. D-glucose, inulin, D-mannitol, D-mannose, L-rhamnose and trehalose are utilized as the sole carbon sources, but L-arabinose, D-cellobiose, dulcitol, D-fructose, myo-inositol, β-lactose, maltose, D-raffinose, D-sorbitol, sucrose, xylitol and D-xylose are not. According to the API ZYM system, *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase (C4), esterase lipase (C8), α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, α-mannosidase, trypsin and valine arylamidase are positive; while, arylamidase, cysteine, α-fucosidase and naphthol-AS-BI-phosphohydrolase are weakly positive; but β-glucuronidase and lipase (C4) are negative. The diagnostic diamino acid in the cell-wall peptidoglycan is *meso*-diaminopimelic acid. The whole-cell hydrolysates contain madurose, galactose, mannose, ribose, rhamnose and glucose. The *N*-acyl type of muramic acid is acetyl. Mycolic acids are not detected. The predominant menaquinone is MK-9(H_4), while the minor amount of MK-9(H_6), MK-9(H_2) and MK-9(H_0) are also present. The phospholipid profile consists of diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), hydroxy-phosphatidylethanolamine (OH-PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylinositolmannosides (PIMs), phosphatidylmonomethylethanolamine (PME), hydroxy-phosphatidylmonomethylethanolamine (OH-PME), an unidentified aminophosphoglycolipid (APGL) and four unknown phospholipids (PLs). The major fatty acids are iso-$C_{16:0}$ and 10-methyl $C_{17:0}$. The genomic DNA G+C content of strain GKU 164^T is 70.4 mol%.

The type strain, GKU 164^T (= BCC 70457^T = NBRC 110400^T), was isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels) collected at Khao Khitchakut National Park, Chantaburi province, Thailand.

3.2 *Actinomadura syzygii* sp. nov. isolated from jambolan plum tree (*Syzygium cumini* L. Skeels)

3.2.1 16S rRNA gene analysis

The almost complete 16S rRNA gene sequence (1507 nt) of strain GKU 157^T was compared with corresponding sequences in public database by EzTaxon-e server. The results indicated that strain GKU 157^T was a member of the genus *Actinomadura*. The type strains showed the highest similarities were *A. chibensis* NBRC 106107^T (98.6 % 16S rRNA gene sequence similarity;20 nt difference at 1440), *Actinomadura bangladeshensis* 3-46-b3^T (98.5 %; 22 nt difference at 1455), *Actinomadura meyerae* A288^T (98.2 %; 26 nt difference at 1436), *A. geliboluensis* A8036^T (98.2 %; 27 nt difference at 1464), *Actinomadura chokoriensis* 3-45-a/11^T (98 %; 29 nt difference at 1459). However, the neighbour-joining and maximum likelihood trees indicated that strain GKU 157^T fell in a lineage with *A. chibensis* NBRC 106107^T, *A. pelletieri* NBRC 103052^T (97.9 %; 31 nt difference at ,444) and *A. meridiana* JCM 17440^T (96.9 %; 44 nt difference at 1430) (Fig. 5 and 6).

Figure 5 Neighbour-joining tree based on almost complete 16S rRNA gene sequences (1,260 nt) showing the relationship between strain GKU 157^T and all recognized species of the genus *Actinomadura*. *Nonomuraea pusilla* ATCC 27296^T (GenBank acession no. D85491) was used as an outgroup. Asterisks denote branches that were also recovered from maximum-likelihood tree. Numbers at branch points indicate bootstrap percentage (based on 1,000 replications); only values > 50 % are showed. Bar, 0.005 substitutions per nucleotide position.

Figure 6 Maximum-likelihood tree based on almost complete 16S rRNA gene sequences (1,260 nt) showing the relationship between strain GKU 157^T and all recognized species of the genus *Actinomadura*. *Nonomuraea pusilla* ATCC 27296^T (GenBank acession no. D85491) was used as an outgroup. Numbers at branch points indicate bootstrap percentage (based on 1,000 replications); only values > 50 % are showed. Bar, 0.005 substitutions per nucleotide position.

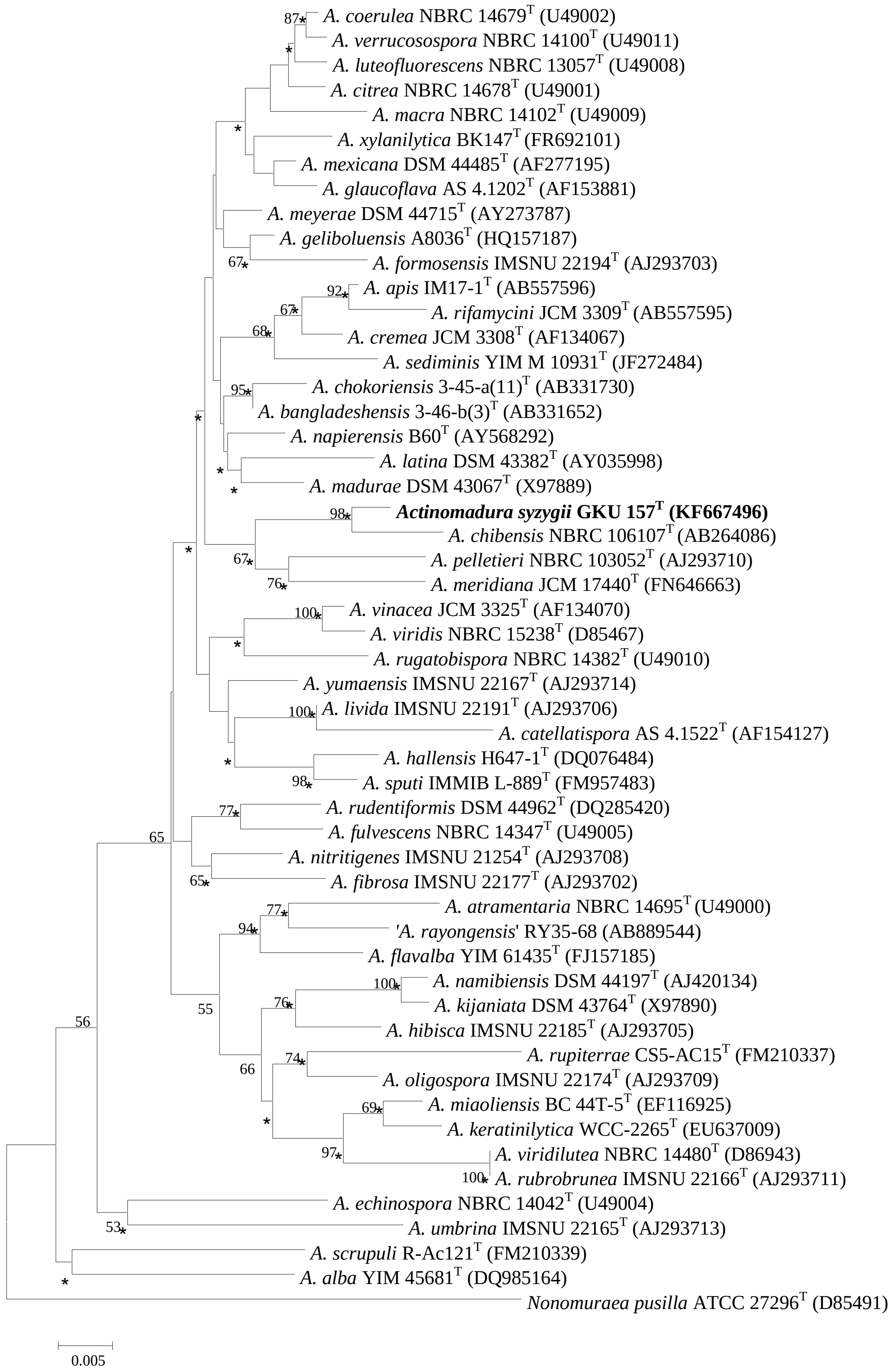
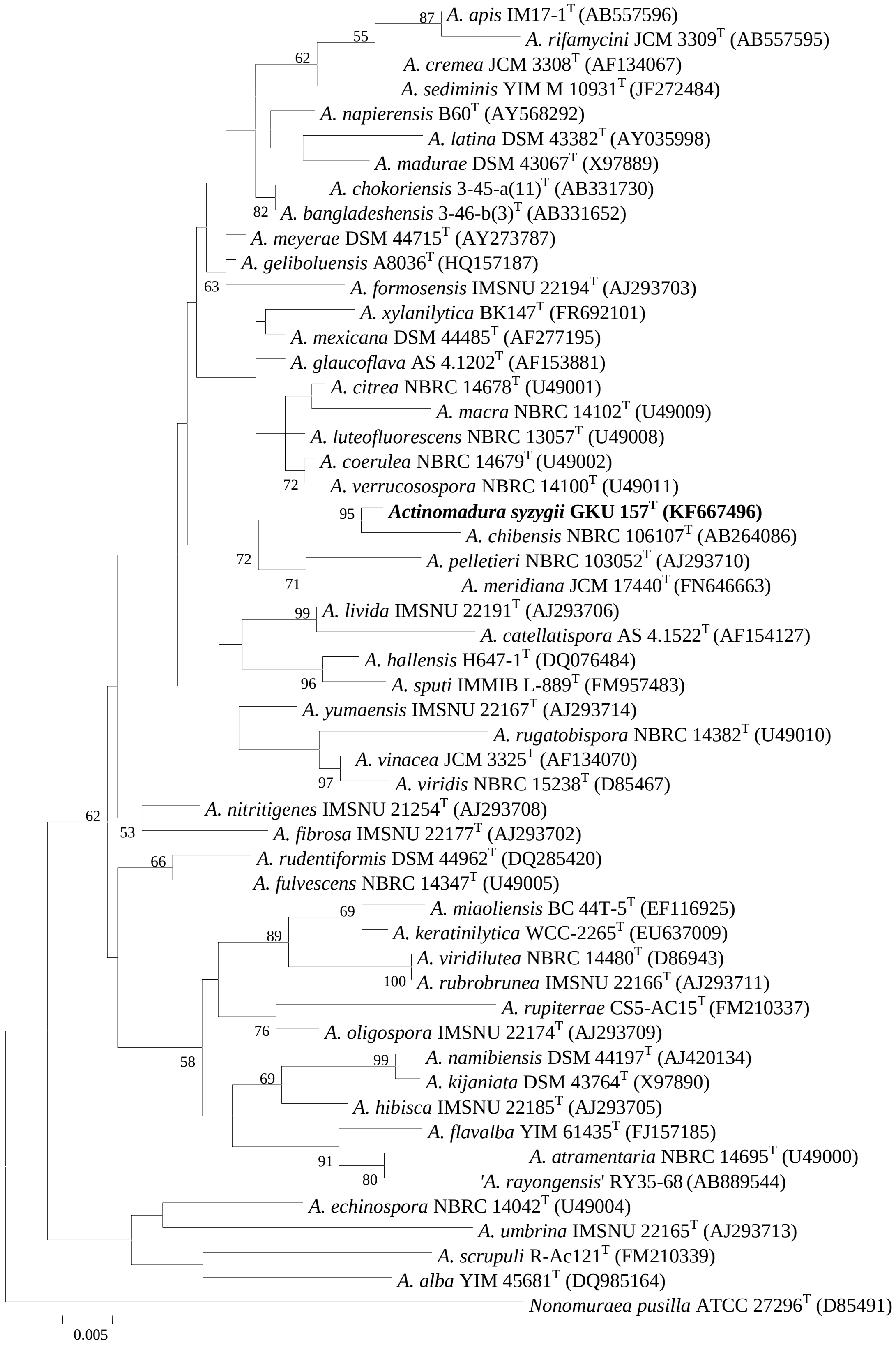

87
A. coerulea NBRC 14679^{T} (U49002)
A. verrucosospora NBRC 14100^{T} (U49011)
A. luteofluorescens NBRC 13057^{T} (U49008)
A. citrea NBRC 14678^{T} (U49001)
A. macra NBRC 14102^{T} (U49009)
A. xylanilytica BK147^{T} (FR692101)
A. mexicana DSM 44485^{T} (AF277195)
A. glaucoflava AS 4.1202^{T} (AF153881)
A. meyerae DSM 44715^{T} (AY273787)
A. geliboluensis A8036^{T} (HQ157187)
67
A. formosensis IMSNU 22194^{T} (AJ293703)
92
A. apis IM17-1^{T} (AB557596)
67
A. rifamycini JCM 3309^{T} (AB557595)
68
A. cremea JCM 3308^{T} (AF134067)
A. sediminis YIM M 10931^{T} (JF272484)
95
A. chokoriensis 3-45-a(11)^{T} (AB331730)
A. bangladeshensis 3-46-b(3)^{T} (AB331652)
A. napierensis B60^{T} (AY568292)
A. latina DSM 43382^{T} (AY035998)
A. madurae DSM 43067^{T} (X97889)
98
Actinomadura syzygii GKU 157^{T} (KF667496)
A. chibensis NBRC 106107^{T} (AB264086)
67
A. pelletieri NBRC 103052^{T} (AJ293710)
76
A. meridiana JCM 17440^{T} (FN646663)
100
A. vinacea JCM 3325^{T} (AF134070)
A. viridis NBRC 15238^{T} (D85467)
A. rugatobispora NBRC 14382^{T} (U49010)
A. yumaensis IMSNU 22167^{T} (AJ293714)
100
A. livida IMSNU 22191^{T} (AJ293706)
A. catellatispora AS 4.1522^{T} (AF154127)
A. hallensis H647-1^{T} (DQ076484)
98
A. sputi IMMIB L-889^{T} (FM957483)
77
A. rudentiformis DSM 44962^{T} (DQ285420)
A. fulvescens NBRC 14347^{T} (U49005)
65
A. nitritigenes IMSNU 21254^{T} (AJ293708)
65
A. fibrosa IMSNU 22177^{T} (AJ293702)
77
A. atramentaria NBRC 14695^{T} (U49000)
94
'A. rayongensis' RY35-68 (AB889544)
A. flavalba YIM 61435^{T} (FJ157185)
100
A. namibiensis DSM 44197^{T} (AJ420134)
76
A. kijaniata DSM 43764^{T} (X97890)
55
A. hibisca IMSNU 22185^{T} (AJ293705)
56
74
A. rupiterrae CS5-AC15^{T} (FM210337)
66
A. oligospora IMSNU 22174^{T} (AJ293709)
69
A. miaoliensis BC 44T-5^{T} (EF116925)
A. keratinilytica WCC-2265^{T} (EU637009)
97
A. viridilutea NBRC 14480^{T} (D86943)
100
A. rubrobrunea IMSNU 22166^{T} (AJ293711)
A. echinospora NBRC 14042^{T} (U49004)
53
A. umbrina IMSNU 22165^{T} (AJ293713)
A. scrupuli R-Ac121^{T} (FM210339)
A. alba YIM 45681^{T} (DQ985164)
Nonomuraea pusilla ATCC 27296^{T} (D85491)
0.005

Figure 5


87
A. apis IM17-1^{T} (AB557596)
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62
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A. madurae DSM 43067^{T} (X97889)
A. chokoriensis 3-45-a(11)^{T} (AB331730)
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A. bangladeshensis 3-46-b(3)^{T} (AB331652)
A. meyerae DSM 44715^{T} (AY273787)
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63
A. formosensis IMSNU 22194^{T} (AJ293703)
A. xylanilytica BK147^{T} (FR692101)
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A. glaucoflava AS 4.1202^{T} (AF153881)
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A. macra NBRC 14102^{T} (U49009)
A. luteofluorescens NBRC 13057^{T} (U49008)
A. coerulea NBRC 14679^{T} (U49002)
72
A. verrucosospora NBRC 14100^{T} (U49011)
95
Actinomadura syzygii GKU 157^{T} (KF667496)
A. chibensis NBRC 106107^{T} (AB264086)
72
A. pelletieri NBRC 103052^{T} (AJ293710)
71
A. meridiana JCM 17440^{T} (FN646663)
99
A. livida IMSNU 22191^{T} (AJ293706)
A. catellatispora AS 4.1522^{T} (AF154127)
A. hallensis H647-1^{T} (DQ076484)
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A. sputi IMMIB L-889^{T} (FM957483)
A. yumaensis IMSNU 22167^{T} (AJ293714)
A. rugatobispora NBRC 14382^{T} (U49010)
A. vinacea JCM 3325^{T} (AF134070)
97
A. viridis NBRC 15238^{T} (D85467)
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A. nitritigenes IMSNU 21254^{T} (AJ293708)
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A. fibrosa IMSNU 22177^{T} (AJ293702)
66
A. rudentiformis DSM 44962^{T} (DQ285420)
A. fulvescens NBRC 14347^{T} (U49005)
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A. miaoliensis BC 44T-5^{T} (EF116925)
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A. keratinilytica WCC-2265^{T} (EU637009)
A. viridilutea NBRC 14480^{T} (D86943)
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A. rubrobrunea IMSNU 22166^{T} (AJ293711)
A. rupiterrae CS5-AC15^{T} (FM210337)
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A. hibisca IMSNU 22185^{T} (AJ293705)
A. flavalba YIM 61435^{T} (FJ157185)
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A. atramentaria NBRC 14695^{T} (U49000)
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A. echinospora NBRC 14042^{T} (U49004)
A. umbrina IMSNU 22165^{T} (AJ293713)
A. scrupuli R-Ac121^{T} (FM210339)
A. alba YIM 45681^{T} (DQ985164)
Nonomuraea pusilla ATCC 27296^{T} (D85491)
0.005

Figure 6

3.2.2 Morphological and physiological characteristics

Strain GKU 157^T grew well on all of the media tested (Table 7 and 8). Substrate mycelia were well developed and the colors were variable depending on the growth medium. Aerial mycelia were observed on ISP 2, ISP 3, ISP 4 and oatmeal-nitrate agar. Pale pink aerial mycelium was found on ISP 2, ISP 3 and ISP 4 media, with abundant sporulation on ISP 4 medium. No soluble pigments were produced on any of the media tested. Strain GKU 157^T produced abundantly branched, non-fragmenting substrate mycelium and aerial hyphae, which differentiated into spore chains. Hooked to short spiral (1 turn) spore chains consisted of about 20 non-motile spores with warty surface (Fig. 7). The temperature and pH range for growth of strain GKU 157^T were observed at 14–40 °C and pH 6–11, with optimum growth at 20–34 °C and pH 8–10. Strain GKU 157^T could be differentiated from its phylogenetically closely related type strains by culture characteristics, pH and NaCl for growth tolerance, acid production from carbohydrates, gelatin liquefaction, coagulation and peptonization of milk, H_2S production, utilization of carbon sources and enzyme activities (Table 7 and 8). Additional phenotypic properties of strain GKU 157^T are presented in the species description.

Table 7 Comparison of the phenotypic properties of strain GKU 157^T and closely related type species of the genus *Actinomadura* with validly published names. Species: 1, GKU 157^T; 2, *A. chibensis* NBRC 106107^T; 3, *A. pelletieri* NBRC 103052^T; 4, *A. meridiana* JCM 17440^T. All data were obtained in this study. +, positive; -, negative; w, weakly positive.

Characteristic	**1**	**2**	**3**	**4**
Spore morphology				
Arrangement	Hooked to spiral	Hooked to spiral	Hooked to spiral	Hooked
Number of spore (per chain)	About 20	About 20	2-6	About 20
Colony characteristics on ISP 2 r				
Growth	Good	Good	Good	Moderate
Aerial mycelium	Pale pink	White	None	None
Substrate mycelium	Light coral red	Mustard	Light coral rose	Bamboo
Diffusible pigment	None	Amber	None	Golden brown
Growth at pH 11	+	+	+	-
Growth at 5_% Na-Cl (w/v)	-	+	-	-
Catalase activity	w	+	+	+
Oxidase activity	+	+	w	+
Acid production from:				
Adonitol	-	-	+	-
D-cellobiose	-	+	-	-

Characteristic	1	2	3	4
D-glucose	+	+	-	+
D-mannose	-	-	-	+
L-rhamnose	-	+	-	-
Gelatin liquefaction	-	+	-	-
Milk coagulation	-	w	+	+
Milk peptonization	-	w	+	+
H_2S production	-	-	-	+
Hypoxanthine decomposition	-	w	-	-
Utilization of:				
D-cellobiose	+	+	-	-
D-mannose	-	-	-	+
D-sorbitol	-	+	-	-
D-trehalose	+	w	w	+
Dulcitol	-	w	-	-
L-rhamnose	-	+	-	-
Maltose	w	-	-	-
Enzyme activities of:				
α-glucosidase	+	+	+	-
β-glucosidase	-	+	-	-

Table 8 Additional culture characteristics of strain GKU 157^T and closely related type species of the genus *Actinomadura* after incubation at 27°C for 21 days. Species: 1, GKU 157^T; 2, *A. chibensis* NBRC 106107^T; 3, *A. pelletieri* NBRC 103052^T; 4, *A. meridiana* JCM 17440^T. All data were obtained in this study.

Medium	Growth and characteristics	1	2	3	4
ISP 3	Growth	Good	Good	Poor	Moderate
	Aerial mycelium	Pale pink	White	None	None
	Substrate mycelium	Bright coral rose	Light wheat	Light apricot	Light beige
	Diffusible pigment	None	None	None	None
ISP 4	Growth	Good	Good	Poor	Good
	Aerial mycelium	Pale pink	Cream	None	None
	Substrate mycelium	Pale peach	Light citron gray	Pastel pink	Pale yellow
	Diffusible pigment	None	None	None	None
ISP 5	Growth	Good	Good	Poor	Moderate
	Aerial mycelium	None	White	None	None
	Substrate mycelium	Bamboo	Light beige	Light apricot	Oatmeal

Medium	Growth and characteristics	1	2	3	4
	Diffusible pigment	None	Bright yellow	None	None
ISP 6	Growth	Good	Good	Moderate	Moderate
	Aerial mycelium	None	None	None	None
	Substrate mycelium	Orange	Bamboo	Mustard	Light mustard tan
	Diffusible pigment	None	None	None	None
ISP 7	Growth	Good	Good	Moderate	Good
	Aerial mycelium	None	Eggshell	None	None
	Substrate mycelium	Light apricot	Oatmeal	Baby pink	Light melon yellow
	Diffusible pigment	None	Bright maize	None	None
GYM	Growth	Good	Good	Good	Good
	Aerial mycelium	None	Light rose beige	None	None
	Substrate mycelium	Light coral red	Cocoa brown	Bright cherry red	Seal brown
	Diffusible pigment	None	Bright orange	None	Amber
MBA	Growth	Good	Good	Good	Good
	Aerial mycelium	None	None	None	None
	Substrate mycelium	Russet orange	Light beige	Oatmeal	Tan
	Diffusible pigment	None	None	None	None
Oatmeal-agar	Growth	Good	Good	Good	Good
	Aerial mycelium	Pale peach	Oyster white	None	White
	Substrate mycelium	Baby pink	Oyster white	Oyster white	Oyster white
	Diffusible pigment	None	None	None	None

Figure 7 Scanning electron micrograph of hooked to short spiral spore chains with warty surface of strain GKU 157^T grown on ISP 4 medium at 28 °C for 21 days. Bar, 1 μm.

3.2.3 Chemotaxonomic characteristics

Chemotaxonomic analyses revealed that strain GKU 157^T exhibited characteristics which were typical of members of the genus *Actinomadura*. The strain contained *meso*-diaminopimelic acid as the dignostic diamino acid and whole-cell sugars were galactose, glucose, madurose, mannose and ribose showing that its possessed cell-wall type III (Lechevalier & Lechevalier, 1970b) and whole-cell sugar pattern B (Lechevalier, 1968). The *N*-acyl type of muramic acid in peptidoglycan was acetyl. Mycolic acids were absent. The predominant menaquinone was MK-9(H_6) (69 %), while the minor amount of MK-9(H_4) (14 %), MK-9(H_8) (11 %), MK-9(H_2) (5 %) and MK-9(H_0) (1 %) were also detected. The diagnostic phospholipids were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylinositolmannoside (PIM) and two unknown phospholipids (PLs) (Fig. 8), corresponded to phospholipid type I (Lechevalier *et al.*, 1977). The major cellular fatty acids were $C_{16:0}$ (29.5 %), iso-$C_{16:0}$ (19.7 %), $C_{18:1}$ ω9c (9.5 %), $C_{18:0}$ (7.4 %), 10-methyl $C_{18:0}$ (tuberculostearic acid; 6.9 %), corresponded to fatty acid type 3a (Kroppenstedt *et al.*, 1985); while the minor amount of a summed feature 3 ($C_{16:0}$ ω7c and/or $C_{16:1}$ ω6c; 4.5 %), $C_{17:1}$ ω8c (4.1 %), $C_{17:0}$ (3.9 %), a summed feature 9 (10-methyl $C_{16:0}$ and/or iso-$C_{17:1}$ ω9c; 2.4 %), iso-$C_{18:0}$ (2.4 %), a summed feature 8 ($C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c; 1.9 %), $C_{17:1}$ ω6c (1.7 %), $C_{18:3}$ ω6c (1.7 %) and $C_{14:0}$ (1.6 %) were also presented. The genomic DNA G+C content was 73.1 mol%.

3.2.4 DNA-DNA hybridisation

Comparison of genotypical, morphological and phenotypical characteristics of strain GKU 157^T to its closest phylogenetically neighbors evidently distinguished strain GKU 157^T from the others (Table 7 and 8). Strain GKU 157^T harbored long spore chain with warty surface similar to those of *A. chibensis* NBRC 106107^T and *A. meridiana* JCM 17440^T, while *A. pelletieri* NBRC 103052^T carried short spore chain. However, similarity of 16S rRNA gene sequences between strain GKU 157^T and *A. meridiana* JCM 17440^T and *A. pelletieri* NBRC 103052^T were lower (97.9 % and 96.9 %, respectively) than that of *A. chibensis* NBRC 106107^T (98.6 %). Taken together of 16S rRNA gene sequencing and phylogenetic analyses, strain GKU 157^T was mostly related to *A. chibensis* NBRC 106107^T with 98 % and 95 % boothstrap values of neighbour-joining and maximum likelihood algorithms supported, respectively (Fig. 5 and 6). Therefore, the validity of a novel species status of strain GKU 157^T was confirmed by DNA–DNA hybridization by fluorometrically determined using photobiotin-labelled DNA probes and microplate wells (Ezaki *et al.*, 1989). Level of DNA–DNA relatedness value between strain GKU 157^T and *A. chibensis* NBRC 106107^T was examined from two independent determinations and revealed 48.8±2.1% which was clearly below the 70 % value considered to be the threshold for the definition of bacterial species (Wayne *et al.*, 1987). Based on all evidences, strain GKU 157^T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura syzygii* sp. nov. is proposed.

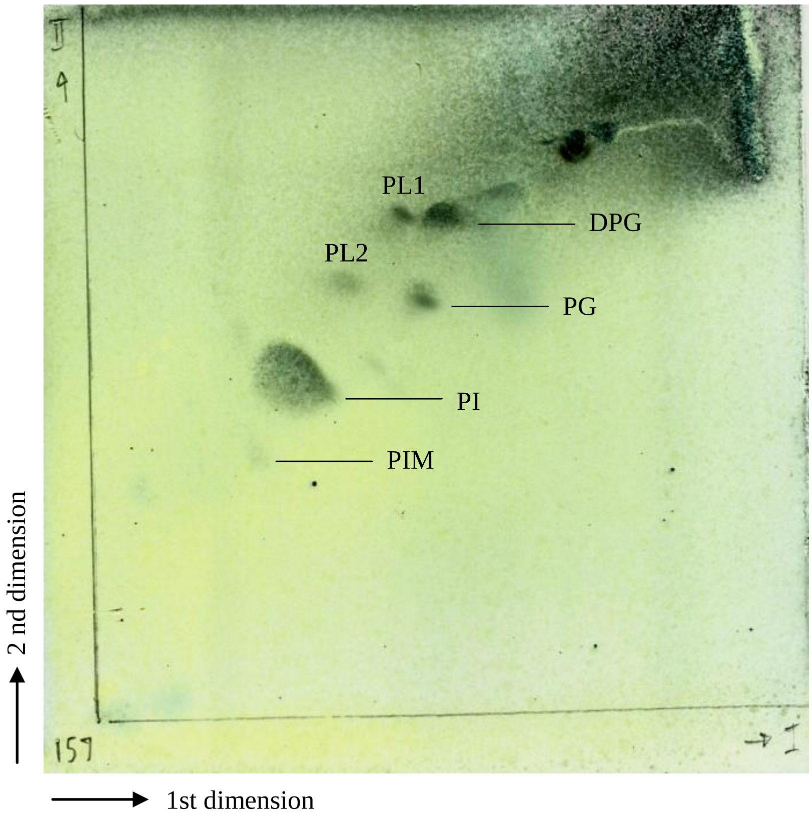


Figure 8 Phospholipid profile by two-dimensional TLC detected with phosphomolybdic acid of strain GKU 157^T. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositolmannoside and PLs (PL1 and PL2), unknown phospholipids.

3.2.5 Description of *Actinomadura syzygii* sp. nov.

Actinomadura syzygii (sy.zy'gi.i. N.L. gen. n. syzygii of *Syzygium cumini* L. Skeels, the jambolan plum tree from which the type strain was isolated).

Aerobic, Gram-stain-positive, non-acid-alcohol-fast, non-motile actinomycete that forms an extensively branched, non-fragmenting substrate and aerial mycelium. Abundant aerial mycelium is present on ISP 4 medium, which differentiated into hooked to short spiral chains about 20 non-motile spores with warty surface. No soluble pigment is produced on any of the media tested. The optimal temperature for good growth occurs at 20–34 °C and pH at 8–10. The NaCl tolerance range for growth is up to 4 % (w/v). Oxidase, DNase and nitrate reduction are positive, while catalase is weakly positive, but urease, citrate utilization, gelatin liquefaction, milk coagulation and peptonization, H_2S and melanin production are negative. Acid is produced from D-glucose, but not from adonitol, D-cellobiose, D-galactose, D-mannose, D-sorbitol, L-arabinose, L-rhamnose and maltose. Starch, casein and L-tyrosine are degraded, but xanthine and hypoxanthine are not. D-cellobiose, D-glucose and D-trehalose are utilized as the sole carbon sources, while maltose is weakly utilized, but D-mannose, D-raffinose, D-sorbitol, D-xylose, dulcitol, inulin, L-arabinose, L-rhamnose, myo-inositol and sucrose are not. According to the API ZYM system, acid phosphatase, alkaline phosphatase, cysteine arylamidase, esterase (C4), esterase lipase (C8), leucine arylamidase, lipase (C4), *N*-acetyl-*β*-glucosaminidase, naphthol-AS-BI-phosphohydrolase, trypsin, valine arylamidase, *α*-chymotrypsin and *α*-glucosidase are positive, but *α*-fucosidase, *α*-galactosidase, *α*-mannosidase, *β*-galactosidase, *β*-glucosidase and *β*-glucuronidase are negative. The cell-wall contains *meso*-diaminopimelic acid and the whole-cell sugars are galactose, glucose. madurose, mannose and ribose. The *N*-acyl type of muramic acid is acetyl. Mycolic acids are absent. The predominant menaquinone is MK-9(H_6) with the minor amounts of MK-9(H_4), MK-9(H_8), MK-9(H_2) and MK-9(H_0). The phospholipid profile contains phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylinositolmannoside (PIM) and two unknown phospholipids (PLs). The major cellular fatty acids are $C_{16:0}$, iso-$C_{16:0}$, $C_{18:1}$ ω9c, $C_{18:0}$, 10-methyl $C_{18:0}$ (tuberculostearic acid). The genomic DNA G+C content of the type strain is 73.1 mol%.

The type strain, GKU 157^T (= BCC 70456^T = NBRC 110399^T), was isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels) collected at Khao Khitchakut National Park, Chantaburi province, Thailand.

4. Localisation and visualization of endophytic actinomycetes in plant

4.1 Intergeneric conjugation efficiency

pIJ10257 was successfully transferred *Streptomyces* sp. GMKU944 by intergeneric conjugation between *E. coli* ET12567/(pUZ8002/pIJ10257) and mycelia of the strain GMKU944. Highest conjugation frequency for *Streptomyces* sp. GMKU944 was obtained when mycelium from 24-h-old culture. (10^{-3} per CFU, Table 9). Conjugation frequencies were slightly decreased when used 36-h-old and 48-h-old culture (10^{-3}-10^{-7} per CFU, Table 9).

We also determined the effect of media on conjugation efficiency. We found that the MS supplemented with 10 mmol/l $MgCl_2$ gave highest conjugation frequency (1.35×10^{-3} per CFU, Table 9). The result was in agreement with recently report that MS supplemented with 10 mmol/l $MgCl_2$ has been used as conjugative medium for several *Streptomyces* species (Flett *et* *al*., 1997; Fouces *et al*., 2000; Kitani *et al*., 2000; Nikodinovic *et al*., 2003; Paranthaman and Dharmalingum, 2003; Enŕiquez *et al*., 2006). In this work, conjugal transfer on MS supplemented with 10 mmol/l $MgCl_2$ was chosen for conjugal transfer of *Streptomyces* sp. GMKU944 as it gave the highest conjugation efficiency.

Table 9 Effect of mycelium growth of *Streptomyces* sp. GMKU944 on conjugation efficiency with *E. coli* ET12567 (pUZ8002 /pIJ10257)

Time (h)	Conjugation frequency*		
	MS + 10 mM $MgCl_2$	ISP4 + 10 mM $MgCl_2$	TSA + 10 mM $MgCl_2$
24	1.35×10^{-3}	2.18×10^{-3}	1.58×10^{-3}
36	2.17×10^{-3}	3.67×10^{-3}	3.40×10^{-3}
48	6.10×10^{-3}	3.66×10^{-4}	5.15×10^{-5}

*Values are presented as the number of transconjugants per colony forming units (CFU) of recipient, and each value represents the average efficiency from three independent experiments.

4.2 Verification of the ex-conjugants

The presence of pIJ10257*egfp* in ex-conjugants was determined by PCR of a 1.5 kb fragment of *egfp* gene (Fig. 9). The result indicated that the ex-conjugants were real. Moreover, the presence of chomosomally integrated plasmid in *Streptomyces* sp. GMKU944 did not effect on its phenotype, the ex-conjugants showed phenotypic characteristics including colony morphology, spore formation and growth rate as same as the wild type strain. When visualized under fluorescent microscope, the ex-conjugants showed high level of fluorescence (Figure 10). It indicated that *egfp* gene driving by constitutive *ermE* promoter could express in *Streptomyces* sp. GMKU944.

4.3 Validation of endophytic property

Streptomyces sp. GMKU 944/*egfp* alone and the mixture with *Rhizobium* sp. were re-inoculated into germinated seeds of wattle tree. The results showed that *Streptomyces* sp. GMKU 944/*egfp* alone could not induce nodulation compared to mixed culture inoculation with *Rhizobium* sp. (Figure 11). Furthermore, the endophyte revealed no effect to growth and nodulation of wattle tree. The result was in agreement with Trujillo *et al*. (2010) who reported that endophytic actinomycete, *Micromonospora* spp., gave no effect to nodulation on *Lupinus angustifolius*. No symptom was detected in all plantlets which indicated that *Streptomyces* sp. GMKU 944 was not pathogenic bacteria. In addition, *Streptomyces* sp. GMKU 944 could be re-isolated from the roots of wattle tree suggested that it was indeed true endophyte. The absence of actinomycetes in non-inoculated seedlings indicated that these seeds did not contain endophytic actinomycetes, therefore, they may be exogenous and obtained directly from soil.

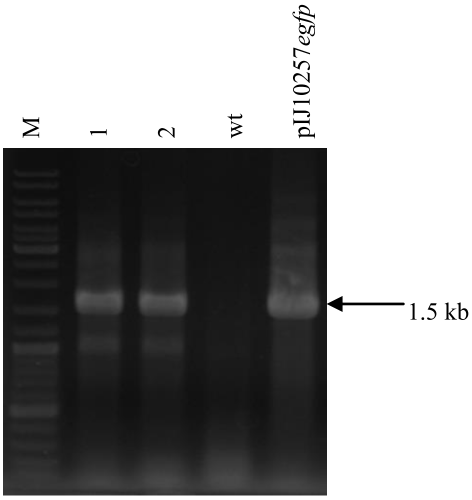


Figure 9 Verification of ex-conjugants by PCR with 10257seqF and 10257 seqR primers. Lane M, 1 kb ladder mix (Fermentus); lane 1-2, ex-conjugants; lane wt, wild type strain; lane pIJ10257*egfp*, pIJ10257*egfp* plasmid DNA.

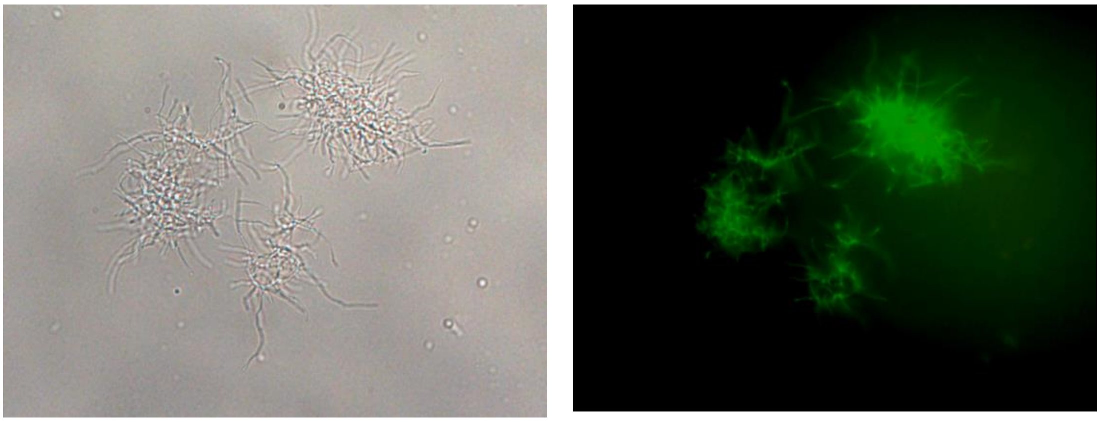


Figure 10 The ex-conjugant of EGFP-tagged *Streptomyces* sp. GMKU944 expressing enhanced green fluorescent protein (EGFP). Cells growing in ISP2 broth at 3 days were visualized using fluorescent microscope (magnificent 400x).

4.4 Localization of endophytic actinomycete in root tissues

Two month old plantlets inoculated with eGFP-tagged *Streptomyces* sp. GMKU944 were cut using Cryo microtome. The sections were visualized under laser scanning confocal microscope with excitation wavelength at 465 to 495 nm which gave emission wavelength at 515 to 555 nm (for eGFP detection). The results showed that the fluorescence could be observed on internal plant structure especially cell wall of plant which indicated the auto-fluorescent of the plant tissue. The problem was overcome by merging of two micrographs of eGFP detection (show in green) and plant auto-fluorescence detection (an excitation wavelength of 420 to 460 nm; show in red; which the pure culture of eGFP-tagged strain shown no fluorescence detection; micrograph not shown). The merged micrograph revealed micro-colonies of eGFP-tagged *Streptomyces* sp. GMKU944 appeared mainly at phoem parenchyma cells and some at xylem parenchyma cells (Figure 12).

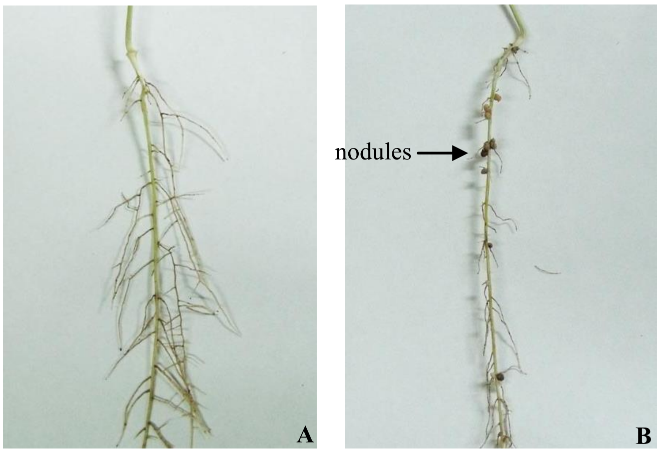


Figure 11 Two month old inoculated wattle tree plantlets after inoculated with *Streptomyces* sp. GMKU944/*egfp* alone (A) and with mixture of *Streptomyces* sp. GMKU944/*egfp* and *Rhizobium* sp. DASA36075 (B).

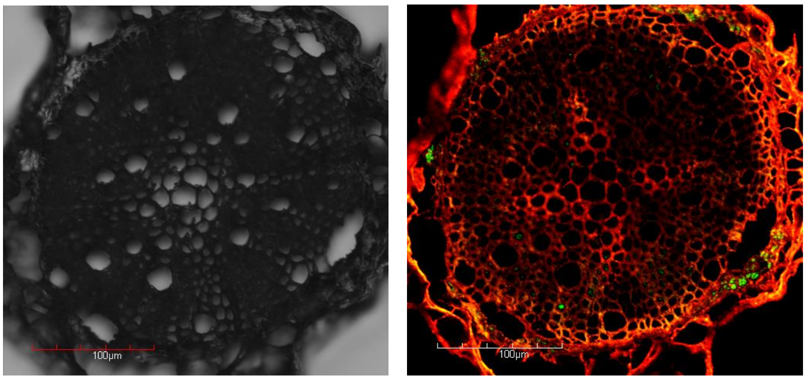


Figure 12 The root section of eGFP-tagged *Streptomyces* sp. GMKU944 inoculated plantlets of wattle tree visualized under laser scanning confocal microscope with phase-contrast field (A).

The merge of two micrographs of eGFP detection (shown in green) and plant autofluorescence (shown in red) (B). Bar 100 um.

4.5 Colonization of endophytic actinomycete on root surface

SEM micrographs revealed that presence of filamentous bacteria in the surface of nodule and root structure of 2 month old plantlets of wattle tree which inoculated with *Streptomyces* sp. GMKU944 and *Rhizobium* sp. DASA36075 (Figure 13 and 14). The hyphal size (~1µm) and sporulated structure of *Streptomyces* sp. GMKU944 was observed (Figure 13). The hyphal of *Streptomyces* sp. GMKU944 were also observed inside the crushed root tissue (Figure 14). SEM and confocal micrographs suggested that this endophytic actinomycete is closely associated with plant.

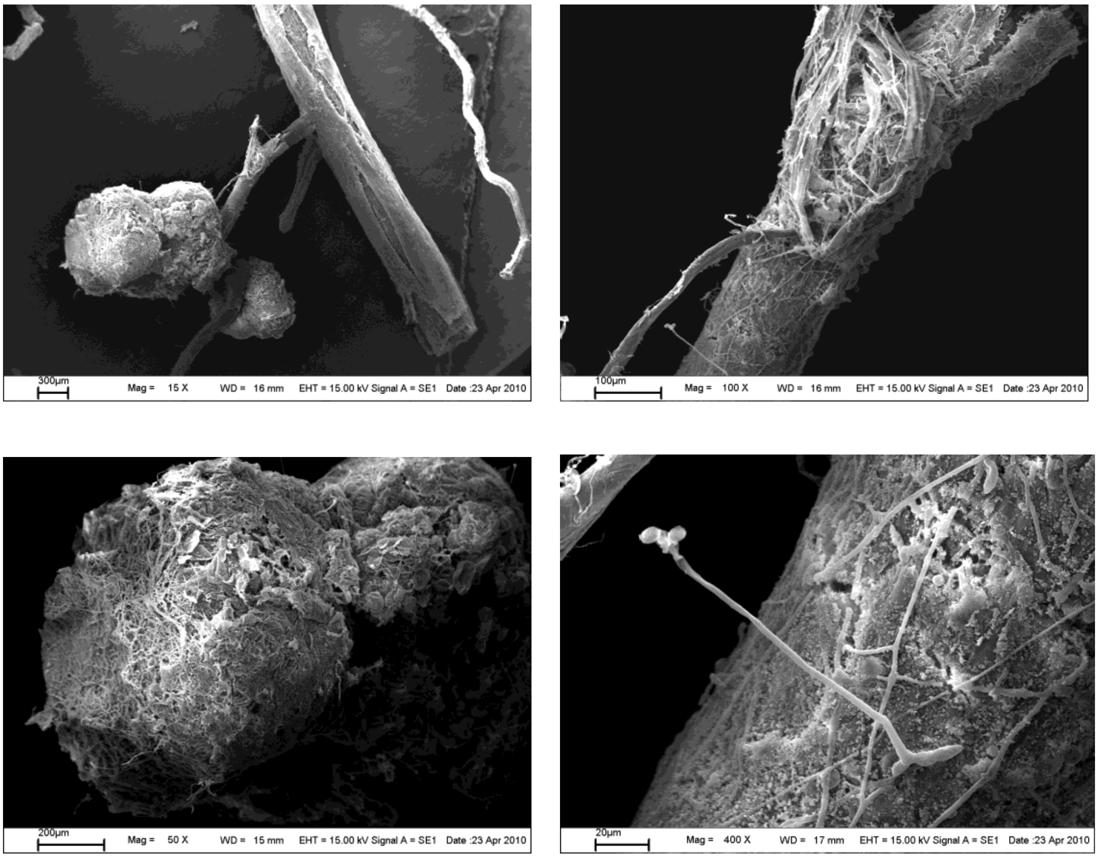


Figure 13 SEM micrographs of root and nodule surface of wattle tree inoculated with *Streptomyces* sp. GMKU944 and *Rhizobium* sp. DASA36075 (left top panel; bar = 300 µm). *Streptomyces* hyphal colonization found on root surface (right top panel; bar = 100 µm; right bottom; bar = 20 µm). *Streptomyces* hyphal colonization found on nodule surface (left bottom panel; bar = 10 µm).

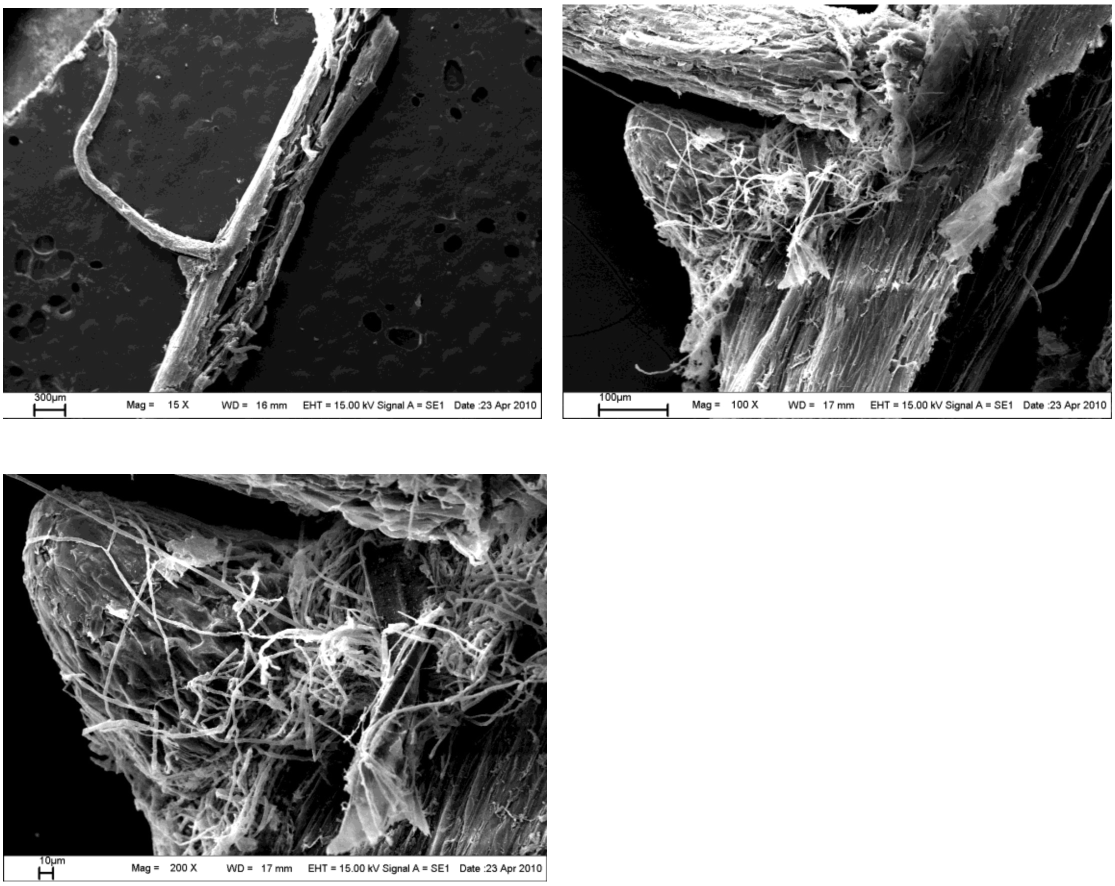


Figure 14 SEM micrographs of the 2 month old root surface of wattle tree plantlets inoculated with eGFP-tagged *Streptomyces* sp. GMKU944 (left top panel; bar = 300 µm). *Streptomyces* hyphal colonization found on root surface and interior of crushed root (right top panel; bar = 100 µm and left bottom panel; bar = 10 µm).

5. Interaction of endophytic actinomycetes with plant

5.1 Siderophore producing endophytic *Streptomyces*

5.1.1 Construction of siderophore-deficient mutant

To investigate the effect of bacterial siderophores on plant growth, a siderophore-deficient mutant was constructed in order to compare its ability to that of the *Streptomyces* sp. GMKU 3100 wild type. The *desD* gene was targeted for gene disruption since it codes for a siderophore synthetase which catalyzes the key step in desferrioxamine biosynthesis in *S. coelicolor* (Barona-Gómez et al., 2004). Based on the published sequences of the *desD*-like genes available in databases, specific primers for *desD* were successfully designed. A partial *desD*-like gene was amplified from the genome of *Streptomyces* sp. GMKU 3100 to give a fragment of 1.2 kb (Fig. 15c). Conceptual translation of the partial gene (GenBank accession number JX204383) revealed 99% identity to DesD of *S. albus* (accession number

ZP_06590848). Insertional inactivation of *desD* in the siderophore biosynthetic gene cluster of *Streptomyces* sp. GMKU 3100 was performed by introduction of pIJ8671/*desD* into the strain by intergeneric conjugation. pIJ8671/*desD* was therefore integrated at the homologous position between the partial *desD*-like gene in the plasmid and the intact *desD* in the chromosome by single cross-over recombination (Fig. 15b). Siderophore-deficient mutants were screened and characterized by (i) resistance to thiostrepton; (ii) absence of siderophore production on CAS agar (Fig. 15a); (iii) presence of a 0.8-kb amplicon of the thiostrepton resistance gene (Fig. 15d); and (iv) absence of a 5.4-kb long amplicon present in pIJ8671/*desD* (Fig. 15e). In addition, the mutants showed a morphology on MS agar in growth of mycelium and spore formation similar to that of wild type. However, slightly slower growth of the mutants was observed compared to the wild type (i.e. late spore formation).

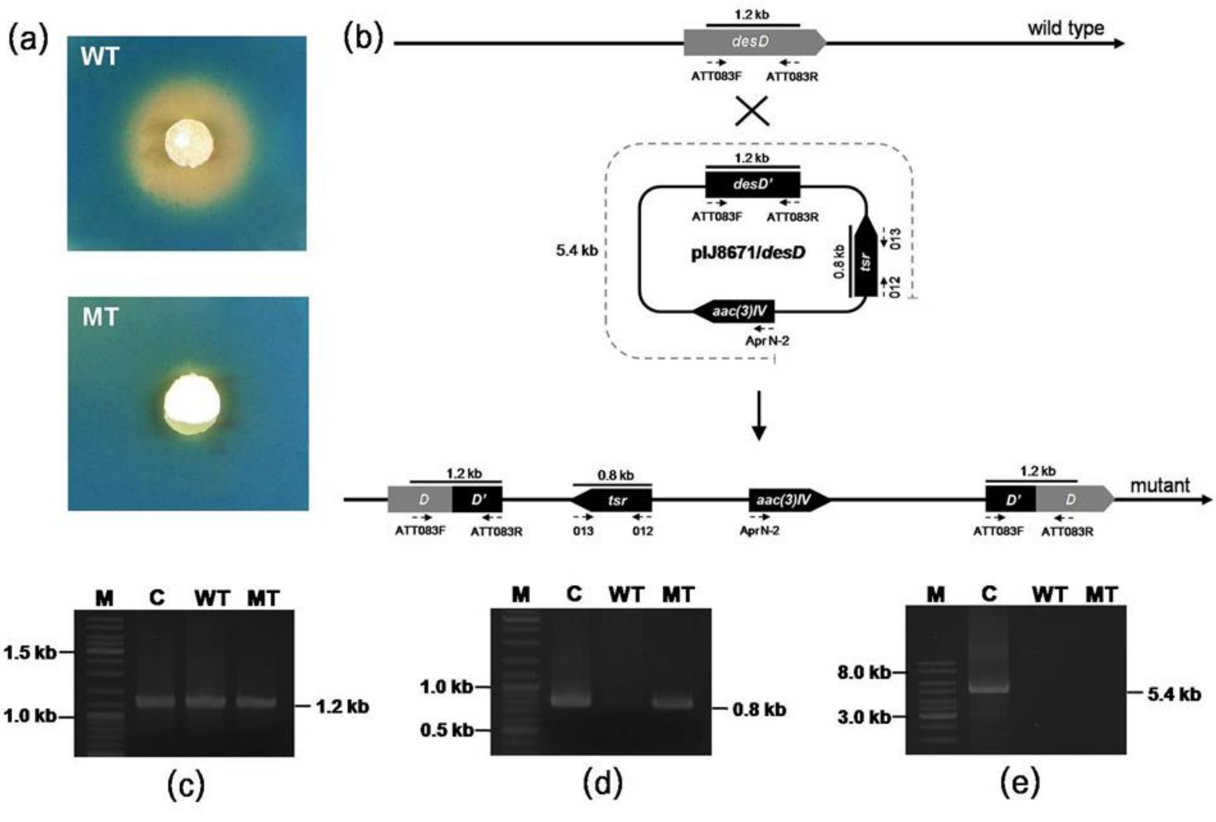


Figure 15 Characterization of *Streptomyces* sp. GMKU 3100 and the siderophore-deficient mutant. (a) Orange halo of siderophore production on CAS agar of the wild type (above) and the mutant (below). (b) Illustration of the insertion inactivation of the *desD* gene by single cross-over recombination. Small arrows indicate primers used for amplification tests. (c) PCR amplification tests for the 1.2-kb *desD* gene sequence using primers, ATT083F and ATT083R. (d) PCR amplification tests for the 0.8-kb thiostrepton resistance gene sequence using primers, ATT012 and ATT013. (e) PCR amplification tests for the 5.4-kb long amplicon in pIJ8671/*desD*

using primers ATT012 and Apr N-2. M, 1-kb ladder; C, pIJ8671/*desD*; WT, *Streptomyces* sp. GMKU 3100; MT, siderophore-deficient mutant.

5.1.2 Effect of siderophore on plant growth promotion

Streptomyces sp. GMKU 3100 and its siderophore-deficient mutant were investigated for their ability for plant growth enhancement by re-inoculation of both strains into seedlings of rice and mungbean with and without 10 µM ferric citrate (Fe-citrate). In addition, the wild type and the mutant could be re-isolated from the inoculated 14-day rice plants and 28-day mungbean plants in every condition. Growth parameters (root and shoot lengths, root and shoot fresh weights, and root and shoot dry weights) of rice and mungbean plants inoculated with the wild type and the mutant strains were recorded after 14 and 28 days, respectively. Plants inoculated with *Streptomyces* sp. GMKU 3100 appeared statistically significantly ($p < 0.05$) greater in plant growth parameters compared with uninoculated plants and plants inoculated with the mutant, both with and without Fe-citrate (Fig. 16 and 17). In the presence of Fe-citrate, the root and shoot lengths, and root and shoot fresh/dry weights of rice and mungbean plants increased with GMKU 3100 inoculation even more remarkably than without Fe-citrate (Fig. 16 and 17). Compared with the uninoculated plants and siderophore mutant treated plants, GMKU 3100 obviously built up the number of roots, increasing the root fresh weight in both rice and mungbean plants (Fig. 16a and 17a). However, the untreated plants and siderophore-deficient mutant inoculated plants showed equal plant growth parameters with no statistically significant difference (Fig. 16 and 17).

With Fe-citrate, the percentage increase in root and shoot lengths for GMKU 3100-inoculated rice plants was 125 and 160% greater, respectively, compared with the untreated controls (Fig. 16b). The percent increase in root dry and fresh weights for GMKU 3100-inoculated rice plants were 140 and 320% greater, respectively, compared with the untreated plants (Fig. 16d). The percentage increase in shoot dry and fresh weights for GMKU 3100-inoculated rice plants were about 200% greater than that of the controls (Fig. 16d). Compared with the uninoculated controls, GMKU 3100 enhanced the lengths 115 and 120% higher in roots and shoots of mungbean plants, respectively in the presence of Fe-citrate (Fig. 17b). GMKU 3100-inoculated mungbean plants had root dry and fresh weights about 200% higher than the untreated controls (Fig. 17d). In the same condition, shoot dry and fresh weights of GMKU 3100-inoculated mungbean plants increased 190 and 170%, respectively, compared with uninoculated plants (Fig. 17d).

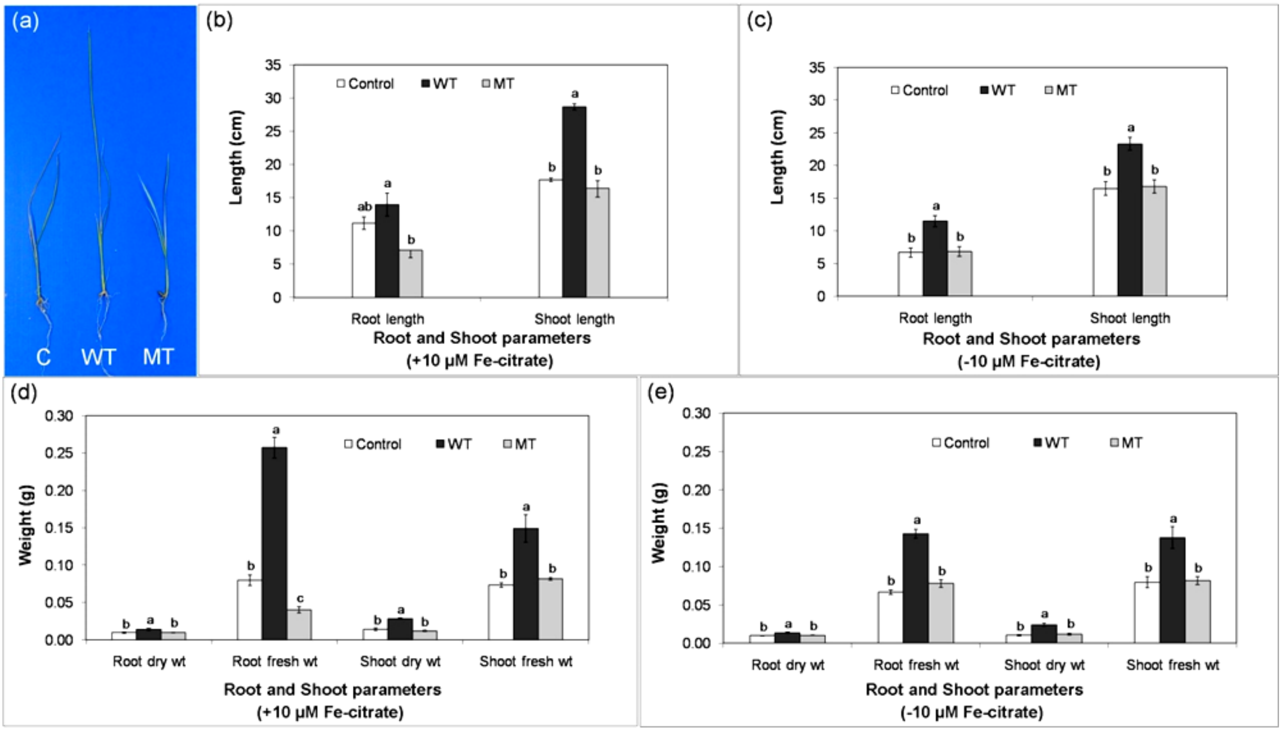


Figure 16 Plant growth parameters of rice plants (*Oryza sativa* L. cv. *KDML105*) inoculated with *Streptomyces* sp. GMKU 3100 and the siderophore-deficient mutant after 14 days. (a) 14-day rice plants; (b) root and shoot lengths with 10 µM Fe-citrate; (c) root and shoot lengths without 10 µM Fe-citrate; (d) root dry/fresh weights and shoot dry/fresh weights with 10 µM Fe-citrate; (e) root dry/fresh weights and shoot dry/fresh weights without 10 µM Fe-citrate; C, uninoculated plant (control); WT, *Streptomyces* sp. GMKU 3100; MT, siderophore-deficient mutant. Data are the mean of 10 replicates. Means designated with different letters are significantly different (*p*=0.05). Error bars show standard deviation (*n=10*).

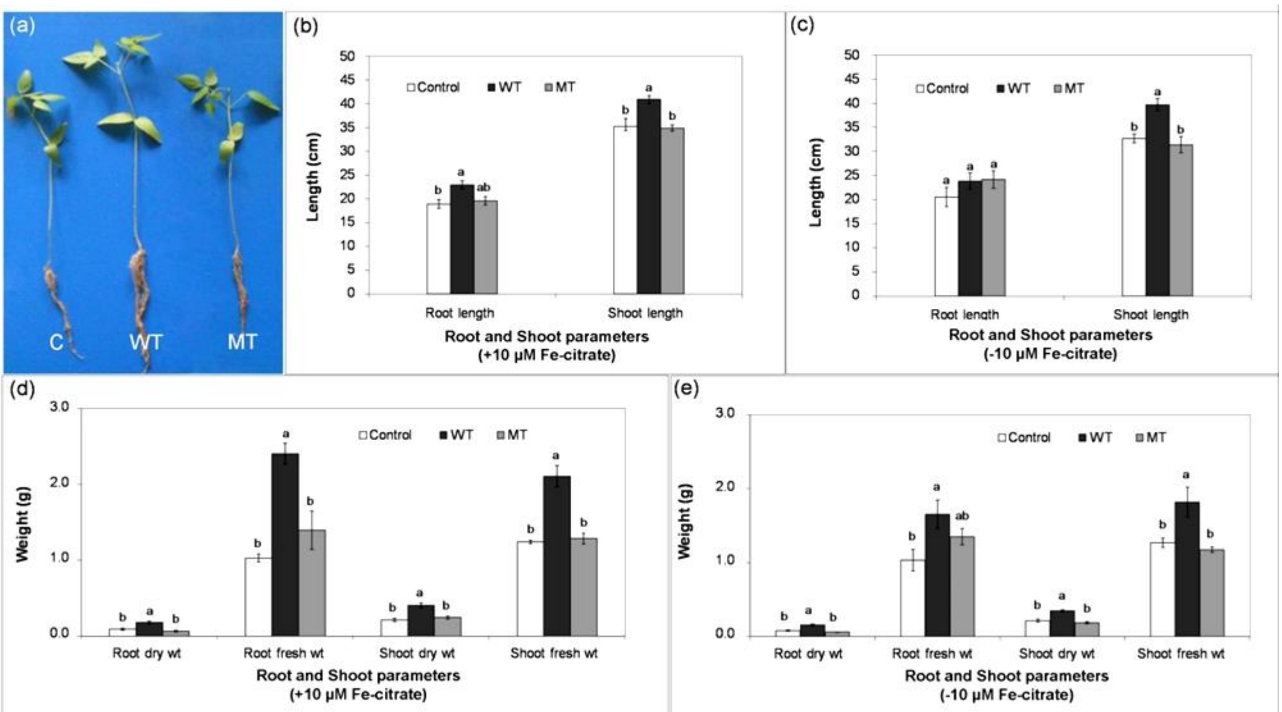


Figure 17 Plant growth parameters of mungbean plants (*Vigna radiata* (L.) Wilczek cv. CN72) inoculated with *Streptomyces* sp. GMKU 3100 and the siderophore-deficient mutant after 28 days. (a) 28-day mungbean plants; (b) root and shoot lengths with 10 µM Fe-citrate; (c) root and shoot lengths without 10 µM Fe-citrate; (d) root dry/fresh weights and shoot dry/fresh weights with 10 µM Fe-citrate; (e) root dry/fresh weights and shoot dry/fresh weights without 10 µM Fe-citrate; C, uninoculated plant (control); WT, *Streptomyces* sp. GMKU 3100; MT, siderophore-deficient mutant. Data are the mean of 10 replicates. Means designated with different letters are significantly different (p=0.05). Error bars show standard deviation ($n=10$).

5.2 Chitinase producing endophytic actinomycetes

A total of one hundred and thirty-three endophytic actinomycetes (GMKU and GKU) isolated from medicinal plants in Thailand were screened for chitinase production potential on CCA plates. Among them, eighty endophytic actinomycetes isolates (60.2 %) were found chitinase activity. Thirty isolates showed high chitinase activity (clear zone ≥ 5.0 mm.), twenty-two isolates showed moderate activity (3.0-4.9 mm.) and twenty-eight isolates showed low activity (1.1-2.9 mm.) (Table 10).

Table 10 Chitinase activity, chi18A and chi19 genes and antagonistic activity of endophytic actinomycetes

No.	Isolate no.	Identification	Chitinase activity	Cell-wall degrading activity	*chi18A* gene	*chi19* gene	Antagonistic activity
GMKU culture collection							
1	GMKU 301	*Streptomyces*	+ + +	+ +	+	+	+ + +
2	GMKU 302	*Streptomyces*	+ +	+ + +	+	+	+
3	GMKU 303	*Promicromonospora*	+ + +	+ + +	-	+	ND
4	GMKU 305	*Streptomyces*	-	ND	ND	ND	ND
5	GMKU 306	*Streptomyces*	+	+ + +	-	-	ND
6	GMKU 307	*Streptomyces*	+	+ + +	-	-	ND
7	GMKU 308	*Streptomyces*	-	ND	ND	ND	ND
8	GMKU 309	*Streptomyces*	-	ND	ND	ND	ND
9	GMKU 311	*Streptomyces*	-	ND	ND	ND	ND
10	GMKU 312	*Streptomyces*	+	+ + +	+	+	+
11	GMKU 313	*Streptomyces*	+	+	+	+	+ +
12	GMKU 314	*Streptomyces*	-	ND	ND	ND	ND
13	GMKU 315	*Streptomyces*	-	ND	ND	ND	ND
14	GMKU 316	*Streptomyces*	+	+ + +	-	-	ND
15	GMKU 317	*Microbispora*	+ + +	+ +	-	-	ND
16	GMKU 318	*Microbispora*	+ + +	+ + +	-	-	ND
17	GMKU 319	*Streptomyces*	+	+ + +	-	-	ND
18	GMKU 320	*Streptomyces*	-	ND	ND	ND	ND
19	GMKU 322	*Streptomyces*	+ + +	+ + +	+	+	+
20	GMKU 323	*Streptomyces*	-	ND	ND	ND	ND
21	GMKU 324	*Microbispora*	+ + +	+ + +	-	-	ND
22	GMKU 325	*Nonomuraea*	-	ND	ND	ND	ND
23	GMKU 326	*Micromonospora*	+ +	+ +	+	+	-
24	GMKU 327	*Nocardia*	-	ND	ND	ND	ND
25	GMKU 328	*Micromonospora*	+ + +	+ + +	-	-	ND
26	GMKU 329	*Actinomadura*	-	ND	ND	ND	ND
27	GMKU 330	*Micromonospora*	+ + +	+ + +	+	+	+
28	GMKU 331	*Actinomadura*	+ + +	+ + +	-	-	ND
29	GMKU 333	*Streptomyces*	+	+	-	-	ND
30	GMKU 334	*Streptomyces*	+ + +	+	-	-	ND
31	GMKU 335	*Streptomyces*	+ +	+	+	+	+ +
32	GMKU 336	*Streptomyces*	-	ND	ND	ND	ND
33	GMKU 337	*Streptomyces*	+ + +	+	+	+	+ +
34	GMKU 340	*Microbispora*	+ + +	+ + +	+	+	-
35	GMKU 341	*Streptomyces*	+ + +	+ +	+	+	+ +
36	GMKU 342	*Streptomyces*	+ + +	+	+	+	+
37	GMKU 343	*Streptomyces*	+ +	+ + +	-	-	ND
38	GMKU 344	*Streptomyces*	-	ND	ND	ND	ND
39	GMKU 345	*Streptomyces*	-	ND	ND	ND	ND

No.	Isolate no.	Identification	Chitinase activity	Cell-wall degrading activity	*Chi18A* gene	Chi19 gene	Antagonictic activity
40	GMKU 346	*Nocardiopsis*	-	ND	ND	ND	ND
41	GMKU 347	*Microbispora*	-	ND	ND	ND	ND
42	GMKU 348	*Micromonospora*	-	ND	ND	ND	ND
43	GMKU 350	*Microbispora*	+ + +	+ + +	-	-	ND
44	GMKU 351	*Actinomadura*	+	-	ND	ND	ND
45	GMKU 352	*Streptomyces*	-	ND	ND	ND	ND
46	GMKU 353	*Micromonospora*	+	+ + +	+	-	-
47	GMKU 354	*Streptomyces*	+ +	+ + +	+	+	+ + +
48	GMKU 355	*Streptomyces*	+ + +	+ +	+	+	+ + +
49	GMKU 356	*Actinomadura*	-	ND	ND	ND	ND
50	GMKU 357	*Microbispora*	+ + +	+ + +	-	-	ND
51	GMKU 358	*Micromonospora*	+ + +	+ + +	+	-	ND
52	GMKU 359	*Actonoallomurus*	-	ND	ND	ND	ND
53	GMKU 360	*Streptomyces*	-	ND	ND	ND	ND
54	GMKU 361	*Streptomyces*	+	+ +	+	+	+ +
55	GMKU 362	*Microbispora*	+ +	+ + +	+	+	+
56	GMKU 363	*Microbispora*	+	+	+	+	+
57	GMKU 364	*Microbispora*	+	+ + +	+	-	+
58	GMKU 365	*Streptomyces*	+ +	+	+	+	+ +
59	GMKU 366	*Microbispora*	+ +	+ + +	-	-	ND
60	GMKU 368	*Microbispora*	+ +	+ + +	-	-	ND
61	GMKU 369	*Microbispora*	+ + +	+ + +	-	-	ND
62	GMKU 370	*Actinoallomurus oryzae sp. nov.*	+ +	+ + +	+	+	+ +
63	GMKU 372	*Microbispora*	+ + +	+ + +	+	+	-
64	GMKU 377	*Saacharopolyspora*	+ +	-	ND	ND	ND
65	GMKU 378	*Microbispora*	+	+ + +	+	-	+
GKU culture collection							
1	GKU 101	*Streptomyces*	+ + +	+ + +	+	+	+ +
2	GKU 102	*Streptomyces*	-	ND	ND	ND	ND
3	GKU 103	*Streptomyces*	-	ND	ND	ND	ND
4	GKU 104	*Streptomyces*	+ +	+ +	+	+	+ + +
5	GKU 105	*Streptomyces*	+ + +	+ +	+	-	+ +
6	GKU 106	*Streptomyces*	-	ND	ND	ND	ND
7	GKU 107	*Streptomyces*	+ + +	+ +	+	+	+
8	GKU 108	*Streptomyces*	+ + +	+ + +	+	+	+
9	GKU 109	*Streptomyces*	+ + +	+ + +	+	+	+
10	GKU 110	*Streptomyces*	-	ND	ND	ND	ND
11	GKU 111	*Streptomyces*	+ +	+	-	-	ND
12	GKU 112	*Streptomyces*	+ + +	+ + +	+	+	+
13	GKU 113	*Streptomyces*	-	ND	ND	ND	ND
14	GKU 114	*Streptomyces*	-	ND	ND	ND	ND
15	GKU 115	*Streptomyces*	-	ND	ND	ND	ND

No.	Isolate no.	Identification	Chitinase activity	Cell-wall degrading activity	*Chi18A* gene	Chi19 gene	Antagonictic activity
16	GKU 116	*Streptomyces*	+	+ + +	-	-	ND
17	GKU 117	*Streptomyces*	-	ND	ND	ND	ND
18	GKU 118	*Streptomyces*	-	ND	ND	ND	ND
19	GKU 119	*Actinomadura*	-	ND	ND	ND	ND
20	GKU 120	*Actinomadura*	-	ND	ND	ND	ND
21	GKU 121	*Streptomyces*	+ +	+ + +	-	-	ND
22	GKU 122	*Streptomyces*	+ +	+ + +	-	-	ND
23	GKU 123	*Streptomyces*	-	ND	ND	ND	ND
24	GKU 124	*Streptomyces*	+ +	+ + +	+	+	+
25	GKU 125	*Actinomadura*	+ + +	+ + +	+	-	ND
26	GKU 126	*Actinomadura*	+	+ +	+	+	+
27	GKU 127	*Actinomadura*	-	ND	ND	ND	ND
28	GKU 128	*Actinomadura*	+	+ + +	+	+	+
29	GKU 129	*Actinomadura*	-	ND	ND	ND	ND
30	GKU 130	*Actinomadura*	-	ND	ND	ND	ND
31	GKU 131	*Streptomyces*	-	ND	ND	ND	ND
32	GKU 133	*Dactylosporangium*	+ + +	+ + +	+	+	-
33	GKU 134	*Streptomyces*	-	ND	ND	ND	ND
34	GKU 135	*Streptomyces*	-	ND	ND	ND	ND
35	GKU 136	*Streptomyces*	-	ND	ND	ND	ND
36	GKU 137	*Streptomyces*	+	+ + +	+	+	+
37	GKU 138	*Streptomyces*	+	+ +	-	-	ND
38	GKU 139	*Streptomyces*	+	+ +	+	+	+ +
39	GKU 140	*Streptomyces*	+	+ +	+	+	+ +
40	GKU 141	*Streptomyces*	+ +	+ + +	+	+	+ +
41	GKU 142	*Streptomyces*	-	ND	ND	ND	ND
42	GKU 143	*Streptomyces*	-	ND	ND	ND	ND
43	GKU 144	*Streptomyces*	-	ND	ND	ND	ND
44	GKU 145	*Streptomyces*	+ +	+ +	-	-	ND
45	GKU 146	*Streptomyces*	-	ND	ND	ND	ND
46	GKU 147	*Actinomadura*	+	-	ND	ND	ND
47	GKU 148	*Streptomyces*	+	+ + +	-	-	ND
48	GKU 149	*Streptomyces*	+ +	+	-	-	ND
49	GKU 150	*Nonomuraea*	+ +	+	+	+	-
50	GKU 151	*Streptomyces*	+	+ +	+	+	+
51	GKU 152	*Nocardia*	-	ND	ND	ND	ND
52	GKU 153	*Streptomyces*	+ + +	+	-	-	ND
53	GKU 154	*Actinomadura*	+	+ +	+	+	-
54	GKU 155	*Streptomyces*	+	+ + +	-	-	ND
55	GKU 156	*Nocardia*	-	ND	ND	ND	ND
56	GKU 157	*Actinomadura syzygii sp. nov.*	+	+ + +	+	+	+
57	GKU 158	*Streptomyces*	-	ND	ND	ND	ND

No.	Isolate no.	Identification	Chitinase activity	Cell-wall degrading activity	*Chi18A* gene	Chi19 gene	Antagonictic activity
58	GKU 159	*Streptomyces*	+ + +	+ + +	-	-	ND
59	GKU 160	*Streptomyces*	+ +	+ + +	-	-	ND
60	GKU 161	*Streptomyces*	-	ND	ND	ND	ND
61	GKU 162	*Streptomyces*	-	ND	ND	ND	ND
62	GKU 164	*Nonomuraea syzygii sp. nov.*	-	ND	ND	ND	ND
63	GKU 165	*Streptomyces*	+	+ + +	-	-	ND
64	GKU 167	*Actinomadura*	-	ND	ND	ND	ND
65	GKU 168	*Streptosporangium*	-	ND	ND	ND	ND
66	GKU 170	*Streptomyces*	-	ND	ND	ND	ND
67	GKU 171	*Streptomyces*	+ +	+ + +	-	+	+
68	GKU 172	*Streptomyces*	+	+ + +	+	+	+

Note: chitinase and fungal cell wall degrading activity: + + + = clear zone ≥ 5.0 mm., + + = 3.0-4.9 mm., + = 1.1-2.9 mm., - = no activity (≤ 1.0 mm.). Fungal antagonistic activity: + + + = inhibition zone ≥ 10 mm., + + = 1-9 mm., + = ≤ 1.0 mm., - = no activity

5.2.1 Cell-wall degrading activity of chitinase producing actinomycetes

All of the eighty chitinase-positive isolates were determined cell-wall degrading activity on MFA plates. Seventy-six endophytic actinomycetes isolates (96.3 %) were found to exhibited cell-wall degrading activity. Forty-nine isolates showed high cell-wall degrading activity (clear zone ≥ 5.0 mm.), fifteen isolates showed moderate activity (3.0-4.9 mm.) and twelve isolates showed low activity (1.1-2.9 mm.) (Table 10).

5.2.2 Molecular detection of chitinase genes

Seventy-six endophytic actinomycetes isolates that showed both chitinase and cell-wall degrading activity were detected the partial of genes encoding family 18 group A and 19 chitinases. Degenerate primers were designed according to the conserved regions of chitinases available in database and successfully amplified the chitinase genes of actinomycetes. Amplified fragments of family 18 group A and 19 chitinases genes were approximately 500 and 400 bp long, respectively. DNA sequencing of these partial chitinase genes was performed and revealed similarity to those of family 18 group A and 19 chitinases available in GenBank database. As a result, forty-five endophytic actinomycetes carried family 18 group A chitinase gene and forty-two endophytic actinomycetes isolates harboured family 19 chitinase genes. Among them, forty endophytic actinomycetes isolates (57.1 %) carried both genes of family 18 group A and 19 chitinase.

5.2.3 Identification of chitinase-producing actinomycetes

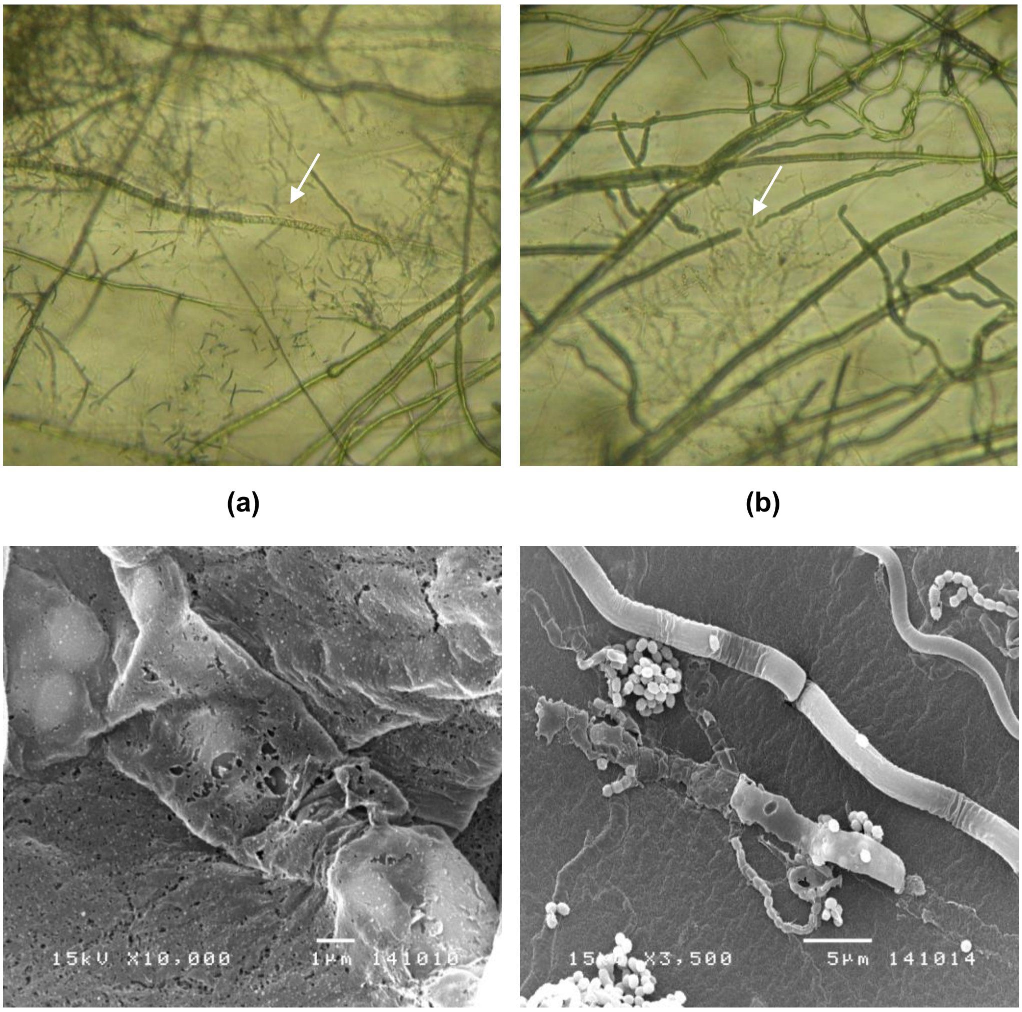
Forty-five endophytic actinomycetes that presented both family 18 group A and 19 chitinase genes were identified by partial 16S rRNA gene sequencing. When 16S rRNA gene sequences were compared to the GenBank and EzTaxon-e databases, they revealed that the chitinase-producing isolates belonged to the genera *Streptomyces* (59.6 %), *Microbispora* (12.8 %), *Actinomadura* (10.7 %), *Micromonospora* (8.5 %), *Actinoallomurus* (2.1 %), *Dactylosporangium* (2.1 %), *Nonomuraea* (2.1 %) and *Promicromonospora* (2.1 %). A novel described species, *Actinoallomurus oryzae* BCC 31373^T (GMKU 370; Indananda *et al*. 2011) and *Actinomadura syzygii* BCC also exhibited high cell-wall degrading activity and carried both chitinase genes. Forty endophytic actinomycetes that carried both chitinase genes consist of twenty-six *Streptomyces*, five *Microbispora*, four *Actinomadura*, two *Micromonospora* and one each from *Actinoallomurus*, *Dactylosporangium* and *Nonomuraea*. Five endophytic actinomycetes that carried only family 18 group A chitinase genes were obtained from two *Micromonospora* and one each from *Actinomadura*, *Microbispora* and *Streptomyces*. Only two endophytic actinomycetes belonging to *Streptomyces* and *Promicromonospora* carried only family 19 chitinase genes.

5.2.4 *In vitro* evaluation of fungal antagonism

All of the forty-five endophytic actinomycetes that carried both family 18 group A and 19 chitinase genes were evaluated for in *vitro* antagonism again *Fusarium moniliforme* DOAC 1224 on SCA plates. Thirty-three strains showed antagonistic potential against the mycelial growth of *F. moniliforme* DOAC 1224. Five strains resulted in strong inhibition (inhibition zone ≥ 10 mm.), twelve strains resulted in moderated inhibition (1-9 mm.) and twenty-one strains resulted in weak inhibition. The maximum inhibition was achieved by *Streptomyces* sp. GMKU 301 (inhibition zone 21 mm.), GMKU 354 (19 mm) and GKU 101 (12 mm).

5.2.5 Microscopic observations of fungal cell wall lysis

For this study, two chitinase producing endophytic actinmycetes were selected. Firstly, *Streptomyces* sp. GMKU 301 produced high activity on CCA and moderate activity on MFA. The strain also had strong antagonistic activity and represented both chitinase genes. Secondly, *Streptomyces* sp. GMKU 322 produced high activity on CCA and MFA. The strain had weak antagonistic activity and represented both chitinase genes. Both chitinolytic *Streptomyces* strains were observed the effect on *F. moniliforme* DOAC 1224 cell wall during dual culture on CCA. Observation of the interaction by light microscope revealed lysis and distortion of the fungal hyphae (Fig. 18a and 18b). Observation by SEM revealed pore information and decomposition of mycelial surface of fungi (Fig. 18c and 18d).



(a) (b)

(c) (d)

Figure 18 Microscopic observation of endophytic streptomycetes and *F. moniliforme* DOAC 1224 interaction during dual culture after 3 days of incubation on CCA. Light micrograph (10X) of damaged mycelia of *F. moniliforme* DOAC 1224 with *Streptomyces* sp. GMKU 301 (a) and *Streptomyces* sp. GMKU 322 (b). Scanning electron micrographs of damaged mycelia of *F. moniliforme* DOAC 1224 with *Streptomyces* sp. GMKU 301 (c, Bar, 1 μm) and *Streptomyces* sp. GMKU 322 (d, Bar, 5 μm).

5.2.6 Evaluation of bicontrol efficacy of chitinolytic actinomycetes

The individual chitinolytic *Streptomyces* sp. GMKU 301 and GMKU 322 were evaluated the biocontrol efficacy against *F. moniliforme* DOAC 1224 in maize plants. The result demonstrated that the application of individual *Streptomyces* sp. GMKU 301 and GMKU 322 significantly inhibited foot rot and wilting diseases of maize plant caused by *F. moniliforme*

DOAC 1224 (Table 11). Moreover, the application of *Streptomyces* sp. GMKU 322 was significantly inhibited disease incidence of foot rot and wilting disease of maize plants more than that of *Streptomyces* sp. GMKU 301. Maize plants that did not treated with *Streptomyces* but inoculated with *F. moniliforme* DOAC 1224 alone rendered approximately 80% foot rot and wilting diseases with the majority of maize plants completely dead (Table 11 and Fig 19 and 20). The maize plants treated with individual *Streptomyces* and inoculated with the pathogen were rendered some infection (Fig. 19).

Table 11 Biocontrol efficacy of chitinolytic *Streptomyces* against foot rot and wilting diseases caused by *F. moniliforme* DOAC 1224 of maize plants at 14-day after inoculation.

Treatment	Disease incidence (%)*	Biocontrol efficacy (%)
Control	83 ± 4.62 a	-
GMKU 301	47 ± 2.31 b	43.38
GMKU 322	28 ± 4.00 c	66.27

*Data are represented as means of three replicates ± SD and designated with differently letters are significantly difference (*p* = 0.05).

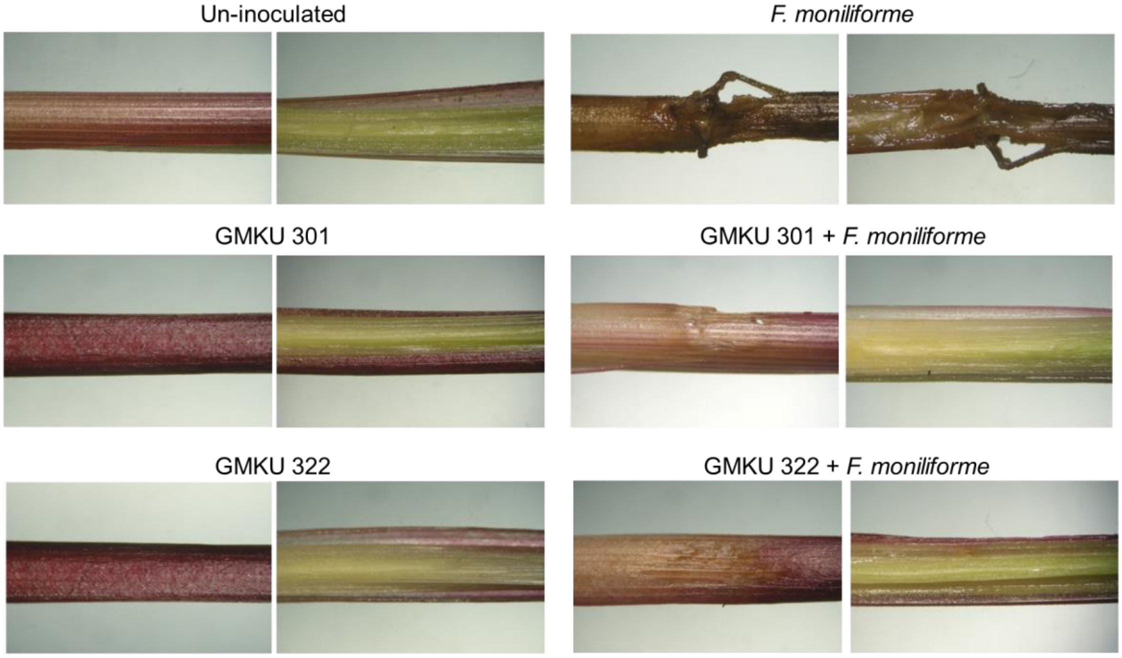


Figure 19 The efficacy of individual *Streptomyces* sp. GMKU 301 and GMKU 322 in the suppression of foot rot and wilting diseases caused by *F. moniliforme* in maize plants at 7 days after inoculation of pathogen examined under stereo-microscope (50X).

Figure 20 Pot experiments illustrating the efficacy of individual *Streptomyces* sp. GMKU 301 and GMKU 322 in the suppression of foot rot and wilting diseases in maize plants at 7 days after inoculation of *F. moniliforme.*

6. Identification of new bioactive compound from endophytic actinomycetes

In this work an endophytic *Microbispora* sp. GMKU363 isolated from a root of Thai medicinal plant 'Lin Ngu Hao' (*Clinacanthus siamensis* Bremek.) had high potential to produce new metabolites. The strain was then identified using 16S rRNA gene sequenceing the result show that was a member of the genus *Microbispora* on the basis of 99.9% 16S rRNA gene sequence (1387 nucleotides; GenBank accession number GU459171) with the *Microbispora mesophila* JCM 3151^T type strain (accession number AF002266).

Microbispora sp. GMKU 363 was cultured in A-11M medium at 30 ˚C for 6 days, and the whole culture was extracted with 1-butanol. The crude extract was screened for novel compounds using reversed-phase column HPLC. The chromatogram (Figure 21) showed 5 target peaks, A, B, C, D and E one target B was compared its UV spectrum to data base (Kindly provided by Prof. Y. Igarashi, Toyama Prefectural University, Japan) and showed high potential to be a novel compound. To get high the yield, strain GMKU 363 was cultured again in

A-11M medium and the whole culture broth was extracted with 1-butanol. The organic solvent was pooled and taken to dryness under rotary evaporation to give a dark brown solid (3g).

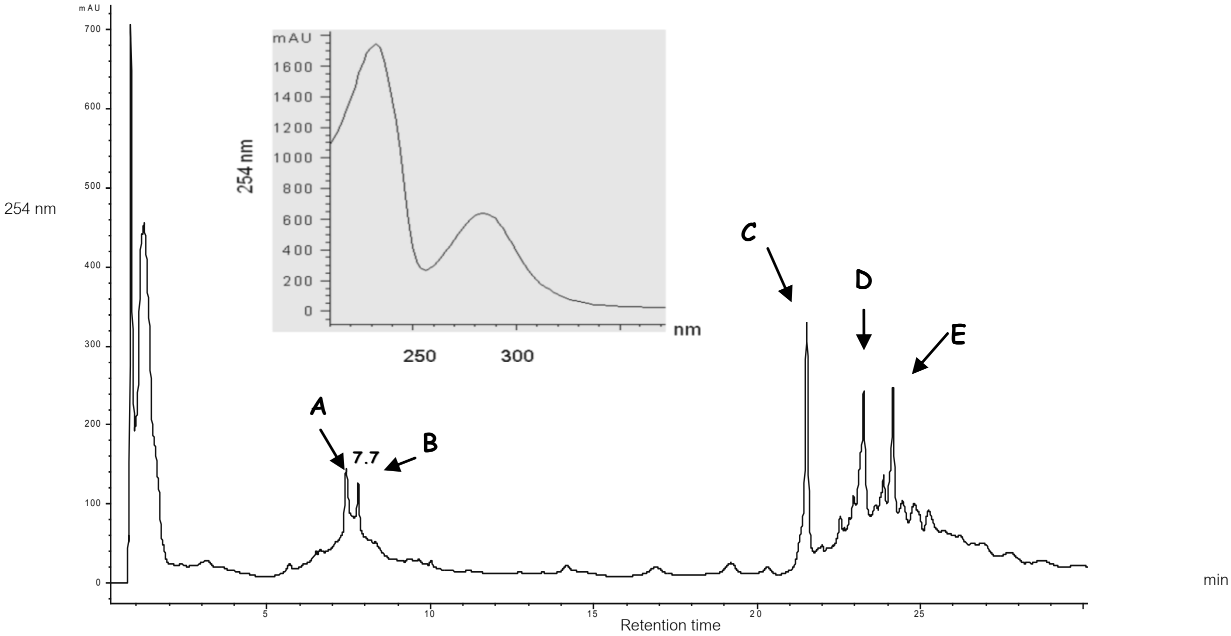


Figure 21 HPLC chromatogram crude extract of *Microbispora* sp. GMKU 363 revealed 5 targets, A, B, C, D and E. UV spectrum of target B was indicated.

The solid was separated by flash column chromatography using silica gel 60 (3 cm diameter). The column was eluted with 120 ml each fraction of chloroform, then gradients of chloroform: MeOH (20:1, 10:1, 4:1, 2:1 and 1:1) and MeOH to give eight fractions C1-C8 (Table 12).

Each fraction was analyzed by HPLC to detect the target B. Fraction C5, a yellow solvent (229 mg) was separated by HPLC purification on a C18 column, follow by dryness under rotary evaporation to give a colorless solid (0.7 mg). Fraction C5 (a colorless solid, 0.7 mg) was identified to be Linfuranone A.

The compound was obtained as an optically active, colorless amorphous powder that gave an $[M + Na]^+$ peak at *m*/*z* 417.2257 in the high resolution ESITOFMS appropriate for a molecular formula of $C_{22}H_{34}O_6$, (calcd for $C_{22}H_{34}O_6Na$, 417.2248), which was consistent with the ^1H and ^{13}C NMR data (Table 13). The IR spectrum of linfuranone A showed absorption bands for hydroxyl (3333 cm^{-1}) and carbonyl (1691 cm^{-1}) functionalities. ^{13}C NMR and HMQC spectral data confirmed the presence of 22 carbons, which were assigned to two oxygenated quaternary

sp^2 carbons, seven olefinic carbons (five are proton-bearing), one quaternary sp^3 carbon, four sp^3 methines (three are oxygen-bearing), three sp^3 methylenes, five methyl carbons.

Table 12 Fractions obtained from the crude extract of *Microbispora* sp. GMKU 363

Fraction No.	**Weight (mg)**	**Physiological characters**
C1	-	Yellow
C2	40	Colorless
C3	-	Pale yellow
C4	696	Red
C5	229	Yellow
C6	-	Yellow
C7	-	Yellow
C8	500	Dark brown

Table 13 ^1H and ^{13}C NMR data for linfuranone A (1) in CD_3OD.

No.	δ_C[a]	δ_H mult (*J* in Hz)[b]	HMBC[b,c]
1	22.3/22.4, CH_3	1.43/1.44, s	2, 3
2	104.37/104.39, qC		
3	205.5, qC		
4	110.3, qC		
5	187.2, qC		
6	38.5, CH_2	2.65, m	4, 5, 7, 8
		2.72, m	4, 5, 7, 8
7	69.6, CH	3.96, m	
8	38.2/38.3, CH_2	1.61, m	7, 9, 10
9	29.9, CH_2	2.18, m	7, 8, 10
		2.24, m	7, 8, 10
10	132.74/132.79,[d] CH	5.59, m	11, 12
11	132.68/132.70,[d] CH	6.06, m	
12	132.00/132.02, CH	6.06, m	
13	136.3/136.4, CH	5.58, m	11, 12
14	41.8, CH	2.31, m	13, 15, 21
15	83.1, C	3.63, d (8.4)	13, 14, 17, 21, 22
16	138.0, qC		
17	133.5, CH	5.35, d (8.4)	18, 22
18	65.2, CH	4.56, dq (8.4, 6.4)	16
19	22.9, CH_3	1.19, d (6.4)	17, 18
20	6.0, CH_3	1.67, s	3, 4, 5
21	18.0, CH_3	0.86, d (7.0)	13, 14, 15
22	11.6, CH_3	1.64, d (1.1)	15, 16, 17

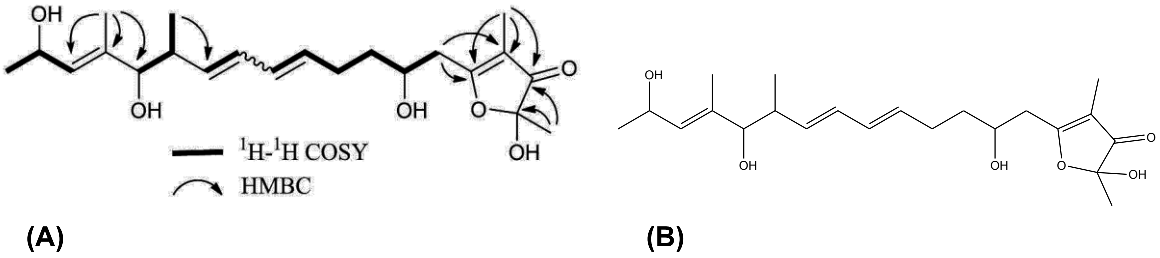
[a] Recorded at 100 MHz.

[b] Recorded at 500 MHz.

[c] HMBC correlations are from proton(s) stated to the indicated carbon.

[d] Interchangeable signals.

Analysis of the COSY spectrum led to four proton-bearing fragments, H-17 to H-19, H-15/H-14/H-21, H-12/H-13, and H-6 to H-11 (Figure 22). The first and second fragments were joined through the quaternary sp^2 carbon C-16 by HMBC correlations from a vinyl methyl H-22 to C-15, C-16, and C-17. This fragment was expanded to include the third fragment on the basis of an HMBC correlation from H-21 to C-13, providing an eight-carbon fragment bearing two oxygen substitutions at C-15 and C-18. The last COSY-defined fragment was connected to the unsaturated ketone substructure bearing an oxygen substitution at position on the basis of a series of HMBC correlations from H-6 to C-4 and C-5, and from a vinyl methyl H-20 to C-3, C-4, and C-5. The carbonyl carbon C-3 was correlated with H-1, which also showed a correlation to the oxygenated carbon C-2 (104.4), establishing the attachment of the two-carbon fragment C-1/C-2 to C-3. The chemical shift of C-2 was suggestive of the bonding of two oxygen atoms to this carbon. Finally, consideration of the molecular formula and the remaining unsaturation degree provided the connectivity between C-11 and C-12 and the placement of an oxygen atom between C-2 and C-5 to establish this molecule as a new member of furanone-containing polyketides. *E* configuration for the double bond between C-16 and C-17 was confirmed by NOEs between H-18 and H-22, and H-15 and H-17. The geometry of C-10/C-11 and C-12/C-13 double bonds could not be assigned due to the proton signal overlapping. With respect to the four stereocenters in the linear chain part, there exist two possible configurations at the hemiketal C-2 carbon. Two signals corresponding to the two diastereomers were observed for H-1 and several carbons. Absolute stereochemistry of the compound is under investigation.



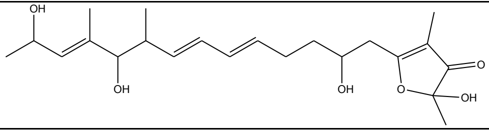
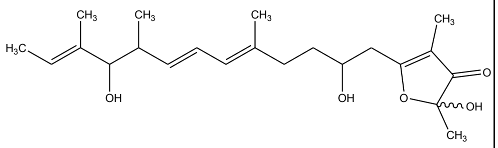
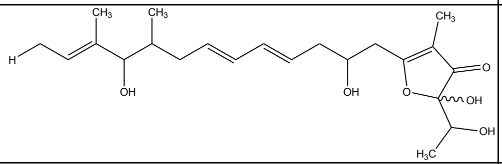
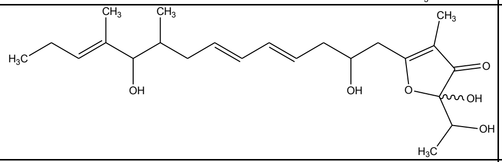
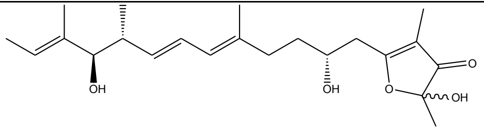
(A) **(B)**

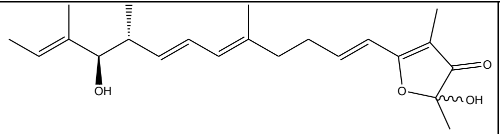
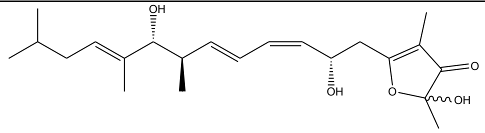
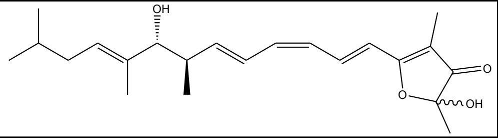
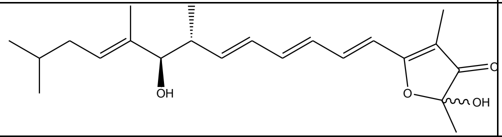
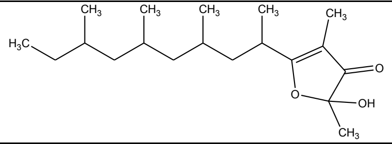
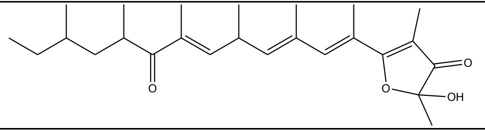
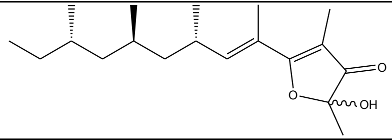
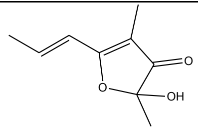
Figure 22 Analysis of the COSY spectrum led to four proton-bearing fragments (A). Structure of the compound linfuranone A (B).

The compound is a relatively rare 3-furanone derived from polyketide with a hemiketal at C-2 and an unsaturated alkyl chain at C-5. There are a few known structurally close metabolites isolated from *Streptomyces* spp., which are E837, E492, E975 and actinofuranones A and B

(Banskota *et al*., 2006 and Cho *et al.,* 2006; Table 14). Further related structures are not from actinobacterial origin, but were isolated from myxobacteria, fungi and marine mollusks (Capon and Faulkner, 1984; Kuroda, *et al*., 1984; Cimino, *et al.,* 1987; Kunze *et. al.,* 2005; Bromley *et al*., 2012; Table 4). E837, E492 and E975 exhibited inhibition against helminth NADH-fumarate reductase and bovine heart NADH oxidase, while actinofuranones had no display of biological activity. Biological screening of linfuranone A in diverse bioassays indicated that this compound was active in an assay designed to screen antidiabetic and antiatherogenic activities using mouse ST-13 preadiopocytes. By the treatment with 50 μM the compound, about 50% of preadipocytes were differentiated into the matured adipocytes and accumulated the lipid droplets. The compound displayed no appreciable activities in antimicrobial and cytotoxic assays.

Table 14 Comparison of a new 5-alkenyl-3,3(*2H*)-furanone, linfuranone A with reference compounds.

Compounds	Structures	Producers	Bioactivity
5-alkenyl-3,3(*2H*)-furanone, linfuranone A		*Microbispora* sp. GMKU 363	-
5-alkenyl-3,3*(2H)* -furanones E-837		*Streptomyces aculeolatus* NRRL 18422	1. Antifungal activity: against *Saccharomyces cerevisiae* 2. Electron transport enzyme inhibition: against NADH-fumarate reductase of *Ascaris suum* (roundworm of pig) and NADH oxidase from bovine heart
5-alkenyl-3,3*(2H)* -furanones E-492		*Streptomyces* sp. Eco86	-
5-alkenyl-3,3*(2H)* -furanones E-975		*Streptomyces* sp. Eco86	-
Actinofuranone A		Marine *Streptomyces* CNQ 766	1. Antifungal activity: against *Mucor hiemalis* and *Trichoderma koningii* (fungal plant pathogen) 2. Cytotoxic activity: against mouse fibroblast cell line

Actinofuranone B		Marine *Streptomyces* CNQ 766	1. Antifungal activity: against *Mucor hiemalis, Trichoderma koningii* and *Ustilago maydis* (fungal plant pathogen) and *Aspergillus fumigatus* 2. Cytotoxic activity: against mouse fibroblast cell line
Aurafuron A		*Stigmatella aurantiaca* DW4/3-1 *Archangium gephyra* Ar 10844	-
(8Z)-aurafuron B		*Stigmatella aurantiaca* DW4/3-1	
(8E)-aurafuron B		*Stigmatella aurantiaca* DW4/3-1	
AS-183		*Scedosporium* sp. SPC-15549	Inhibitor of microsomal Acyl-CoA: cholesterol acyltransferase
Aglajne-2		*Bulla striata* (sea snail)	
Norpectinatone furanone		*Siphonaria lesson* (sea snail)	
2,3-dihydro-2-hydroxy-2,4dimethyl-5-trans-propenylfuran-3-one		*Ascochyta salicorniae*	1. Inhibitor of enzyme tyrosine kinase activity 2. Antiparasitic activity: against *Trypanosoma brucei* subsp. rhodesiense (human sleeping sickness) and *Trypanosoma cruzi* (American trypanosomiasis of Chagas disease) 3. Antifungal activity: against *Microbotryum violaceum* (plant pathogen fungus) and *Eurotium repens*

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Output ที่ได้จากโครงการ

1. ผลงานวิจัยที่ตีพิมพ์ในวารสารระดับนานาชาติ (เอกสารแนบท้าย)

1. Rungin, S., Indananda, C., Suttiviriya, P., Kruasuwan, W., Jaemsaeng, R. and Thamchaipenet, A. (2012). Plant growth enhancing effects by a siderophore-producing endophytic streptomycete isolated from a Thai jasmine rice plant (*Oryza sativa* L. cv. KDML105). **Antonie Van Leeuwenhoek** 102: 463–472. (IF2013 = 2.137)

2. Indananda, C., Igarashi, Y., Ikeda, M., Oikawa, T. and Thamchaipenet, A. (2013). Linfuranone A, a new polyketide from plant-derived *Microbispora* sp. GMKU 363. **J. Antibiot.** 66(11): 675-677. (IF2013 = 2.041)

3. Rachniyom, H., Matsumoto, A., Indananda, C., Duangmal, K., Takahashi, Y. and Thamchaipenet, A. (2015). *Actinomadura syzigii* sp.nov., an endophytic actinomycete isolated from the roots of jambolan plum tree (*Syzygium cumini* L. Skeels). **Int. J. Syst. Evol. Microbiol.** (accepted). (IF2013 = 2.798)

4. Rachniyom, H., Matsumoto, A., Indananda, C., Duangmal, K., Takahashi, Y. and Thamchaipenet, A. (2015). *Nonomuraea syzigii* sp. nov., an endophytic actinomycete isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels). **Int. J. Syst. Evol. Microbiol.** (accepted). (IF2013 = 2.798)

5. Rachaniyom, H. and Thamchaipenet, A. (2015). Endophytic streptomycetes control root rot and wilt disease in maize plants. **Plant Soil**. (in preparation). (IF2013 = 3.235)

2. ผลงานวิจัยที่ตีพิมพ์ในหนังสือระดับนานาชาติ

1. Thamchaipenet, A., Bunyoo, C., Jaemsaeng, R., Lohmaneeratana, K., Rungin S. and Phornphisutthimas, S. (2013). Intergeneric conjugation: a practical method for genetic manipulation in actinomycetes. In: R. K. Gaur and H. K. Gautam (eds.) *Molecular Biology of Bacteria*. Nova Science Publishers, NY. pp. 1-17.

3. การได้รับเชิญเป็นวิทยากรในงานประชุมระดับนานาชาติ

1. Thamchaipenet, A. (2014). Endophytic actinomycetes: novel taxa, bioactive compounds and plant growth enhancement. ***Microbial Resources for environmental and industrial applications Symposium*.** Chulalongkorn University, Bangkok, Thailand, 3 June 2014.
2. Thamchaipenet, A. (2014). Endophytic actinomycetes as plant growth enhancers, stress reducers and pathogen protectors. ***The 1st ASEAN Microbial Biotechnology Conference (AMBC2014)*.** Bangkok International Trade & Exhibition Centre (BITEC), Bangkok, Thailand, 19-21 February 2014.
3. Thamchaipenet, A. (2013). Endophytic actinomycetes isolated from Thai plants and their potential for new taxa, natural products and plant growth enhancing agents. ***The 1st International Conference on Natural Products for Agriculture***. Bangkok, Thailand, 17-21 November 2013.
4. Thamchaipenet, A. (2013). Endophytic actinomycetes act as plant growth enhancers, pathogen protectors and stress reducers. ***Society for General Microbiology (SGM) Spring Conference 2013***, Manchester, UK, 25-28 March 2013.
5. Thamchaipenet, A. (2012). Recent investigation of endophytic actinomycetes isolated from Thai plants reveals plant growth enhancement. ***The 2nd Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation (2nd APMNF)***. Phuket, Thailand. 28-31 October 2012.
6. Thamchaipenet, A. (2012). Diversity of endophytic actinomycetes from Thai tropical plants and their properties of plant growth enhancement. ***The 90th Anniversary Meeting of The Society for Biotechnology, Japan (SBJ)***. Kobe International Conference Center, Kobe, Japan. 23-26 October 2012
7. Thamchaipenet, A. (2012). Novel taxa, bioactive compounds and plant growth enhancement from endophytic actinomycetes isolated from Thai plants. ***The International Conference 2011: The Roles of the Learned Societies in Improving the Quality of Life in the Context of Globalization***. Dusit Thani Hotel, Bangkok, Thailand. 19 June 2012.

8. Thamchaipenet, A. (2012). Investigation of plant growth promoting properties from endophytic actinomycetes from Thai tropical plants. ***International Conference on Microbial, Plant and Animal Research (ICMPAR2012)***. Mody Institute of Technology and Science, Lakshmangarh, India. 29-31 March 2012.

9. Thamchaipenet, A. (2011). Recent investigation and future prospects of endophytic actinomycetes from Thai tropical plants. ***The 16th International Symposium on the Biology of Actinomycetes (ISBA16)***. Puerto Vallarta, Mexico. 11-15 December 2011.

4. การนำเสนอแบบปากเปล่าและโบสเตอร์ของนิสิตปริญญาเอก

1. Kruasuwan and Thamchaipenet (2014). Endophytic bacteria and actinomycetes promote growth of sugarcane plants. ***The 1st ASEAN Microbial Biotechnology Conference (AMBC2014)***. Bangkok International Trade & Exhibition Centre (BITEC), Bangkok, Thailand, 19-21 February 2014.

2. Rachniyom, H., Indananda, C. and Thamchaipenet, A. (2012). Biological control of chitinase-producing endophytic streptomycetes against *Fusarium moniliforme* in maize plants (*Zea mays* L. cv. Nakhonsawan 3). ***The 2nd Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation (2nd APMNF)***. Phuket, Thailand. 28-31 October 2012.

3. Jaemsaeng, R., Indananda, C. and Thamchaipenet, A. (2012). 1-Aminocyclopropane-1-carboxylate (ACC) deaminase production from endophytic actinomycetes and its growth-promoting effect on mungbean plants. ***The 2nd Asian Conference on Plant-Microbe Symbiosis and Nitrogen Fixation (2nd APMNF)***. Phuket, Thailand. 28-31 October 2012.

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5. รางวัลที่ได้รับ

1. SARC Distinguished Scientist Award-2011, Scientific and Applied Research Center, India

6. Research Network

1. Prof. Dr. Yoko Takahashi, Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan
2. Dr. Atsuko Matsumoto, Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan
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ลงนาม ....
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Antonie van Leeuwenhoek (2012) 102:463–472
DOI 10.1007/s10482-012-9778-z

ORIGINAL PAPER

Plant growth enhancing effects by a siderophore-producing endophytic streptomycete isolated from a Thai jasmine rice plant (*Oryza sativa* L. cv. KDML105)

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Received: 14 April 2012 / Accepted: 13 July 2012 / Published online: 27 July 2012

Abstract An endophytic *Streptomyces* sp. GMKU 3100 isolated from roots of a Thai jasmine rice plant (*Oryza sativa* L. cv. KDML105) showed the highest siderophore production on CAS agar while phosphate solubilization and IAA production were not detected. A mutant of *Streptomyces* sp. GMKU 3100 deficient in just one of the plant growth promoting traits, siderophore production, was generated by inactivation of a *desD*-like gene encoding a key enzyme controlling the final step of siderophore biosynthesis. Pot culture experiments revealed that rice and mungbean plants inoculated with the wild type gave the best enhancement of plant growth and significantly increased root and shoot biomass and lengths compared with untreated controls and siderophore-deficient mutant treatments. Application of the wild type in the presence or absence of ferric citrate significantly promoted plant growth of both plants. The siderophore-deficient mutant clearly showed the effect of this important trait involved in plant–microbe interaction in enhancement of growth in rice and mungbean plants supplied with sequestered iron. Our results highlight the value of a substantial understanding of the relationship of the plant growth promoting properties of endophytic actinomycetes to the plants. Endophytic actinomycetes, therefore, can be applied as potentially safe and environmentally friendly biofertilizers in agriculture.

Keywords Endophyte · *Streptomyces* · Siderophore · Plant growth promotion · Rice (*Oryza sativa* L.) · Mungbean (*Vigna radiata* (L.) Wilczek)

Introduction

Endophytic actinomycetes can be defined as those Gram-positive filamentous bacteria that generally inhabit soil and rhizosphere and can colonize the internal tissues of plants without causing any evident damage or morphological changes in the plants (Kunoh 2002; Hasegawa et al. 2006). They interact with plants

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as free-living non-symbiotic bacteria similar to *Pseudomonas* spp. (Mercado-Blanco and Bakker 2007) and could be considered as being involved in a neutral interaction. Reflecting the enormous plant diversity in different niches and ecosystems, many reports indicate that plants are rich reservoirs for diversity and discovery of novel taxa of actinomycetes (Inbar et al. 2005; Hasegawa et al. 2006; Zin et al. 2007; Bascom-Slack et al. 2009; Qin et al. 2011). Endophytic actinomycetes were isolated from live tissues of various type of plants such as wheat (Coombs et al. 2004; Sadeghi et al. 2012), rice (Tian et al. 2004), tomato (Tan et al. 2006; Fialho de Oliveira et al. 2010), banana (Cao et al. 2005), cowpea (Dimkpa et al. 2008), cucumber (El-Tarabily et al. 2009), medicinal plants (Qin et al. 2009), eaglewood (Nimnoi et al. 2010), blue lupin (Trujillo et al. 2010), chickpea (Misk and Franco 2011) and neem tree (Verma et al. 2011).

Recently, there has been an increasing number of reports that these endophytic actinomycetes are clearly beneficial to plants. They protect plants against phytopathogens by the production of antibiotics or chitinolytic enzymes to inhibit fungal pathogens, thus being considered as potential biocontrol agents of plant diseases (Coombs et al. 2004; Tian et al. 2004; Cao et al. 2005; Zin et al. 2007; Quecine et al. 2008; El-Tarabily et al. 2009; Misk and Franco 2011). They also directly promote plant growth such as by the production of phytohormones (auxins, cytokinins and gibberellins), production of siderophores to scavenge ferric iron from the environment, solubilization of inorganic phosphate, fixing nitrogen and suppression of stress ethylene in plant by production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Cao et al. 2005; Tan et al. 2006; Dimkpa et al. 2008; El-Tarabily et al. 2009; Trujillo et al. 2010; Fialho de Oliveira et al. 2010; Misk and Franco 2011; Verma et al. 2011; Sadeghi et al. 2012). However, whether these plant growth enhancers form an active interaction between plant and bacterium is poorly studied. In this work, we investigated plant growth promoting properties by endophytic actinomycetes isolated from Thai rice plant cultivars. We firstly demonstrated a clear link between a genotype and a phenotype of a siderophore producing endophyte that delineated this single trait towards plant growth promotion. We also showed that an endophytic streptomycete isolated from a rice plant could increase growth parameters in different host plants.

Materials and methods

Isolation and identification of endophytic actinomycetes

Endophytic actinomycetes were isolated from healthy Thai rice cultivars, Thai glutinous rice plants (*Oryza sativa* L. cv. RD6) and Thai jasmine rice plants (*O. sativa* L. cv. KDML105) collected from Pathum Thani Rice Research Center, Pathum Thani province, Thailand. The excised plant materials (leaves, stems and roots) were surface-sterilized and endophytic actinomycetes were isolated according to the protocol previously described by Indananda et al. (2010). The plant samples were initially washed in running tap water and sterilized by sequential immersion in 95 % (v/v) ethanol for 10 min and 1 % sodium hypochlorite for 15 min and then the surface-sterilized plant materials were washed in sterile water three times. The leaves, stems and roots were then soaked in 10 % (w/v) $NaHCO_3$ solution for 10 min to retard the growth of endophytic fungi. The plant materials were crushed and the resulting solution was spread onto starch-casein agar (SCA; Küster and Williams 1964) supplemented with 50 mg/ml nalidixic acid, 2.5 U/ml penicillin G, 50 mg/ml nystatin and 50 mg/ml cycloheximide. The crushed leaf, stem and root debris was also placed on SCA. Colonies of endophytic actinomycetes appeared on the agar after incubation at 28 °C for 4–5 weeks. The pure isolates were identified by 16S rRNA gene amplification using the primers and conditions described by Indananda et al. (2010). The 16S rRNA gene sequences of the isolates were determined by direct sequencing of the PCR products. The sequences were compared with those of 16S rRNA genes in the GenBank and EzTaxon-e (Kim et al. 2012) databases to indicate close relationships with valid species.

Determination of phosphate solubilization, IAA and siderophore production

Phosphate solubilization potential of endophytic actinomycetes was determined by growing actinomycetes in tryptic soy broth (TSB) in a rotary shaker at 200 rpm, 28 °C for 5 days. 100 µl of cell culture was dropped onto Pikovskaya agar (Pikovskaya 1948) containing tricalcium phosphate and incubated at 28 °C for 5 days. The presence of clear zones around the microbial colonies was used as indicator for positive strains.

Indole-3-acetic acid (IAA) was assayed based on the colorimetric method described by Glickmann and Dessaux (1995) with some modifications. Endophytic actinomycetes were inoculated into 5 ml TSB supplemented with tryptophan (500 µg/ml) in a rotary shaker at 200 rpm, 28 °C in the dark for 7 days. The cultures were centrifuged at 12,000 rpm for 5 min and 1 ml supernatant was mixed with 2 ml Salkowski's reagent (Gordon and Weber 1951) and incubated for 30 min at room temperature. Development of a pink color indicated IAA production.

Siderophore assay was done using the chrome azurol S (CAS) assay (Schwyn and Neilands 1987). An agar plug of 5-day growth of endophytic actinomycetes on YM agar was placed onto a CAS agar plate and incubated at 28 °C for 2 days. A positive strain was indicated by an orange halo around the agar plug.

Construction of siderophore-deficient mutant

A partial *desD*-like gene was amplified from genomic DNA of *Streptomyces* sp. GMKU 3100 using specific primers, ATT083F (5′-TGCTTCGTCGCCAACAACGG-3′) and ATT083R (5′-CTGGAGCCGGTTGAGGCAGGA-3′). The PCR reaction was carried out in a final volume of 20 µl containing 0.2 mM of each dNTP, 5 % DMSO, 10 pmol of each primer, 2 µl of 10× *Taq* DNA polymerase buffer and 0.5 units of *Taq* DNA polymerase (Fermentas, USA). The PCR program was as follows: 3 min at 95 °C for 1 cycle; 30 s at 95 °C, 30 s at 68 °C, 1 min and 30 s at 72 °C for 30 cycles and 4 min at 72 °C for 1 cycle. The PCR fragment was confirmed by DNA sequencing and then cloned into an integrating vector, pIJ8671 (Sun et al. 1999) to obtain pIJ8671/*desD* prior to transformation of *E. coli* ET12567(pUZ8002) (MacNeil et al. 1992). Intergeneric conjugation was performed according to Phornphisutthimas et al. (2010). The mycelium of a 24-h culture of *Streptomyces* sp. GMKU 3100 was used as recipient to conjugate with donor *E. coli* ET12567(pUZ8002)/pIJ8671/*desD* on mannitol soya agar (MS; Hobbs et al. 1989) containing 10 mmol/l $MgCl_2$. The mutants were selected by thiostrepton resistance. The mutant was tested for siderophore production using the CAS assay described above (Schwyn and Neilands 1987). The mutant was verified to result from integration of pIJ8671/*desD* into the chromosome by amplification of the thiostrepton resistance gene using specific primers, ATT012 and ATT013, and PCR condition as described by Phornphisutthimas et al. (2010). The absence of a 5.4-kb long PCR product was also determined comparing by comparison of the recombinant plasmid pIJ8671/*desD* using specific primers, ATT012 and Apr N-2 (5′-CCCCGGCGGTGTGCT-3′; Choi et al. 2004). The PCR reaction was carried out using 0.1 unit of Phusion High-Fidelity DNA polymerase (Finnzymes, Finland) with 10 % DMSO as follows: 3 min at 98 °C for 1 cycle; 30 s at 98 °C, 30 s at 69.5 °C, 3 min at 72 °C for 30 cycles and 5 min at 72 °C for 1 cycle.

Pot culture growth conditions

Healthy rice seeds (*Oryza sativa* L. cv. KDML105) were immersed in water for 24 h before surface-sterilization. Rice and mungbean seeds (*Vigna radiata* (L.) Wilczek cv. CN72) were then surface-disinfected with 70 % ethyl alcohol for 1 min followed by a 1 % solution of sodium hypochlorite for 5 min. The seeds were washed six times for 1 min each with sterile distilled water. The seeds were then transferred onto sterilized moistened tissue paper in a tissue culture flask and incubated at room temperature in the dark for 2 days for seed germination. The pruned-root dip method (Musson et al. 1995) was used to inoculate the seedlings. The roots were trimmed using a sterilized scalpel and the seedlings were then immediately placed in sterile glass beakers containing a 10^8 CFU/ml spore suspension of *Streptomyces* sp. GMKU 3100 and its mutant and soaked for 4 h. Ten seedlings were used for each experiment and replicated twice. The seedlings were then transferred to a pot containing autoclaved vermiculite overnight at room temperature. Hoagland's solution (Hoagland and Arnon 1950) supplemented with and without a less complex iron source, 10 µM ferric citrate, was added to the pot in order to assess the role of bacterial siderophores (Sharma et al. 2003). The pots were placed in the greenhouse and supplied with water once a day for 2–4 weeks.

Evaluation of shoot and root length and biomass

After 14- and 28-day growth of rice and mungbean plants, respectively, in the greenhouse, they were surface-sterilized and examined for endophytes according to the protocol previously described (Indananda et al. 2010). Root and shoot lengths, root and shoot fresh weights, and root and shoot dry weights were observed. All measurements of root and shoot parameters were conducted on ten seedlings for each condition. The one-way ANOVA and Tukeys multiple range tests (TMRT) were calculated using SPSS (version 11.5) at $p = 0.05$ to determine the efficacy of wild type, mutant and untreated control in promotion of root and shoot development with and without 10 μM ferric citrate.

Results

Identification of endophytic actinomycetes isolated from rice plants

Nine unique endophytic actinomycetes were isolated from the interior tissues of roots of Thai glutinous rice plants (*Oryza sativa* L. cv. RD6; two isolates, namely GMKU 366 and GMKU 367) and Thai jasmine rice plant (*O. sativa* L. cv. KDML 105; seven isolates, namely GMKU 368, GMKU 369, GMKU 370, GMKU 372, GMKU 3100, GMKU 3101 and GMKU 3102). No actinomycete was isolated from leaves or stems of rice plants. The 16S rRNA gene sequences compared to the Genbank database suggested that the endophytes belonged to the genera *Streptomyces* (GMKU 3100, GMKU 3101 and GMKU 3102) and *Microbispora* (GMKU 366, GMKU 368, GMKU 369 and GMKU 372). A novel described genus, *Actinophytocola oryzae* BCC 31372^T (GMKU 367; Indananda et al. 2010), and a novel described species, *Actinoallomurus oryzae* BCC 31373^T (GMKU 370; Indananda et al. 2011), were also discovered and validated in this collection. In addition, we found that *Microbispora* spp. appeared on SCA in larger numbers compared to other actinomycetes.

Plant growth promotion determination

The endophytic actinomycetes were determined for their plant growth promotion abilities on phosphate solubilization, and production of IAA and siderophores. None of them could solubilize phosphate or produce IAA. Five strains could produce only siderophores (GMKU 366, GMKU 367, GMKU 370, GMKU 3100 and GMKU 3102). Among these strains, *Streptomyces* sp. GMKU 3100 showed the widest orange halo of siderophore production on CAS agar (Fig. 1a). The complete 16S rDNA sequence of strain GMKU 3100 revealed 99.9 % identity (1,506 nucleotides; GenBank accession number JQ922248) with the *Streptomyces somaliensis* NBRC 12916^T (accession number AB184243) and *Streptomyces albidoflavus* DSM 40455^T (accession number Z76676) type strains. This strain was selected for further investigation of siderophore production related to its ability to facilitate plant growth.

Siderophore-deficient mutant

To investigate the effect of bacterial siderophores on plant growth, a siderophore-deficient mutant was constructed in order to compare its ability to that of the *Streptomyces* sp. GMKU 3100 wild type. The *desD* gene was targeted for gene disruption since it codes for a siderophore synthetase which catalyzes the key step in desferrioxamine biosynthesis in *S. coelicolor* (Barona-Gómez et al. 2004). Based on the published sequences of the *desD*-like genes available in databases, specific primers for *desD* were successfully designed. A partial *desD*-like gene was amplified from the genome of *Streptomyces* sp. GMKU 3100 to give a fragment of 1.2 kb (Fig. 1c). Conceptual translation of the partial gene (GenBank accession number JX204383) revealed 99 % identity to DesD of *S. albus* (accession number ZP_06590848). Insertional inactivation of *desD* in the siderophore biosynthetic gene cluster of *Streptomyces* sp. GMKU 3100 was performed by introduction of pIJ8671/*desD* into the strain by intergeneric conjugation. pIJ8671/*desD* was therefore integrated at the homologous position between the partial *desD*-like gene in the plasmid and the intact *desD* in the chromosome by single crossover recombination (Fig. 1b). Siderophore-deficient mutants were screened and characterized by (i) resistance to thiostrepton; (ii) absence of siderophore production on CAS agar (Fig. 1a); (iii) presence of a 0.8-kb amplicon of the thiostrepton resistance gene (Fig. 1d); and (iv) absence of a 5.4-kb long amplicon present in pIJ8671/*desD* (Fig. 1e). In addition, the mutants showed a morphology on MS agar in growth of mycelium and spore formation similar to that of wild type. However, slightly slower growth of the mutants was observed compared to the wild type (i.e. late spore formation).

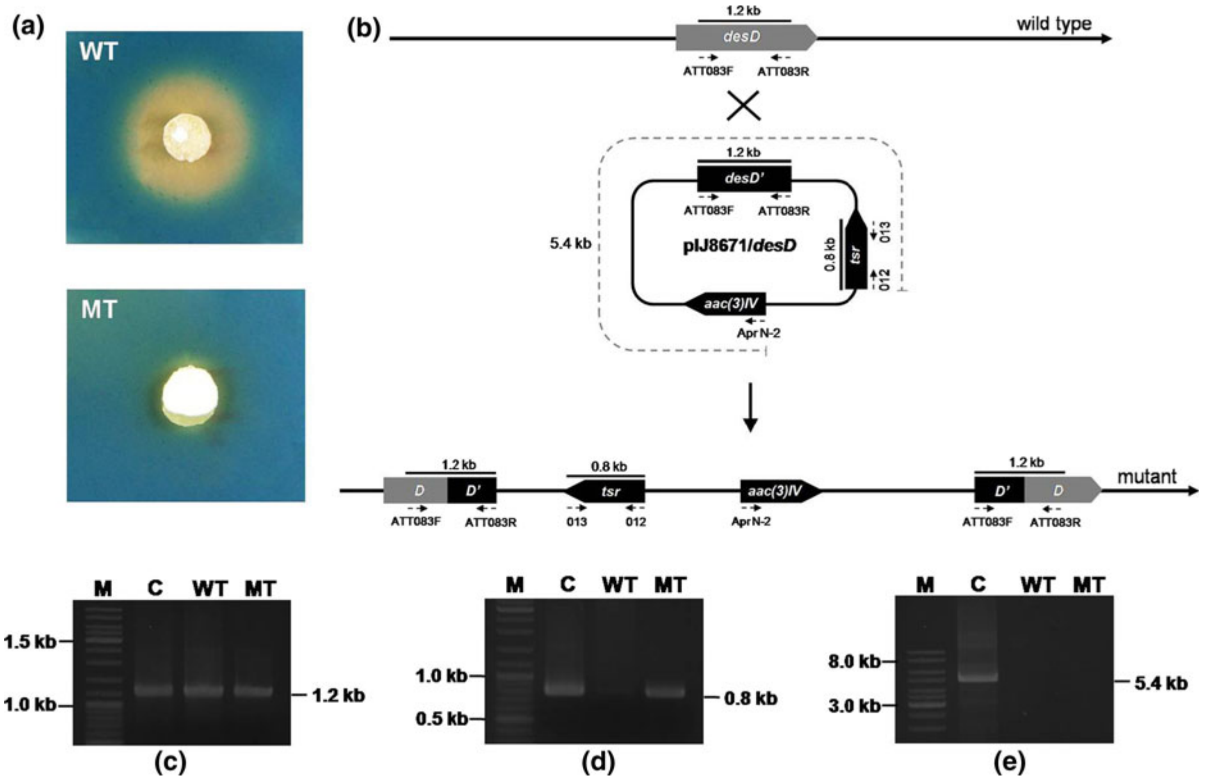

Fig. 1 Characterization of *Streptomyces* sp. GMKU 3100 and the siderophore-deficient mutant. **a** *Orange* halo of siderophore production on CAS agar of the wild type (*above*) and the mutant (*below*). **b** Illustration of the insertion inactivation of the *desD* gene by single cross-over recombination. *Small arrows* indicate primers used for amplification tests. **c** PCR amplification tests for the 1.2-kb *desD* gene sequence using primers, ATT083F and ATT083R. **d** PCR amplification tests for the 0.8-kb thiostrepton resistance gene sequence using primers, ATT012 and ATT013. **e** PCR amplification tests for the 5.4-kb long amplicon in pIJ8671/*desD* using primers ATT012 and Apr N-2. *M* 1-kb ladder, *C* pIJ8671/*desD*, *WT Streptomyces* sp. GMKU 3100, *MT* siderophore-deficient mutant

Effect on plant growth promotion

Streptomyces sp. GMKU 3100 and its siderophore-deficient mutant were investigated for their ability for plant growth enhancement by re-inoculation of both strains into seedlings of rice and mungbean with and without 10 µM ferric citrate (Fe-citrate). In addition, the wild type and the mutant could be re-isolated from the inoculated 14-day rice plants and 28-day mungbean plants in every condition. Growth parameters (root and shoot lengths, root and shoot fresh weights, and root and shoot dry weights) of rice and mungbean plants inoculated with the wild type and the mutant strains were recorded after 14 and 28 days, respectively. Plants inoculated with *Streptomyces* sp. GMKU 3100 appeared statistically significantly ($p < 0.05$) greater in plant growth parameters compared with uninoculated plants and plants inoculated with the mutant, both with and without Fe-citrate (Figs. 2, 3). In the presence of Fe-citrate, the root and shoot lengths, and root and shoot fresh/dry weights of rice and mungbean plants increased with GMKU 3100 inoculation even more remarkably than without Fe-citrate (Figs. 2, 3). Compared with the uninoculated plants and siderophore mutant treated plants, GMKU 3100 obviously built up the number of roots, increasing the root fresh weight in both rice and mungbean plants (Figs. 2a, 3a). However, the untreated plants and siderophore-deficient mutant inoculated plants showed equal plant growth parameters with no statistically significant difference (Figs. 2, 3).

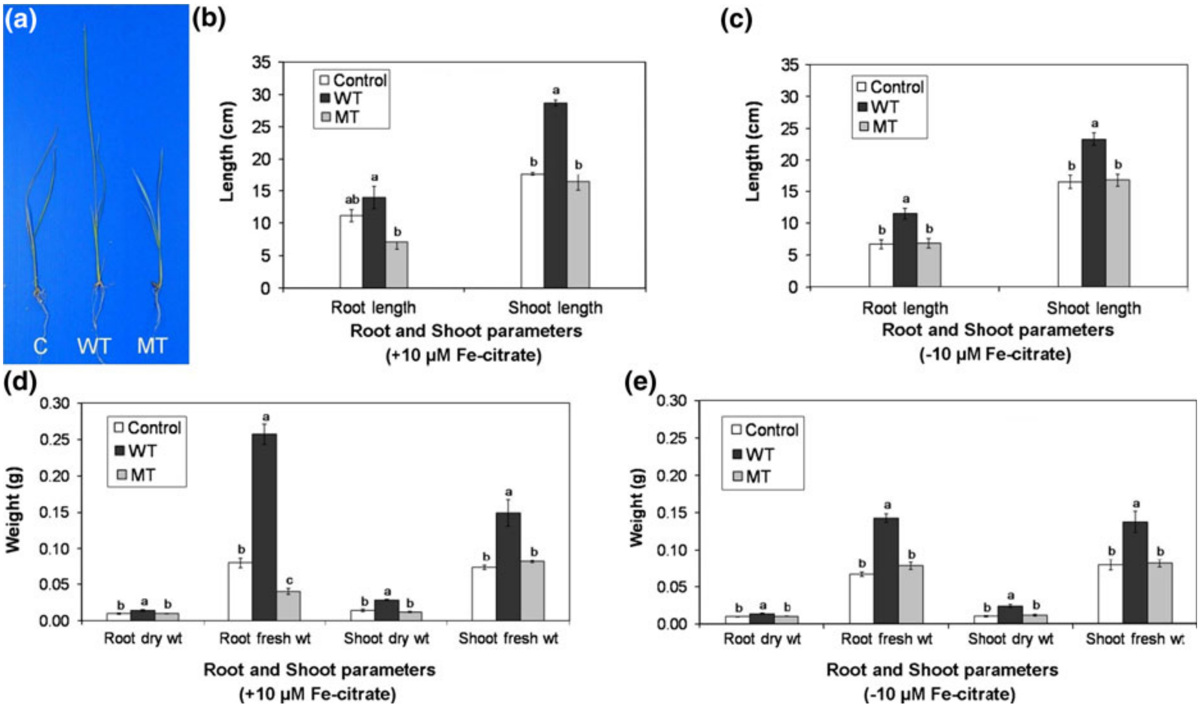

Fig. 2 Plant growth parameters of rice plants (*Oryza sativa* L. cv. KDML105) inoculated with *Streptomyces* sp. GMKU 3100 and the siderophore-deficient mutant after 14 days. **a** 14-day rice plants; **b** root and shoot lengths with 10 μM Fe-citrate; **c** root and shoot lengths without 10 μM Fe-citrate; **d** root dry/fresh weights and shoot dry/fresh weights with 10 μM Fe-citrate; **e** root dry/fresh weights and shoot dry/fresh weights without 10 μM Fe-citrate; *C* uninoculated plant (control), *WT Streptomyces* sp. GMKU 3100, *MT* siderophore-deficient mutant. Data are the mean of 10 replicates. Means designated with *different letters* are significantly different ($p = 0.05$). *Error bars* show standard deviation ($n = 10$)

With Fe-citrate, the percentage increase in root and shoot lengths for GMKU 3100-inoculated rice plants was 125 and 160 % greater, respectively, compared with the untreated controls (Fig. 2b). The percent increase in root dry and fresh weights for GMKU 3100-inoculated rice plants were 140 and 320 % greater, respectively, compared with the untreated plants (Fig. 2d). The percentage increase in shoot dry and fresh weights for GMKU 3100-inoculated rice plants were about 200 % greater than that of the controls (Fig. 2d). Compared with the uninoculated controls, GMKU 3100 enhanced the lengths 115 and 120 % higher in roots and shoots of mungbean plants, respectively in the presence of Fe-citrate (Fig. 3b). GMKU 3100-inoculated mungbean plants had root dry and fresh weights about 200 % higher than the untreated controls (Fig. 3d). In the same condition, shoot dry and fresh weights of GMKU 3100-inoculated mungbean plants increased 190 and 170 %, respectively, compared with uninoculated plants (Fig. 3d).

Discussion

Endophytic bacteria could be found within different parts such as leaves, stems, roots and seeds inside rice plants (Mano and Morisaki 2008). However, more diverse actinobacteria were detected from roots than other parts of rice plants (Tian et al. 2007). In our work, endophytic actinomycetes could only be isolated from the roots. It was suggested that roots are the most abundant sources of actinomycete endophytes (Sardi et al. 1992; Taechowisan et al. 2003). This may be due to the fact that endophytic populations of the root originate from the rhizosphere (Germida et al. 1998). The presence of endophytic actinomycetes inside the root tissues has an important role with regard to plant development and may also protect against soil-borne pathogens (Sardi et al. 1992). Several genera of actinomycetes were isolated from rice plants such as *Actinoplanes*, *Amycolatopsis*, *Dactylosporangium*, *Frankia*, *Micromonospora*, *Rhodococcus* and *Streptomyces* (Tian et al. 2007; Naik

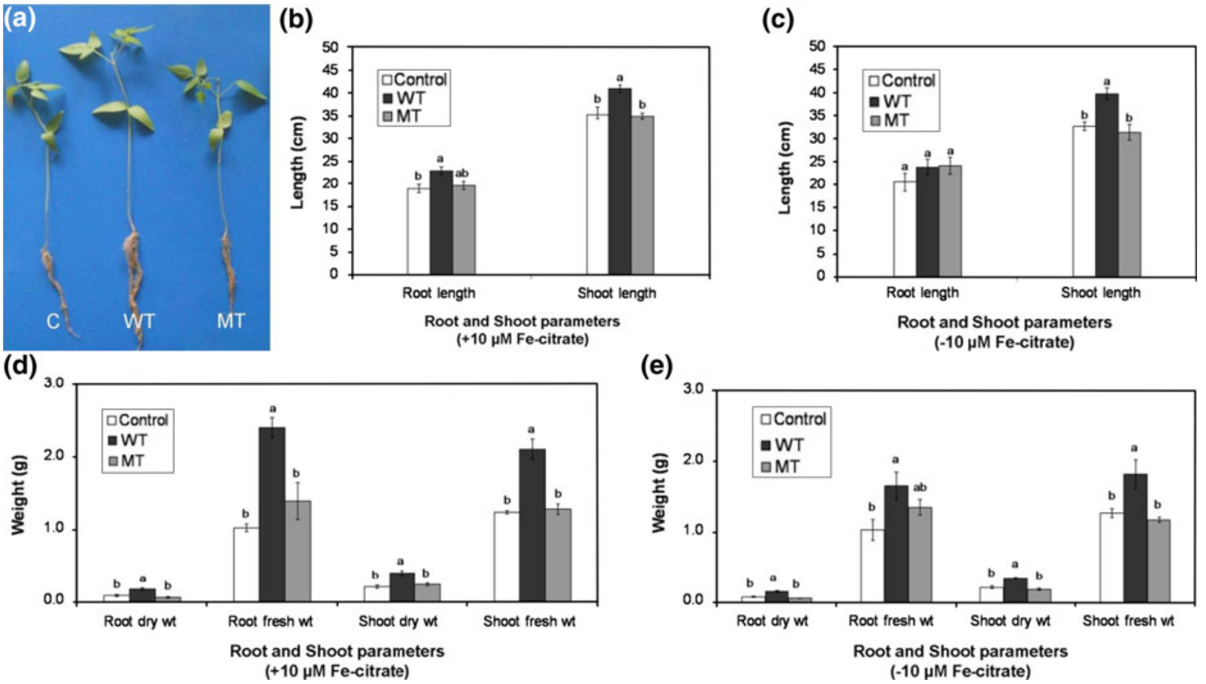

Fig. 3 Plant growth parameters of mungbean plants (*Vigna radiata* (L.) Wilczek cv. CN72) inoculated with *Streptomyces* sp. GMKU 3100 and the siderophore-deficient mutant after 28 days. **a** 28-day mungbean plants; **b** root and shoot lengths with 10 μM Fe-citrate; **c** root and shoot lengths without 10 μM Fe-citrate; **d** root dry/fresh weights and shoot dry/fresh weights with 10 μM Fe-citrate; **e** root dry/fresh weights and shoot dry/fresh weights without 10 μM Fe-citrate; *C* uninoculated plant (control), *WT Streptomyces* sp. GMKU 3100, *MT* siderophore-deficient mutant. Data are the mean of 10 replicates. Means designated with different letters are significantly different ($p = 0.05$). *Error bars* show standard deviation ($n = 10$)

et al. 2009). However, we found different genera of non-streptomycetes (*Actinoallomurus*, *Actinophytocola* and *Microbispora*) isolated from rice plants compared with previous reports. Besides, we observed a large number of colonies of *Microbispora* spp. from our isolation. It seems that *Microbispora* spp. predominantly occupy root tissues of rice plants. Although rather few strains were isolated from roots of Thai rice plants, it is noteworthy that one new genus and one new species were proposed (Indananda et al. 2010; 2011) out of nine strains, which indicated that Thai rice plants are rich bio-resources for discovery of new taxa of endophytic actinomycetes. These results are in agreement with the finding that rice represents a rich reservoir for a wide diversity of actinobacteria (Tian et al. 2007).

It was confirmed that *Streptomyces* sp. GMKU 3100 was truly endophytic because it could be isolated from surface-sterilized rice and mungbean plants up to 14 and 28 days, respectively, after inoculation. This indicated that the endophytic streptomycete tested was able to localize and multiply within plant tissues without causing any obviously deleterious effects to both host plants, but influencing plant development. Furthermore, the endophytic streptomycete isolated from cereal plants could effectively colonize leguminous plants such as mungbean and could remarkably enhance growth of mungbean plants in the similar way as in the plant from which it was originally isolated. It was previously concluded that actinobacteria are not host-specific colonizers, but can adapt themselves to live as free-living endophytes (Misk and Franco 2011). We suggest that endophytic actinomycetes may migrate back and forth between the rhizosphere and the inside of the plants and can neutrally colonize and help plant growth in a wide host range of plants.

Recently, endophytic actinomycetes have been recognized as new members of the plant growth promoting bacterial community (PGPB) due to their properties of plant growth enhancement and protection of plants from infectious diseases (El-Tarabily et al. 2009; Misk and Franco 2011; Verma et al. 2011).

However, phosphate solubilization and IAA production were not detected in our endophytic actinomycetes isolated from rice. Only siderophore production could be detected from five strains (56 %), which could exhibit extensive rice growth-promotion benefits. Siderophores are relatively low molecular weight ferric iron specific chelating agents produced by bacteria and fungi under iron-limiting conditions (Neilands 1995). Soil *Streptomyces* spp. have been reported to produce certain kinds of siderophores (Imbert et al. 1995; Barona-Gómez et al. 2006). Microbial siderophores have a positive correlation with plant growth promotion; therefore, production of siderophores is one of the key factors that should be considered for primary screening (Crowley et al. 1991; Glick et al. 1999). There were reports on screening for siderophore production from endophytic actinomycetes (Nimnoi et al. 2010; Fialho de Oliveira et al. 2010; Ruanpanun et al. 2010) and on the relationship between plant growth enhancing effects and biocontrol of plant pathogens (Cao et al. 2005; Tan et al. 2006; Misk and Franco 2011; Verma et al. 2011; Sadeghi et al. 2012) but little work has been done on the effect of the single trait of siderophore production on plant growth parameters (Dimkpa et al. 2008).

In the work firstly reported here, a clear link between a genotype and a phenotype of a siderophore producing endophytic streptomycete related to plant growth enhancement. A mutant of *Streptomyces* sp. GMKU 3100 deficient in siderophore production was constructed. The key gene, *desD*, coding for a siderophore synthetase catalyzing the final step of desferrioxamine biosynthesis in *S. coelicolor* (Barona-Gómez et al. 2004) was targeted for gene disruption. The insertional inactivation of this gene, therefore, yielded a mutant in which production of siderophores was completely abolished. The previous disruption of *desD* in *S. coelicolor* resulted in a mutant that did not produce desferrioxamines because the final oligomerization step in the biosynthetic pathway was disrupted, causing the abrogation of the production of desferrioxamines (Barona-Gómez et al. 2004). The feasibility of *desD* gene disruption in endophytic *Streptomyces* sp. GMKU 3100 gives new possibility to study the biological role of the single trait of bacterial siderophore production with the host plants.

In this study, pot culture experiments were carried out to evaluate the effect of the siderophore-producing endophytic *Streptomyces* sp. GMKU 3100 on plant growth promotion in rice and mungbean plants in comparison with the siderophre-deficient mutant. When the wild type was applied, it gave the best enhancement of plant growth and significantly increased root and shoot biomass and lengths compared with the untreated control and the siderophore-deficient mutant. Furthermore, application of the wild type in the presence or absence of Fe-citrate significantly promoted plant growth of both rice and mungbean plants. The results suggested that the enhancement of rice and mungbean plant growth was solely due to siderophore production from *Streptomyces* sp. GMKU 3100 since the treatments which received the inoculated siderophore-deficient mutant were even less effective as the uninoculated control in reduction of biomass and elongation of roots and shoots. The production of siderophores is one of the important mechanisms used by plant growth-promoting bacteria to promote plant growth (Crowley et al. 1991; Glick et al. 1999). The increase in plant growth of rice and mungbean plants by the endophytic *Streptomyces* sp. GMKU 3100 in our study is supported by other observations where siderophore-producing endophytic actinomycetes were also shown to enhance plant growth, as in tomato (Tan et al. 2006), banana (Cao et al. 2005), cowpea (Dimkpa et al. 2008); chickpea (Misk and Franco 2011), neem tree (Verma et al. 2011) and wheat (Sadeghi et al. 2012). However, the effects of plant growth from previous reports are not derived from siderophores alone but combined with other plant growth promoting properties such as IAA production and phosphate solubilization. Furthermore, their data emphasize the role of plant growth promoting actinomycetes on the antagonistic effect to phytopathogens of plants. In our study, we generated a mutant of the endophytic *Streptomyces* sp. GMKU 3100 deficient in the single trait of plant growth promotion. The siderophore-deficient mutant could be used to clearly evaluate the effect of this important trait involved in plant–microbe interaction in enhancement of growth in rice and mungbean plants. It is evident from our results that siderophore-producing endophytic *Streptomyces* sp. GMKU 3100 increased root and shoot biomass and lengths of rice and mungbean plants by supplying the plants with sequestered iron.

Production of siderophores is also important for antagonism to phytopathogens and improving growth of the plant (Tan et al. 2006). It was suggested that siderophore-producing endophytic actinomycetes also have very significant antagonistic activity against plant pathogens (Cao et al. 2005; Tan et al. 2006; Dimkpa et al. 2008; Misk and Franco 2011; Verma et al. 2011; Sadeghi et al. 2012). Therefore, it will be useful to investigate further the antagonistic effect of this wild type strain and the mutant against rice diseases. More understanding of plant growth-promoting properties of endophytic actinomycetes suggest that these bacteria merit further investigation for potentially safe and environmentally friendly biofertilizers which can help us limit the use of chemical fertilizers in agriculture.

Acknowledgments We thank David Hopwood for very helpful comments. SR was granted a scholarship from the Graduate School, Kasetsart University. The research work was supported by Thailand Research Fund under grant number DBG5480002 and partly funded by Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University (CASTNAR, NRU-KU).

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In: Molecular Biology of Bacteria
Editors: R. Kumar Gaur and H. K. Gautam

ISBN: 978-1-62618-189-2

Chapter 1

Intergeneric Conjugation: A Practical Method for Genetic Manipulation in Actinomycetes

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Abstract

The system for intergeneric conjugation has been developed for many actinomycete species including *Streptomyces* and rare actinomycetes. *E. coli* donor strains generally used are the methylation proficient strain, S17-1 harbouring *tra* genes on its chromosome and the methylation-deficient strain, ET12567 containing *tra* genes in a self-transmissible plasmid pUB307 or a non-transmissible plasmid pUZ8002. Integrative plasmids used are normally non-replicative plasmids carrying the *oriT* for the propose of gene disruption (e.g. pSET151, pIJ8671) or with additional ϕC31 *attP-int* system in the case of gene expression (e.g. pSET152, pIJ8600). Actinomycete recipients used for mating with *E. coli* could be in spore suspension with or without pre-germination process, or mycelia in liquid culture. Several agar media including MS and ISP 4 were generally used for

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streptomycete strains while other more specific media were proposed for rare actinomycetes. Concentration of $MgCl_2$, ratio of donor and recipient and other parameters are significantly important to increase conjugation efficiency. The intergeneric conjugation approach has become a powerful method to genetically manipulate actinomycete species with high efficiency. It turns out to be a common tool to characterise functions of genes/gene clusters in these pharmaceutically important bacteria.

Introduction

Actinomycetes are high G+C content (74 mol%) filamentous Gram-positive bacteria found abundantly in soil, sand and sediments. They have a complex life cycle with an extended substrate mycelia on which aerial hyphae is mostly formed with exospores. Genus *Streptomyces* in particular is a key producer of valuable secondary metabolites including anti-bacterials, anti-fungals, anti-cancers, immunosuppressants, herbicides and insecticides used nowadays in medicine, pharmaceutics and agriculture [1, 2]. Actinomycetes, therefore, are the most attractive microorganisms to study genes involved in biosynthesis of such important secondary metabolites. Recently, genome analysis of *Streptomyces* chromosomes revealed that they carry vast numbers of uncharacterised genes and biosynthetic gene clusters for undiscovered secondary metabolites [3]. Therefore, the establishment of efficient genetic manipulation systems to introduce homologous/heterologous DNAs into actinomycete species has become a critical requirement for achieving functional analysis of the genes/gene clusters of interest.

Genetic manipulation in actinomycetes was developed intensively for the genus *Streptomyces*. Recombinant plasmids construction for gene expression and disruption/replacement can be generally transferred into streptomycete hosts using polyethylene glycol (PEG) mediated protoplast [4]. The procedure is based on the protocol defined for *S. coelicolor* and *S. lividans* [5]. However, this method is time-consuming procedure as it requires high productivity of protoplast formation and regeneration to achieve good transformation efficiency. With a number of different streptomycete species, the method has to be optimised e.g. mycelial age, concentration of the lysozyme to give good protoplast formation and conditions for protoplast regeneration. PEG mediated protoplast transformation does not easily applicable to every species and frequently results in low efficiency particularly with rare actinomycete strains. In addition, due to the strong host restriction modification system of *Streptomyces* [6], it is essential to find a proficient method to transfer DNA with high efficiency. Although high efficient transformation by electroporation was proposed to avoid the problematic protoplast formation and regeneration [7, 8], it requires luxurious equipment and has limited application not to every species of actinomycetes.

Nowadays, the method generally employed for genetic manipulation in *Streptomyces* and other genera is plasmid-mediated intergeneric conjugation with *E. coli* as a donor [9]. This method is proved to be simple and less time consuming and likely to be applied to various actinomycete species. However, the efficiency of this system varies significantly from one species to another, and it is necessary to improve experimental procedures for a new species.

Bacterial Conjugation

Conjugation is a common phenomenon in both Gram-negative and Gram-positive bacteria. It requires close physical contact between donor and recipient cells and is usually mediated by plasmid-encoded proteins which provide transfer (*tra*) functions [10, 11, 12].

The conjugation by self-transmissible plasmid is related in a mechanism sense to that of F plasmid in *E. coli*. Donor creates F pilus to make cell-to-cell contact to recipient. The mechanism is initiated by creating a single-stranded nick at the origin of transfer (*oriT*). The 5'-end of the nicked DNA is transported linearly through the F pilus into the recipient cell where it is replicated; while, the unnicked DNA is replicated in the donor cells [13].

Naturally, some self-transmissible plasmids could transfer among the same bacterial species but not to other different species. However, some plasmids were able both to transfer and to replicate in effectively different species. Some conjugation phenomena with non-pilus mechanisms were also found in Gram-positive bacteria including *Streptomyces* [10].

Conjugation within *Streptomyces* Species

The conjugation process with non-pilus mechanism naturally appears in *Streptomyces*. It is a very distinct process from other bacteria in its molecular mechanism and its visible phenotypic appearance. It occurs through intermycelial transfer from the hyphal tips of a plasmid carrying donor to recipient [14, 15]. It seems that such single-stranded DNA transfer that appears in all conjugation systems in unicellular bacteria does not involve in *Streptomyces* conjugation.

Conjugative plasmid was transferred as a double-stranded DNA molecule [15, 16] by *tra* gene products, e.g. *traB* for pSN22 [17] and *traSA* for pSAM2 [16] during mating [18]. Then the plasmid is intramycelial spread through a septum to the next cell of the recipient mycelium. At this point, the growth is retarded in order to obtain sufficient copy number of the plasmids for the next spreading to another compartment of the mycelium [14, 19]. This delay causes a pock formation, a visible retardation zone of plasmid-acquiring recipient cells grown in a confluent lawn of plasmid-free recipients [20, 21].

Intergeneric Conjugation between *E. coli* and *Streptomyces*

Conjugal transfer from *E. coli* to Gram-positive bacteria with F plasmid-like system was demonstrated by Trieu-Cuot et al. [22]. In 1989, intergeneric conjugation from *E. coli* to *Streptomyces* was firstly described [9] and later has been developed and applied to several genera of *Actinomycetales*. This method has proved to be easy, convenient and less time-consuming compared to the original PEG-mediated protoplast transformation method.

The system of intergeneric conjugation between these two taxonomically different microorganisms is absolutely required an *oriT* and *tra* genes for transfer functions in the donor *E. coli* host. It is hypothetically explained that a plasmid was transferred from *E. coli* to *Streptomyces* by single-stranded DNA forms generated by *oriT*-endonuclease function of donor [10]. This transfer method then could overcome problems of the restriction barrier to foreign DNA in *Streptomyces* hosts by the transfer of single-stranded DNA [23].

E. coli Donor and Mobilisable Plasmids

E. coli donor strains generally used for intergeneric conjugation are the methylation proficient strain, S17-1 harbouring RP4 *tra* genes on its chromosome [9], the methylation-deficient strain, ET12567 (*dam-13::Tn9 dcm-6 hsdM*) [24] containing *tra* genes in a self-transmissible plasmid pUB307 [23] and ET12567 carrying a non-transmissible plasmid pUZ8002 [25]. pUZ8002, a helper plasmid RK2 derivative, is not efficiently transferred itself because of a mutation in its own *oriT* but can supply transfer functions to mobilise *oriT*-carrying non-conjugative replicon plasmids [26]. By using methylation deficient donor such *E. coli* ET12567, the restriction barriers of foreign DNA could be reduced and efficiency of DNA introduction could be highly increased in *Streptomyces* host [6, 23].

The plasmids used for intergeneric conjugal transfer are mobilisable plasmids that required *tra* genes function of F plasmid located on either host chromosome (e.g. *E. coli* S17-1) or helper plasmid (e.g. pUZ8002). There is no evidence yet whether a pilus mechanism is required for mating pair formation between *E. coli* and *Streptomyces*.

Non-replicative plasmids are used on propose of integration of genetic materials into *Streptomyces* chromosomes. These plasmids carry the origin of replication for *E. coli* and the *oriT* region of the broad host range conjugative plasmid RK2 [9, 10]. If the plasmids required for gene expression propose, integrase gene (*int*) and attachment site (*attP*) of *Streptomyces* phage ϕC31 were inserted in order to drive site-specific recombination at *attB* site of *Streptomyces* chromosome [27, 28]. These plasmids, for example, are pIJ8600 (*ori*pUC, *oriT*, ϕC31 *attP-int*, Apr^r, Thio^r, *tipA*p) [29], pSET152 (*ori*pUC, *oriT*, ϕC31 *attP-int*, Apr^r) [28], pIJ6902 (*ori*pUC, *oriT*, ϕC31 *attP-int*, Apr^r, Thio^r, *tipA*p) [30] and pIJ10257 (*ori*pUC, *oriT*, ϕBT1 *attB-int*, Hyg^r, *ermE*p*) [31]. If the plasmids are to use for integration by homologous recombination, no *int* and *attP* of ϕC31 was required e.g. pIJ8671 (*ori*pUC, *oriT*, Apr^r, Thio^r, *tipA*p) [29] and pSET151 (*ori*pUC, *oriT*, Thio^r) [32].

The non-replicative recombinant plasmid is forced to integrate at the target homologous gene to achieve disruption. Some shuttle plasmids were designed for replication purpose in *Streptomyces* then the origin of replication for both *E. coli* and *Streptomyces* was inserted such as pJN100 (*ori*pUC, *ori*pIJ101, *oriT*, Apr^r, *snpA*p) [33].

Pre-Treatments for Streptomycete-Actinomycete Recipients

Pre-germination of spores of *Streptomyces* recipients by heat treatment is critical for intergeneric conjugation procedure [5, 23]. The temperature and time used for spore pre-germination treatment effect on the efficiency of conjugation in individual species. In general, pre-germination of *Streptomyces* spores activated by heat treatment at 50ºC for 10 min before mating with an *E. coli* donor was recommended [5, 9, 23]. The spore pre-germination of *S. lividans* at this condition increased the efficiency of conjugation by 5-10 times [9]. It was practically used for spores of *S. albus*, *S. anibioticus*, *S. aureofaciens*, *S. bambergiensis*, *S.*

fradiae, S. gresius, S. hygroscopicus, S. lavendulae, S. lividans, S. purpureus, S. rimosus ATCC 23955, *S. venezuelae* ATCC 21405, *S. viridochromogenes* [34], *S. avermitilis* [35], *S. clavuligerus* [36], *S. coelicolor* [23], *S. diastatochromogenes* [37], *S. ipomoeae* [38], *S. nodusus* [39], *S. pristinaespiralis* [40], *S. sioyaensis* [41] and a marine *Streptomyces* sp. M095 [42] (Table 1). This condition could, furthermore, use for spore pre-germination of some rare actinomycetes such as *Amycolatopsis japonicum* [37] and *Saccharopolyspora erythraea* [35] (Table 2). However, heat treatment at 50 ºC for 10 min was not an optimal condition for pre-germination of spores for every species. To vary the conditions for each strain to maintain good spore viability for high conjugation efficiency, is highly recommended. For instance, requirement of 10 min heat treatment of spores of *S. lavendulae* FRI-5 [43], a rimocidin-producing *S. rimosus* R7 (ATCC 10970) as well as an oxytetracycline-producing *S. rimosus* M4018 [44] was at 40ºC, while *S. natalensis* was at 45ºC [45].

Some strains required longer pre-germination time such as 50 ºC for 25 min for *S. conglobatus, S. routienii* [33], *S. nodosus* [46] and *Saccharopolyspora spinosa* [33]. The medermycin-producing *Streptomyces* sp. AM-7161 even required very long 1.5–3.0 h incubation time at 50 ºC for optimal spore treatment [47]. In the case of heat-sensitive strains, e.g. *S. virginiae* [34], *Actinoplanes teichomyceticus* [48] and *Kitasatospora setae* [49], spores were used at room temperature without heat treatment. In our current study, most of *Streptomyces* strains achieved a high conjugation efficiency (10^{-2}–10^{-5} exconjugants per recipients) when spores were heat treated at 40ºC for 10 min. e.g. *S. rimosus* R7 and M4018 [44], chloramphenicol-producing *S. venezuelae* ATCC 10712 and some endophytic streptomycetes including siderophore-producing *Streptomyces* sp. GMKU 169 and *Streptomyces* sp. GMKU 3100 [50] (Table 1).

However, some strains could give reasonably high conjugation frequency without pre-germination of spores. For example, spores of *S. rimosus* R7 [44], *S. venezuelae* ATCC 10712 and an ACC deaminase-producing endophytic *Streptomyces* sp. GMKU 336 could be used immediately at room temperature without heat treatment (Table 1). There were also some other reports that no heat treatment of spores was required for *S. ambofaciens* [51], *S. albulus* [52] and *S. toyocaensis* [53]. This indicated that the heat treatment step was not prerequisite for every streptomycete species. In contrast, few exconjugants were obtained when spores of *S. pristinaespiralis* were not subjected to the heat treatment [40].

In case of poorly or non-sporulated actinomycete strains, vegetative mycelia could be considered as alternative recipients instead of spores. It is noteworthy that mycelia of such strains must appear in fine particle or fragmented not forming any pellet or clump in liquid culture otherwise mechanical homogenisation or sonication should be applied. Exponential growth (24-h) of mycelia in tryptic soy broth (TSB) of *S. rimosus* R7, M4018 [44], *S. venezuelae* ATCC 10712, endophytic *Streptomyces* sp. GMKU 336 and *Streptomyces* sp. GMKU 3100 [50] appeared naturally dispersed and moderately fragmented under the light microscope. Thus, no mechanical application was needed before mating with *E. coli* as suggested for mycelia of poorly sporulating *S. peucetius* [54].

Table 1. Summary of intergeneric conjugation parameters between *E. coli* and streptomycete strains

Streptomycete strains	Properties	Spore treatment/ mycelium	*E. coli**/plasmid	Agar medium +10 mM $MgCl_2$	Conjugation efficiency	References
S. albus G	*Sal*GI	50°C, 10 min	S17-1/pTO1	MS	5×10^{-4}	[33]
S. ambofaciens ATCC 15154	Spiramycin	No heat (RT)	pSET152	AS-1	1.4×10^{-2}	[51]
S. albulus IFO14147	ε-Poly-L-lysine	No heat (RT)	S17-1/pLAE003	ATCC no. 5 +40 mM $MgCl_2$	4×10^{-7}	[52]
S. antibioticus ATCC 23879	Oleandomycin	50°C, 10 min	S17-1/pTO1	MS	3×10^{-5}	[33]
S. aureofaciens VKPM AC 755	Chlortetracycline				5×10^{-4}	
S. avermitilis ATCC 31272	Avermectin	50°C, 10 min	S17-1	YSP	NA	[34]
S. bambergiensis ATCC 13879	Moenomycin	50°C, 10 min	S17-1/pTO1	MS	1×10^{-5}	[33]
S. clavuligerus ATCC 27064	Clavulanic acid	50°C, 10 min	pALF297	MS	1×10^{-4}	[35]
S. coelicolor A3(2)	Actinorhodin	50°C, 10 min	ET12567 (pUB307)/ pDJ70	MS	1.1×10^{-1}	[23]
S. couglobatus	-	50°C, 25 min	pJN100	MS	1×10^{-6}	[45]
S. diastatochromogenes Tu6028	Polyketomycin	50°C, 10 min	GM2163/SET152	MS	1.6×10^{-4}	[36]
S. fradiae B-45 VKPM AC 542	Tylosin	50°C, 10 min	S17-1/pTO1	MS	1×10^{-4}	[33]
S. griseus Kr.15 VKPM AC 115	Grisin				2×10^{-4}	
S. hygroscopicus ATCC 21705	Bialaphos Hygromycin				1×10^{-3}	
S. hygroscopicus ATCC 10976					1×10^{-3}	
S. ipomoeae 91-03	Sweet potato pathogen	50°C, 10 min	S17-1/pSET152	ISP 4 +20 mM $MgCl_2$	4.1×10^{-5}	[37]
S. lavendulae ATCC 19777	Phytobacteriomycin	50°C, 10 min	S17-1/pTO1	MS	1×10^{-4}	[33]
S. lividans TK64	-				1×10^{-3}	
S. lavandulae FRI-5	γ-Butylolactone IM-2	40°C, 10 min	pSET152	ISP 2	1.6×10^{-5}	[42]
S. lincolnensis ATCC 25466	Lincomycin	3-d mycelium	pUWL201apr	MS +20 mM $MgCl_2$	1.1×10^{-4}	[55]
S. natalensis ATCC 27448	Pimaricin	45°C, 10 min	pHZ1358	MS	8.55×10^{-5}	[44]
S. nodosus ATCC 14899	Amphotericin	50°C, 25 min	pSOK101	MS	5×10^{-5}	[38]
S. peucetius ATCC 29050	Daunorubicin Doxorubicin	Heat sensitive 18-h mycelium	pSET152	MS	1.5×10^{-4}	[54]

**E. coli* ET12567 (pUZ8002) unless indicated, NA = not available.

Streptomycete strains	Properties	Spore treatment/ mycelium	*E. coli**/plasmid	Agar medium +10 mM $MgCl_2$	Conjugation efficiency	References
S. pristinaespiralis ATCC 25486	Pristinamycin	50°C, 10 min	pSET152	MS +30 mM $MgCl_2$	1.36×10^{-3}	[39]
S. purpureus ATCC 21405	-	50°C, 10 min	S17-1/pTO1	MS	1×10^{-4}	[33]
S. rimosus ATCC 23955	Oxytetracycline	50°C, 10 min	S17-1/pTO1	MS	1×10^{-4}	[33]
S. rimosus R7 (ATCC10970)	Rimocidin	40°C, 10 min	pIJ8600	TSA (Oxoid)	4.21×10^{-3}	[43]
S. rimosus M4018	Oxytetracycline				5.48×10^{-2}	
S. routientii	-	50°C, 25 min	pJN100	MS	2×10^{-6}	[45]
S. sioyaensis NRRL-B5408	Siomycin	50°C, 10 min	ET12567 (pUB307)/pSOK101	ISP 3	1.1×10^{-5}	[40]
S. toyocaensis A80937	Glycopeptide A47934	No heat (RT)	S17-1/pOJ436	R2	2.2×10^{-4}	[53]
S. venezuelae ATCC 10595	Chloramphenicol	50°C, 10 min	S17-1/pTO1	MS	2×10^{-4}	[33]
S. venezuelae ATCC 10712	Chloramphenicol	40°C, 10 min	pSET152	TSA (Oxoid), MS	6.9×10^{-4}	This work
S. virginiae ATCC 13161	Virginiamycin	Heat sensitive (RT)	S17-1/pTO1	MS	2×10^{-3}	[33]
S. viridochromogenes Tu 494	Bialaphos	50°C, 10 min	S17-1/pTO1	MS	3×10–4	[33]
Streptomyces sp. AM-7161	Medermycin	50°C, 1.5-3 h	pSET152	G+Y/GYM	NA	[47]
Streptomyces sp. M095	Holomycin	50°C, 10 min	pIJ8600	MS	1.9×10^{-4}	[41]
Streptomyces sp. GMKU 169	Endophyte/Siderophore	40°C, 10 min	pIJ8600	MS	3.85×10^{-5}	This work
Streptomyces sp. GMKU 336	Endophyte/ACC	RT	pIJ8671	MS	3.61×10^{-5}	This work
Streptomyces sp. GMKU 3100	deaminase	40°C, 10 min	pIJ8600	MS	4.5×10^{-4}	[50]
Streptomyces sp. GMKU 944	Endophyte/Siderophore Endophyte	24-h mycelium	pIJ10257	ISP 4	2.18×10^{-3}	This work

**E. coli* ET12567 (pUZ8002) unless indicated, NA = not available.

Table 2. Summary of intergeneric conjugation parameters between *E. coli* and rare actinomycetes

Actinomycete strains	Properties	Spore treatment/ mycelium	*E. coli**/plasmid	Agar medium +10 mM $MgCl_2$	Conjugation efficiency	References
Actinoplanes teichomyceticus NBCR 13999	Teicoplanin	Heat sensitive (RT)	pSET152	ISP 2 +40 mM $MgCl_2$	4.9×10^{-3}	[48]
Amycolatopsis japonicum DSM 44213	Biodegradable EDTA isomer EDDS	50°C, 10 min	GM2163/pSET152	MS	$<1\times10^{-7}$	[36]
Kitasatospora setae IFO 14216	Bafilomycin B1	No heat (RT)	pSET152	ISP 2 +30 mM $MgCl_2$	1.7×10^{-6}	[49]
Microbispora corallina NRRL 30420	Microbisporicin	48-h mycelium	pSET152	V0.1	NA	[61]
Micromonospora aurantiaca W2b	Polyisoprene rubber degrading	3-d mycelium	pGM446	MS	3.6×10^{-3}	[58]
Micromonospora griseorubida A11725	Mycinamicin II	5-d mycelium	pGM446	MR0.1S	NA	[59]
Micromonospora rosaria IFO13697	Rosamicin	5-d mycelium	pSET152	R2YE	NA	[60]
Micromonospora sp. 40027	Fortimicin A	36-h mycelium	pSET152	Bennet	NA	[57]
Micromonospora sp. GMKU 358	Endophyte/ PLA degradation	36-h mycelium	pIJ8600	V0.1	1.04×10^{-3}	This work
Nonomuraea sp. ATCC 39727	Teicoplanin-like glycopeptide A40926	24-h mycelium	pSET152	V0.1	0.8×10^{-3}	[62]
Saccharopolyspora erythraea ATCC 11635	Erythromycin	50°C, 10 min	S17-1	YSP	NA	[34]
Saccharopolyspora spinosa	Spinocin A/D	50°C, 25 min	pJN100	ABB13	5×10^{-7}	[45]

**E. coli* ET12567 (pUZ8002) unless indicated, NA = not available.

All above strains were able to use either spores or mycelia for intergeneric conjugation and gave equally high efficiency. It resembled *S. fradiae* that using mycelium as a recipient gave no different efficiency from its recipient spores [28]. However, the conjugation of recipient mycelia of *S. toyocaensis* gave lower efficiency than those of recipient spores [53]. It was observed that poorly sporulating endophytic *Streptomyces* sp. GMKU 944 could achieve high conjugation efficiency by using mycelium (Table 1). Similar to a non-spore forming *S. rimosus* R6-593, a mutant of oxytetracycline-producing *S. rimosus* R6, vegetative mycelium was used successfully as recipient instead of spores [44]. On the contrary, an attempt to use mycelium of *S. natalensis* for recipient was unsuccessful [45]. In general, young mycelia (18–24 h) were preferred for intergeneric conjugation [44, 54] but *S. lincolnensis* needs 3-day growth before mating [55].

Utilising mycelia as recipients has been increasingly reported particularly from rare actinomycetes that produced low amounts of spores or none including *Actinomadura*, *Arthrobacter*, *Micromonospora*, *Nocardia*, *Nonomuraea* and *Rhodococcus* [34, 56]. Endophytic *Micromonospora* sp. GMKU 358 carrying poly L-lactic acid (PLA) degradation and fortimicin producing *Micromonospora* sp. 40027 [57] were successfully manipulated by using 36-h mycelial growth as recipient (Table 2). Some species of *Micromonospora* required a longer period of growth of mycelia up to 3-5 days [58, 59, 60] (Table 2). However, optimum condition for *Microbispora corallina* was 48-h mycelial growth [61] while *Nonomuraea* sp. ATCC 39727 required 24-h mycelium [62]. Since mycelia are relatively simple to prepare comparison of spores, it is convincingly advised to use mycelia instead of spores for routinely conjugation experiments for both streptomycetes and non-streptomycetes.

Conjugation Media

One of the important factors affecting the efficiency of intergeneric conjugation is the type of medium used for conjugation. It was demonstrated that conjugation of *E. coli* occurred at a higher frequency on solid than liquid medium [63]. Liquid medium was, however, attempted as a conjugation medium, but it was not successful for intergeneric conjugation between *E. coli* and *Streptomyces* [9]. There were several solid media earlier proposed for intergeneric conjugation such as AS1, LA, R2, SR6, SR12, TSA and YSP [9, 28, 34, 35, 51, 53]. AS1 and R2 agar media were successfully used for *S. ambofaciens* [51], *S. fradiae* [28] and *S. toyocaensis* [53]. However, some of these agar media were inappropriate for conjugation since *E. coli* would outgrow covered the whole plate after overnight incubation. After overlaying with nalidixic acid to get rid of *E. coli*, scraping a lawn of *E. coli* was rather tedious. MS agar medium [64] supplemented with 10 mM $MgCl_2$ was generally recommended as conjugative medium used effectively in many *Streptomyces* species (Table 1) including some non-streptomycetes (Table 2). However, low number or lack of exconjugants sometimes occurred in some streptomycete strains using MS agar as in the cases of *S. ipomoeae* [38] and *S. sioyaensis* [41] where ISP 4 and ISP 3 [65] were respectively replaced.

Although the MS medium supplemented with 10 mM $MgCl_2$ was efficiently used for conjugation experiment in *S. rimosus*, a number of false exconjugants was occasionally obtained due to the soybean component in MS agar that absorbs selective antibiotics and, thus lowers the actual concentration [44]. Therefore, TSA (Oxoid) containing 10 mM $MgCl_2$ was selected and gave 10 fold higher efficiency of conjugation than MS and ISP 4 [44]. Furthermore, TSA (Oxoid) could give equivalent conjugation efficiency to MS agar when used for *S. venezuelae* ATCC 10712 (Table 1). Recent investigation on conjugation media revealed other suitable media for some certain species such as ATCC no. 5 and GYM for *S. albulus* [52] and a medermycin-producing *Streptomyces* sp. AM-7161 [47], respectively.

For rare actinomycetes, finding an appropriate conjugation medium for each species is significantly important. Bennet agar was used for *Micromonospora* sp. 40027 [57], while MR0.1S and R2YE were used for *M. griseorubida* [59] and *M. rosaria* [60], respectively. Conjugative medium giving high efficiency for *M. aurantiaca* was MS (10^{-3}) but low efficiency was observed when used TS and R5 media [58]. However, endophytic *Micromonospora* sp. GMKU 358 revealed very few exconjugants on MS medium but gave high efficiency on V0.1 medium (Table 2) which was used successfully for *Microbispora corallina* [61] and *Nonomuraea* sp. ATCC 39727 [62]. ISP 2 with different concentration of $MgCl_2$ was employed for *Actinoplanes teichomyceticus* and *Kitasatospora setae* [48, 49]. Some species required specific medium such as ABB13 for *Saccharopolyspora spinosa* [33].

Conjugation media are routinely supplemented with 10 mM $MgCl_2$. Although no clear evidence regarding the function of $MgCl_2$, optimal concentration of magnesium is essential for intergeneric conjugation of some certain species [49]. If low conjugation efficiency (10^{-8}–10^{-9}) was obtained using standard protocol [5], concentration of $MgCl_2$ should be optimised to increase the efficiency. It is noteworthy that high concentrations of $MgCl_2$ could severely inhibit growth and spore formation [49]. *S. ipomoeae* required 20 mM $MgCl_2$ in ISP 4 to reach good efficiency [38], while *S. lincolnensis* needed 20 mM $MgCl_2$ in MS [55]. 30 mM $MgCl_2$ was practically used for *S. pristinaespiralis* and *Kitasatospora setae* and could increase 10 times efficiency [40, 49]. *S. albulus* and *Actinoplanes teichomyceticus* required concentration of $MgCl_2$ as high as 40 mM in their conjugal media to achieve desired efficiency [48, 52].

Intergeneric Conjugation Procedure and Related Parameters

Intergeneric conjugation performs by mating an appropriate ratio of an exponential *E. coli* donor (OD_{600} = 0.4–0.6) and recipient spores/mycelia of actinomycetes and selecting the exconjugants on the agar medium with suitable antibiotics [5]. The conjugation efficiency was calculated as the number of exconjugants per recipients. The number of exconjugants per recipients about 10^{-2}–10^{-4} is considered as high efficiency, while 10^{-7}–10^{-9} is supposed as low. Actinomycete strains should be examined for their resistances to antibiotics corresponding to the resistance genes located in the target mobilisable plasmids. Apramycin and thiostrepton resistance genes were commonly used as resistant markers in those plasmids [28, 29, 30, 32, 33].

The spore concentration of *Streptomyces* should be adjusted from 10^3 to 10^8 to obtain discrete colonies on the conjugation plates [23]. For instance, 10^3–10^4 spores concentration of *S. rimosus* was used for mating to avoid patchy lawns on conjugation media that gave some false exconjugants [44]. In the case of using mycelia, concentration of mycelia should also be adjusted. For example, exponential phase mycelium of *S. rimosus* at 10^4 cfu was mated with *E. coli* donor [44].

The ratio of recipient to donor is another factor affected to the efficiency of conjugation. For example, the efficiency of conjugation was increased from 10^{-8} to 10^{-5} exconjugants/recipients when decreasing the donor/recipient ratio for conjugal transfer of pSET152 from *E. coli* ET12567 (pUZ8002) to *S. lavendulae* FRI-5 on ISP 2 medium, but did not affect the frequency of conjugation on MS medium [43]. Similar results were observed in *S. natalensis* [45] and *Kitasatospora setae* (10^{-8} to 10^{-6}) [49] where an excess of donor cells is required to achieve the highest conjugation frequency. However, decreasing the donor/recipient ratio affected the efficiency of conjugation between *E. coli* S17-1 (pOJ436) and *S. toyocaensis* on R2 medium [53]. The conjugation frequency significantly enhanced when number of donor cells of *S. lincolnensis* was increased [55]. In contrast to what occured in *S. coelicolor* and *S. nodosus*, where the number of donor cells per recipient did not affect the efficiency of DNA transfer [23, 39].

After mating on agar medium, incubation period for sufficient growth of actinomycetes at 28–30 ºC is necessary before flooding the agar plates with nalidixic acid and other selective antibiotics. In general, about 16–20 h of incubation is suitable for several streptomycete strains. Rare actinomycetes may take longer incubation time such as endophytic *Micromonospora* sp. GMKU 358 required 24-h incubation before drug overlay.

The incubation temperature also effected the growth of exconjugants in some species. For example, incubation at 37 ºC of conjugation plates between *E. coli* S17-1 and *S. fradiae* gave three times higher efficiency than at 29 ºC [9]. Vector type and size also affected the efficiency of conjugation. In general, the conjugation with small plasmids (approx. 5 kb) has 100–1000 times higher efficiency than that with large plasmids (approx. 20 kb) or cosmids (40–50 kb) [28]. It is advised to test the efficiency of various types of plasmids for effective conjugation for each actinomycete species.

Applications of Intergeneric Conjugation in Actinomycetes

Conjugal transfer by mobilisable plasmids into actinomycete hosts continue to be important in genetic analysis and are routinely used to transfer recombinant plasmids for gene expression or target gene disruption. This genetic transfer system from *E. coli* to actinomycetes enable simple and prospective approach to identify and analyse the function of biosynthetic pathways for medicinally interesting metabolites and precisely manipulate the biosynthesis by pathway engineering of homologous and heterologous genes [61, 66].

Disruption of some target genes through intergeneric conjugation makes verification of the function of a particular gene/gene cluster possible. For instance, disruption of *mycAV* gene in *Micromonospora griseorubida* indicated that it encoded one of the multifunctional mycinamicin PKS genes required during mycinamicin production [59]. When *mycAV* gene was transferred back to the mutant through intergeneric conjugation, complementation was successful [59]. An expression integrative plasmid, pJN100, was used in a complementation experiment of a mutant of *S. griseus* disrupted in the *nonM* gene encoding a reductase essential for nonactin biosynthesis [33]. Disruption of *desD*-like gene in siderophore producing endophytic *Streptomyces* sp. GMKU 3100 could demonstrate the important function of this siderophore trait to plant growth enhancement in rice and mungbean plants [50].

Intergeneric conjugation is used to introduce a gene/gene cluster of interest into the chromosome of an actinomycete host via site specific recombination through actinophage attachment-integrase system located on the integrative plasmids. By this approach, the resistance gene *ptr* copies based on pSET152 was introduced into pristinamycin producing *S. pristinaespiralis* and revealed a dramatic increase in pristinamycin production [40]. New mycinosyl rosamicin-derivatives were obtained when mycinose biosynthesis genes presented in *Micromonospora griseorubida* were introduced via intergeneric conjugation into the rosamicin producing *M. rosaria* IFO13697 [60]. It was demonstrated function of genes responsible for stimulation of NPP biosynthesis of *Pseudonocardia autotrophica* by successfully expression of those genes under the influence of the strong constitutive *ermE** promoter in the integrative expression pSET152 vector [67]. After conjugal transfer system was established for the rimocidin producing *S. rimosus* R7, pIJ8655 containing the *egfp* gene under the control of the thiostrepton-inducible *tipA* promoter was introduced and revealed a high level of fluorescence [44].

Furthermore, a polyester-degrading hydrolase gene from *Thermobifida* sp. BCC23166 was introduced and expressed in *S. rimosus* R7 under *tipA* promoter in pIJ8600 [68]. The biosynthetic gene cluster for microbisporicin, a potent lantibiotic produced by *Microbispora corallina*, was identified by genome scanning [61]. *MibA* gene in the gene cluster was replaced with an apramycin resistance cassette by PCR targeting method [69] and the construct was introduced into *M. corallina* by intergeneric conjugation from ET12567 pUZ8002, and the result indicated that *mibA* gene is essential for microbisporicin biosynthesis [61]. There have been more research works carrying on using intergeneric conjugation procedure to transfer gene/gene cluster and study of gene/gene cluster functions in several actinomycete species.

Conclusion

The development of intergeneric conjugation for genetic exchange system in actinomycetes has become a powerful method to manipulate genes/gene clusters of these pharmaceutically important bacteria. To study the conjugative system of a particular strain, nature of recipients as spores/mycelia has to be examined. Types of *E. coli* donor and mobilisable plasmids must be selected for suitable conjugal transfer with actinomycete species.

Other parameters including the type of conjugation media and concentration of magnesium chloride should be investigated to achieve high conjugation efficiency. Such developed gene transfer system will allow the performance of genetic analysis (e.g. gene expression, target gene disruption) in desired host. The advantages of this genetic transfer system from *E. coli* to actinomycetes allow identification and functional analysis of biosynthetic pathways for pharmaceutically interesting metabolites and the target manipulation of the biosynthesis by pathway engineering of homologous and heterologous genes. Conjugative genetic exchange will provide the means to modify and/or construct strains for novel biotechnological applications.

Acknowledgments

Work from our laboratory related to the material discussed in this chapter was supported by the National Center for Genetic Engineering and Biotechnology (BIOTEC), NSTDA, Thailand Research Fund (TRF), Kasetsart University Research and Development Institute (KURDI) and Center for Advanced Studies in Tropical Natural Resources, National Research University-Kasetsart University (CASTNAR, NRU-KU).

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The Journal of Antibiotics (2013) 66, 675–677
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www.nature.com/ja

NOTE

Linfuranone A, a new polyketide from plant-derived *Microbispora* sp. GMKU 363

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The Journal of Antibiotics (2013) **66,** 675–677; doi:10.1038/ja.2013.67; published online 3 July 2013

Keywords: endophyte; furanone; *Microbispora*; polyketide

Actinomycetes are well-known producers of an enormous variety of secondary metabolites, many of which have beneficial applications in the field of medicine and agriculture. More recently, endophytic actinomycetes residing in plants revealed the potential sources of biodiversity carrying a variety of bioactive metabolites and acting as potential biocontrol agents.[1–3] Particularly, endophytic actinomycetes isolated from tropical plants have been examined to possess significant biosynthetic potential, particularly *polyketide synthase* and *nonribosomal peptide synthetase* genes.[4] We have recently reported a new polyketide compound from an endophytic actinomycete isolated from a Thai medicinal plant collected at the Eastern Botanical Garden (Khao Hin Son), Chachoengsao province, Thailand.[5] Here, we now report a new furanone-containing polyketide, linfuranone A (Figure 1), produced by an endophytic actinomycete isolated from a Thai medicinal plant collected at the same location.

An endophytic *Microbispora* sp. GMKU363 was isolated from a root of Thai medicinal plant 'Lin Ngu Hao' (*Clinacanthus siamensis* Bremek.) according to the reported protocol.[6] The strain was identified as a member of the genus *Microbispora* on the basis of 99.9% 16S ribosomal RNA gene sequence identity (1387 nucleotides; GenBank accession number GU459171) with the *Microbispora mesophila* JCM 3151^T type strain (accession number AF002266).

Strain GMKU363 was cultured on Bn-2 slant agar medium consisting of soluble starch 0.5%, glucose 0.5%, meat extract (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) 0.1%, yeast extract (Difco Laboratories, Detroit, MI, USA) 0.1%, NZ-case (Wako Pure Chemical Industries, Ltd., Osaka, Japan) 0.2%, NaCl 0.2%, $CaCO_3$ 0.1%, agar 1.5% and was inoculated into 500-ml K-1 flasks each containing 100 ml of the V-22 seed medium (pH 7.0) consisting of soluble starch 1%, glucose 0.5%, NZ-case 0.3%, yeast extract 0.2%, triptone (Difco Laboratories) 0.5%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05% and $CaCO_3$ 0.3%. The cultures were cultivated on a rotary shaker (200 r.p.m.) at 30 °C for 4 days. The seed culture (3 ml) was transferred into 500-ml K-1 flasks each containing 100 ml of the A-11M production medium (pH 7.0) consisting of soluble starch 2.5%, glucose 0.2%, yeast extract 0.5%, polypeptone (Wako Pure Chemical Industries, Ltd.) 0.5%, NZ-amine 0.5%, $CaCO_3$ 0.3% and Diaion HP-20 (Mitsubishi Chemical Co.) 1%. The cultures inoculated in flasks were cultured on a rotary shaker (200 r.p.m.) at 30 °C for 6 days, and the whole culture broth was extracted with 100 ml of 1-butanol on each flask by shaking for an additional hour. The organic layer was evaporated to give 3.0 g of crude extract from 1.5 l of culture. The crude extract (3.0 g) was subjected to silica gel column chromatography with a step gradient of $CHCl_3$-MeOH (1:0, 20:1, 10:1, 4:1, 2:1, 1:1 and 0:1 v/v). The concentration of the fraction eluted with a 2:1 mixture of $CHCl_3$-MeOH provided 0.23 g of dark viscous oil, which was further purified by preparative HPLC (Cosmosil AR-II, San Diego, CA, USA, $250 \times 10\,mm^2$) using 30% MeCN in distilled water at 4 ml min^{-1} to give linfuranone A (2.0 mg, $t_R = 12.1$ min).

Linfuranone A (Figure 1) was obtained as an optically active ($[\alpha]_D^{22}$ −9.9 (*c* 0.16, MeOH)), colorless and amorphous compound that gave an $[M+Na]^+$ peak at *m/z* 417.2257 in the high resolution electrospray ionization time-of-flight mass spectrometry appropriate for a molecular formula of $C_{22}H_{34}O_6$, (calculated for $C_{22}H_{34}O_6Na$, 417.2248), which was consistent with the ^1H and ^{13}C NMR data (Table 1). The IR spectrum of linfuranone A showed absorption bands for hydroxyl (3333 cm^{-1}) and carbonyl (1691 cm^{-1}) functionalities. The UV spectrum showed absorption maxima at 282 (ε 23 600) and 232 (ε 75 300) in MeOH. ^{13}C NMR and HMQC spectral data confirmed the presence of 22 carbons, which were assigned to two oxygenated-quaternary sp^2 carbons, seven olefinic carbons (five are proton-bearing), one quaternary sp^3 carbon, four sp^3 methines (three are oxygen-bearing), three sp^3 methylenes and five methyl carbons.

Analysis of the COSY spectrum led to the identification of four proton-bearing fragments, H-17–H-19, H-15/H-14/H-21, H-12/H-13 and H-6–H-11 (Figure 1). HMBC correlations were detected from the

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Received 21 March 2013; revised 12 May 2013; accepted 23 May 2013; published online 3 July 2013

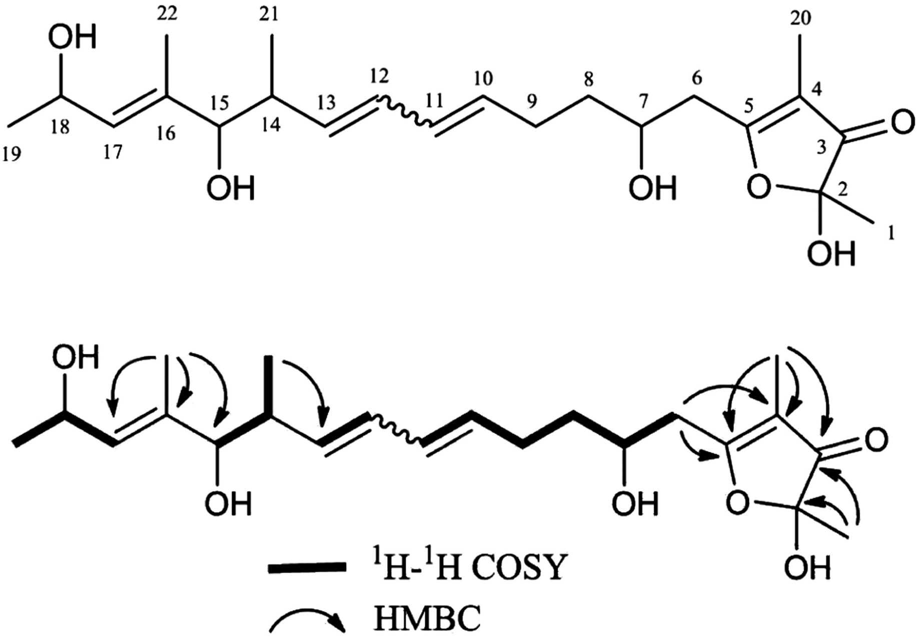

Figure 1 Structure of linfuranone A, ^{1}H–^{1}H COSY and key HMBC correlations.

Table 1 ^{1}H and ^{13}C NMR data for linfuranone A in CD_3OD

No	δ_C[a]	δ_H *mult (J in Hz)*[b]	*HMBC*[b,c]
1	22.3/22.4, CH_3	1.43/1.44, s	2, 3
2	104.37/104.39, qC		
3	205.5, qC		
4	110.3, qC		
5	187.2, qC		
6	38.5, CH_2	2.65, m	4, 5, 7, 8
		2.72, m	4, 5, 7, 8
7	69.6, CH	3.96, m	
8	38.2/38.3, CH_2	1.61, m	7, 9, 10
9	29.9, CH_2	2.18, m	7, 8, 10
		2.24, m	7, 8, 10
10	132.74/132.79,[d] CH	5.59, m	11, 12
11	132.68/132.70,[d] CH	6.06, m	
12	132.00/132.02, CH	6.06, m	
13	136.3/136.4, CH	5.58, m	11, 12
14	41.8, CH	2.31, m	13, 15, 21
15	83.1, CH	3.63, d (8.4)	13, 14, 17, 21, 22
16	138.0, qC		
17	133.5, CH	5.35, d (8.4)	18, 22
18	65.2, CH	4.56, dq (8.4, 6.4)	16
19	22.9, CH_3	1.19, d (6.4)	17, 18
20	6.0, CH_3	1.67, s	3, 4, 5
21	18.0, CH_3	0.86, d (7.0)	13, 14, 15
22	11.6, CH_3	1.64, d (1.1)	15, 16, 17

[a] Recorded at 100 MHz.
[b] Recorded at 500 MHz.
[c] HMBC correlations are from proton(s) stated to the indicated carbon.
[d] Interchangeable signals.

singlet methyl protons H-22 to C-15, C-16 and C-17, establishing the connectivity from C-17 to C-15 and the methyl substitution at the quaternary sp^2 carbon C-16. This fragment was connected to the H-12/H-13 fragment on the basis of an HMBC correlation from H-21 to C-13, providing an eight-carbon fragment bearing two oxygen substitutions at C-15 and C-18. The last COSY-defined fragment was connected to the α,β-unsaturated ketone substructure bearing an oxygen substitution at β-position on the basis of a series of HMBC correlations from H-6 to C-4 and C-5, and from a vinyl methyl H-20 to C-3, C-4, and C-5. The carbonyl carbon C-3 was correlated with H-1, which also showed a correlation to the oxygenated carbon C-2 (δ 104.4), establishing the attachment of the two-carbon fragment C-1/C-2 to C-3. The chemical shift of C-2 was suggestive of the bonding of two oxygen atoms to this carbon. Finally, considering of the molecular formula and the remaining unsaturation degree provided the connectivity between C-11 and C-12, and the placement of an oxygen atom between C-2 and C-5 to establish this molecule as a new member of furanone-containing polyketides. *E* configuration for the double bond between C-16 and C-17 was confirmed by NOEs between H-18 and H-22, and H-15 and H-17. The geometry of C-10/C-11 and C-12/C-13 double bonds could not be assigned due to the proton signal overlapping. As two possible configurations exist at the C-2 hemiketal carbon, two signals corresponding to the two diastereomers were observed for H-1 and several carbons (Table 1). Absolute stereochemistry of linfuranone A is under investigation.

Linfuranone A is a relatively rare 3-furanone derived from polyketide, with a hemiketal at C-2 and an unsaturated alkyl chain at C-5. There are a few known structurally close metabolites isolated from *Streptomyces* spp., which are E837, E492, E975 and actinofuranones A and B.[7,8] Further related structures are not from actinobacterial origin, but were isolated from myxobacteria, fungi and marine molluscs.[9–13] E837, E492 and E975 exhibited inhibition against helminth NADH-fumarate reductase and bovine heart NADH oxidase,[7] whereas actinofuranones had no display of biological activity.[8] Biological screening of linfuranone A in diverse bioassays indicated that this compound was active in an assay designed to screen antidiabetic and antiatherogenic activities using mouse ST-13 pre-adiopocytes.[14] By the treatment with 50 μM linfuranone A, 47% of pre-adipocytes were differentiated into the matured adipocytes and accumulated the lipid droplets. Linfuranone A displayed no appreciable activities in antimicrobial and cytotoxic assays.

ACKNOWLEDGEMENTS

CI was awarded PhD scholarship from the Commission on Higher Education, Ministry of Education, Thailand. This study was supported by Thailand Research Fund under grant number DBG5480002 and Faculty of Science, Burapha University.

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International Journal of Systematic and Evolutionary Microbiology (2015), **65**, 000–000 **DOI** 10.1099/ijs.0.000085

Nonomuraea syzygii sp. nov., an endophytic actinomycete isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels)

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1 A novel endophytic actinomycete, designated strain GKU 164^T, was isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels), collected at Khao Khitchakut National Park, Chantaburi province, Thailand. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strain formed a distinct clade within the genus *Nonomuraea*, and was most closely related to *Nonomuraea monospora* PT708^T (98.77 % 16S rRNA gene sequence similarity) and *Nonomuraea thailandensis* KC-061^T (98.73 %). Strain GKU 164^T formed a branched substrate and aerial hyphae that generated single spores with rough surfaces. The cell wall contained *meso*-diaminopimelic acid. The whole-cell sugars were madurose, galactose, mannose, ribose, rhamnose and glucose. The *N*-acyl type of muramic acid was acetyl. The predominant menaquinone was MK-9(H_4) with minor amounts of MK-9(H_6), MK-9(H_2) and MK-9(H_0). The phospholipid profile contained diphosphatidylglycerol, phosphatidylethanolamine, hydroxy-phosphatidylethanolamine, phosphatidylglycerol, phosphatidylinositol, phosphatidylinositolmannosides, phosphatidylmonomethylethanolamine, hydroxy-phosphatidylmonomethylethanolamine, an unidentified aminophosphoglycolipid and four unknown phospholipids. The major fatty acids were iso-$C_{16:0}$ and 10-methyl $C_{17:0}$. The genomic DNA G+C content was 70.4 mol%. Significant differences in the morphological, chemotaxonomical, and biochemical data together with DNA–DNA relatedness values between strain GKU 164^T and type strains of closely related species, clearly demonstrated that strain GKU 164^T represents a novel species of the genus *Nonomuraea*, for which the name *Nonomuraea syzygii* sp. nov. is proposed. The type strain is GKU 164^T (=BCC 70457^T=NBRC 110400^T).

The genus *Nonomuria* (sic) was firstly proposed by Zhang *et al.* (1998) belonging to the family *Streptosporangiaceae*, but the genus name was later corrected to *Nonomuraea* by Chiba *et al.* (1999). Members of the genus *Nonomuraea* form extensively branched substrate and aerial mycelia. The aerial mycelia differentiate into either hooked, spiral or straight chains of spores, or form a single spore, which shows a folded, irregular, smooth, warty or rough surface ornamentation (Quintana *et al.*, 2003; Kämpfer *et al.*, 2005; Zhao *et al.*, 2011; Zhang *et al.*, 2014b). The type species of the genus is *Nonomuraea pusilla* (Nonomura & Ohara, 1971; Zhang *et al.*, 1998). At the time of writing, the genus comprises ~~38~~ species with validly published names and

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Abbreviations: DPG, diphosphatidylglycerol; ISP, International *Streptomyces* Project; OH-PE, hydroxy-phosphatidylethanolamine; OH-PME, hydroxy-phosphatidylmonomethylethanolamine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PIM, phosphatidylinositolmannoside; PME, phosphatidylmonomethylethanolamine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GKU 164^T is KF667499.

Two supplementary figures and two supplementary tables are available with the online Supplementary Material.

two subspecies, including the newly described *Nonomuraea muscovyensis* (Ozdemir-Kocak *et al.*, 2014) and *Nonomuraea fuscirosea* (Zhang *et al.*, 2014a). Three further species, '*Nonomuraea guangzhouensis*' and '*Nonomuraea harbinensis*' (Wang *et al.*, 2014), and '*Nonomuraea shaanxiensis*' (Zhang *et al.*, 2014b), have also been recently described but the names are not yet validly published. Members of the genus *Nonomuraea* have been isolated from a wide range of natural habitats such as soils, including terrestrial and mangrove rhizospheric soil, coastal sediments and caves, and plants (Wang *et al.*, 2011, 2013, 2014; Xi *et al.*, 2011; Zhao *et al.*, 2011; Nakaew *et al.*, 2012; Zhang *et al.*, 2014b). Some novel species have been also isolated from soil in Thailand, namely *Nonomuraea monospora* (Nakaew *et al.*, 2012) and *Nonomuraea thailandensis* (Sripreechasak *et al.*, 2013), and some species have been found as endophytes, for example *Nonomuraea antimicrobica* (Qin *et al.*, 2009) and *Nonomuraea endophytica* (Li *et al.*, 2011). In this study, we performed a taxonomic analysis of strain GKU 164^{T} using a polyphasic approach, and propose that the strain represents a novel species of the genus *Nonomuraea*.

As part of a program to discover novel endophytic actinomycetes from medicinal plants, strain GKU 164^{T}, was recovered from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels) collected at Khao Khitchakut National Park, Chantaburi province, Thailand. The excised roots were surface-sterilized and endophytic actinomycetes were isolated using the modified method of Indananda *et al.* (2010). The sample roots were cut into small pieces of 2 cm in length and then rinsed with 0.1 % (v/v) Tween 20 solution for 5 min. The root surface was sterilized by soaking in 1 % sodium hypochlorite for 15 min and subsequently immersing in 70 % ethanol for 10 min. Surface-sterilized roots were then washed in sterile water three times. The root materials were then soaked in 10 % (w/v) $NaHCO_3$ solution for 10 min to delay the growth of fungi. The roots were then crushed in 1/4 Ringer's solution and the resulting solution was spread onto starch-casein agar (SCA; Küster & Williams, 1964) supplemented with 100 µg ml^{-1} ampicillin, 2.5 U ml^{-1} penicillin G, 50 µg ml^{-1} amphotericin B and 50 µg ml^{-1} cycloheximide. The remaining root debris was also placed on SCA. Colonies of endophytic actinomycetes appeared on the agar after incubation at 28 °C for 3–4 weeks. Colonies of strain GKU 164^{T} were purified on mannitol-soya (MS) agar (Hobbs *et al.*, 1989). The pure culture was maintained in 20 % (v/v) glycerol suspensions at −80 °C and as lyophilized cells for long-term preservation.

Genomic DNA of strain GKU 164^{T} was extracted by the method of Kieser *et al.* (2000). The 16S rRNA gene was amplified using primers STR1F (5′-TCACGGAGAGTTTGATCCTG-3′) and STR1530R (5′-AAGGAGATCCAGCCGCA-3′) (Kataoka *et al.*, 1997). The PCR program was as follows: initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min 30 s and extension at 72 °C for 1 min, with a final extension step at 72 °C for 4 min. The PCR product was purified using a Gel/PCR DNA Fragment Extraction kit (Geneaid) and then sent to Macrogen (Korea) for DNA sequencing using primers STR1F, STR1530R, 926F (5′-AAACTCAAAGGAATTGACGG-3′) (Tajima *et al.*, 2001) and ATT026R (5′-TGGACTACCAGGGTATCTAATC-3′). The resulting 16S rRNA gene sequence of strain GKU 164^{T} was compared with corresponding sequences of type strains available in the EzTaxon-e server (Kim *et al.*, 2012). A multiple sequence alignment of strain GKU 164^{T} and other related taxa was performed using the CLUSTAL X program version 2.0 (Larkin *et al.*, 2007). Phylogenetic trees were reconstructed using neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) algorithms in MEGA software version 6.0 (Tamura *et al.*, 2013). The topology of the phylogenetic trees was evaluated with bootstrap analysis based on 1000 resamplings (Felsenstein, 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset. The values of pairwise 16S rRNA gene sequence similarity among strain GKU 164^{T} and other related species were determined using the EzTaxon-e sever.

Comparison of the almost-complete 16S rRNA gene sequence (1448 nt) of strain GKU 164^{T} with those of related strains indicated that strain GKU 164^{T} was a member of the genus *Nonomuraea*. The most closely related strains were *N. monospora* $PT708^{\mathrm{T}}$ (98.77 % 16S rRNA gene sequence similarity; 17 nt difference at 1380), *N. thailandensis* KC-061^{T} (98.73 %; 18 nt difference at 1422), *Nonomuraea solani* NEAU-$Z6^{\mathrm{T}}$ (98.68 %; 19 nt difference at 1442), *Nonomuraea rhizophila* YIM 67092^{T} (98.34 %; 24 nt difference at 1444), '*N. shaanxiensis*' NEAU-st1 (98.20 %; 26 nt difference at 1442) and *Nonomuraea rosea* GW 12687^{T} (98.04 %; 27 nt difference at 1375). 16S rRNA gene sequence similarity values of strain GKU 164^{T} with strains of other species of the genus *Nonomuraea* were lower than 98 %. The neighbour-joining tree was in agreement with the maximum-likelihood tree in which strain GKU 164^{T} always formed a closely phyletic line with *N. monospora* $PT708^{\mathrm{T}}$ and *N. thailandensis* KC-061^{T} [Figs 1 and S1 (available in the online Supplementary Material)].

The morphological characteristics of strain GKU 164^{T} were observed by light and scanning electron microscopy (model JSM-5410; JEOL) using cultures grown on Czapek's agar (CZA; Atlas, 2004) at 28 °C for 30 days. Cultural characteristics of strain GKU 164^{T} were determined after incubation at 28 °C for 21 days on modified Bennett's agar (MBA; Jones, 1949), nutrient agar (NA; Difco), yeast extract-starch agar (YS; JCM medium 61), CZA and various International *Streptomyces* Project (ISP) media (Shirling & Gottlieb, 1966): yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7). Colours were designated by comparison with colour strips from the Colour Harmony Manual (Jacobson *et al.*, 1958). The

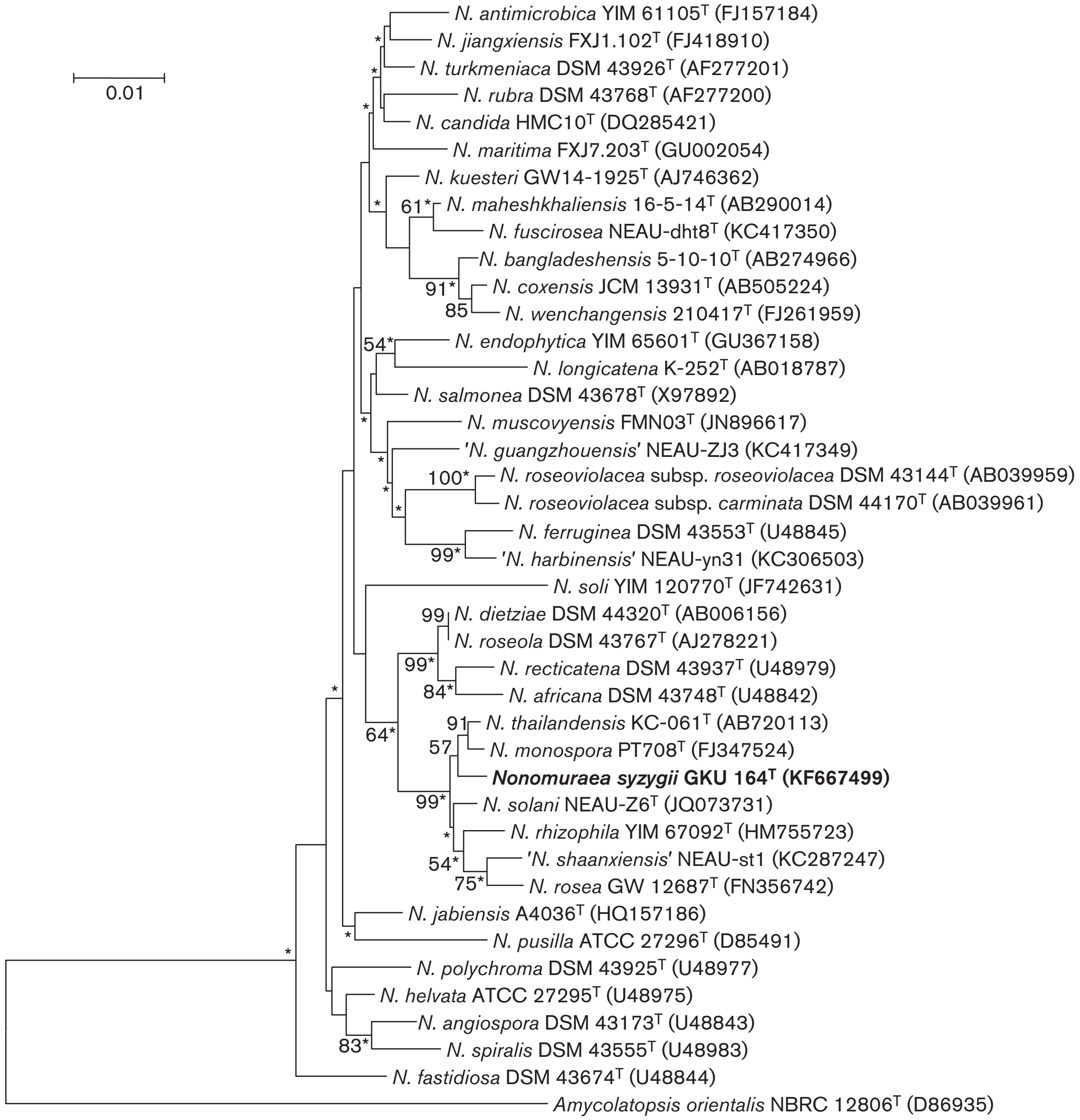

Fig. 1. Neighbour-joining tree based on almost-complete 16S rRNA gene sequences, showing the relationship between strain GKU 164^{T} and all recognized species of the genus *Nonomuraea*. *Amycolatopsis orientalis* NBRC 12806^{T} (GenBank accession no. D86935) was used as an outgroup. Asterisks denote branches that were also recovered from maximum-likelihood tree. Numbers at branch points indicate bootstrap percentages (based on 1000 replications); only values >50 % are shown. Bar, 0.01 substitutions per nucleotide position.

range of temperature (5–50 °C), pH (4–11) and NaCl (1–7 %) for growth tolerance of strain GKU 164^{T} was examined on NA for 14–21 days. Catalase and oxidase activities were observed with 3 % (v/v) hydrogen peroxide solution and 1 % (v/v) tetramethyl-ρ-phenylenediamine solution, respectively. Reduction of nitrate was observed using nitrate broth (Difco). Starch hydrolysis was examined on ISP 4 medium. Urease activity was determined based on a colour change in Stuart's urea agar (Stuart *et al.*, 1945). Gelatin liquefaction was evaluated on glucose-peptone-gelatin medium (2.0 % glucose, 0.5 % peptone, 20 % gelatin; pH 7.0). Coagulation and peptonization of milk were observed in 10 % (w/v) skimmed milk broth (Difco). Hydrolysis of DNA was tested on DNase agar (Difco). Production of H_2S and melanin pigment were determined on ISP 6 and ISP 7 media, respectively. Citrate utilization was tested on Simmons' citrate agar (Difco). Decomposition of casein, xanthine, hypoxanthine and L-tyrosine were evaluated by using the media described by Gordon *et al.* (1974). Utilization of carbon sources was examined on ISP 9 medium (Shirling & Gottlieb, 1966) supplemented with each filter-sterilized carbon source used at a final concentration of 1 % (w/v). Enzyme activity profiles were tested by using the API ZYM system (bioMérieux) according to the manufacturer's instructions.

51 ~~Aerial and~~ substrate mycelia of strain GKU 164^T were developed without fragmentation. Sporangia were not found. Strain GKU 164^T showed good growth on ISP 2, ISP 3, ISP 4, MBA, NA and YS agar, but no growth was observed on ISP 5. Substrate mycelia were abundantly produced on most of the media tested and the colours were variable depending on the growth medium (Table S1). No soluble pigment was detected on any of the media tested. Aerial mycelium was absent on most of the media except for CZA, where poor growth was observed after incubation at 28 °C for 21 days. Non-motile spores were borne singly and the spore surface was rough (Fig. 2). The temperature and pH range for growth of strain GKU 164^T were 10–36 °C and pH 5–9, with optimum growth at 18–32 °C and pH 7–8. Other physiological and biochemical characteristics of strain GKU 164^T were compared to strains of closely related species and revealed the differences from *N. monospora* PT708^T, *N. thailandensis* KC-061^T, *N. solani* NEAU-Z6^T, *N. rhizophila* YIM 67092^T and '*N. shaanxiensis*' NEAU-st1 (Table 1). Strain GKU 164^T possesses single spores similar to those of *N. monospora* PT708^T, *N. solani* NEAU-Z6^T and '*N. shaanxiensis*' NEAU-st1 but the spore surface is rough while others are smooth.

Biomass for chemotaxonomic studies was obtained from cultures grown in yeast extract-glucose broth (1 % yeast extract, 1 % glucose; pH 7.0) at 27 °C for 7 days and then cells were freeze-dried. The isomer of diaminopimelic acid in the cell wall was determined by TLC using whole-cell hydrolysates according to the method of Hasegawa *et al.* (1983). The whole-cell sugars composition was analysed by TLC using the method of Becker *et al.* (1965). The *N*-acyl type of muramic acid in the peptidoglycan was examined by the method of Uchida & Aida (1984). The presence of mycolic acids was monitored by TLC following the procedure of Tomiyasu (1982). Isoprenoid quinones were extracted and purified by using the method of Collins *et al.* (1977) and analysed by LC/MS (JSM-T100LP; JEOL). Analyses of phospholipids and whole-cell fatty acids using the freeze-dried cells were carried out by the service at Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Thailand. Phospholipids in cells were extracted and identified by two-dimensional TLC as described by Minnikin *et al.* (1984). Cellular fatty acids were extracted, methylated and analysed by using the Microbial Identification System (MIDI) according to the method of Sasser (1990) and the manufacturer's instructions. Fatty acid methyl esters were analysed by GLC and using the Microbial Identification software package (Sherlock version 6.1; MIDI database RTSBA6). The G+C content of genomic DNA of strain GKU 164^T, which was extracted according to the method of Marmur (1961), was determined by HPLC according to the procedure of Tamaoka & Komagata (1984).

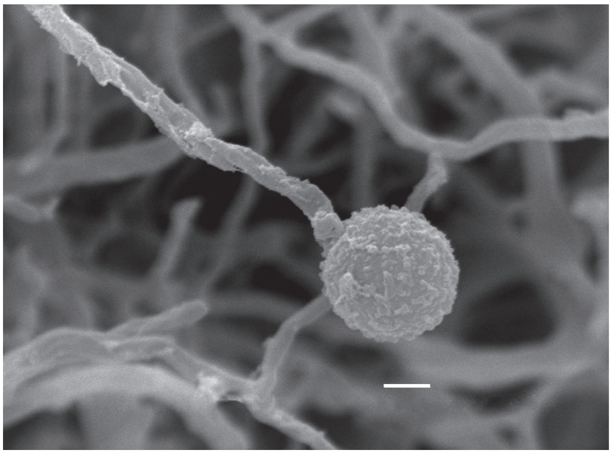

Fig. 2. Scanning electron micrograph of a single spore on aerial mycelia of strain GKU 164^T grown on Czapek's agar at 28 °C for 30 days. Bar, 1 μm.

Chemotaxonomic properties of strain GKU 164^T revealed typical characteristics of members of the genus *Nonomuraea*. The strain contained *meso*-diaminopimelic acid as the diagnostic diamino acid in the cell-wall peptidoglycan. Madurose, galactose, mannose, ribose, rhamnose and glucose were detected as the diagnostic sugars in the whole-cell hydrolysates. The presence of *meso*-diaminopimelic acid and madurose indicated that cell wall and whole-cell sugar of strain GKU 164^T was of type III and type B, respectively (Lechevalier & Lechevalier, 1970). The *N*-acyl type of muramic acid in the peptidoglycan was acetyl. Mycolic acids were not detected. The predominant menaquinone was MK-9(H_4) (76 %), while minor amounts of MK-9(H_6) (10 %), MK-9(H_2) (9 %) and MK-9(H_0) (5 %) were also detected. The diagnostic phospholipids were diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), hydroxy-phosphatidylethanolamine (OH-PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylinositolmannosides (PIMs), phosphatidylmonomethylethanolamine (PME), hydroxy-phosphatidylmonomethylethanolamine (OH-PME), an unidentified aminophosphoglycolipid and four unknown phospholipids (Fig. S2). The phospholipid pattern of strain GKU 164^T corresponded to type IV (Lechevalier *et al.*, 1977). The major cellular fatty acids were iso-$C_{16:0}$ (31.8 %) and 10-methyl $C_{17:0}$ (25.0 %) which were generally similar to those of recognized members of the genus *Nonomuraea* (Qin *et al.*, 2009; Li *et al.*, 2011; Zhao *et al.*, 2011; Nakaew *et al.*, 2012; Sripreechasak *et al.*, 2013); the minor fatty acids are shown in Table S2. The genomic DNA G+C content of strain GKU 164^T was 70.4 mol%.

According to phenotypic and genotypic results, strain GKU 164^T was evidently different from type strains of

Table 1. Comparison of the phenotypic properties of strain GKU 164^T and type strains of closely related species of the genus *Nonomuraea*

Strains: 1, GKU 164^T (data from this study); 2, *N. monospora* PT708^T (this study); 3, *N. thailandensis* KC-061^T (this study); 4, *N. solani* NEAU-Z6^T (Wang *et al.* 20 5); 5, *N. rhizophila* YIM 67092^T (Zhao *et al.* 20); 6, '*N. shaanxiensis*' NEAU-st1 (Z ang *et al.* 2014b, ND, No data available; +, positive; −, negative; w, weakly positive.

Characteristic	1	2	3	4	5	6
Spore morphology						
Arrangement	Single	Single	Spiral	Single	Spiral	Single
Ornamentation	Rough	Smooth	Smooth	Smooth	Rough	Smooth
Number of spores	1	1	>10	1	7-10	1
Growth on ISP 3						
Aerial mycelium	None	Greyish-brown	Reddish-grey	White	White	None
Substrate mycelium	Greyish-red	Cuba	Violet brown	White	Brown–yellow	Yellowish-grey
Soluble pigment	None	Reddish-brown	Brownish-grey	None	None	None
Temperature for growth (°C)	10–36	ND	14–38	20–39	10–37	18–28
pH range for growth	5–9	5–9	5–9	7–9	6–8	6–9
Maximum NaCl tolerance (%)	3	3	3	2	7	2
Nitrate reduction	−	+	+	−	−	+
Starch hydrolysis	+	+	+	+	−	−
Gelatin liquefaction	+	w	−	+	−	+
Urease activity	−	−	−	−	+	−
Oxidase activity	+	+	+	−	−	−
Coagulation of milk	−	−	−	ND	+	+
Peptonization of milk	−	w	w	ND	+	ND
Decomposition of:						
Hypoxanthine	w	w	w	ND	+	ND
L-Tyrosine	+	+	+	+	+	−
Utilization of:						
L-Arabinose	−	+	+	+	−	−
Cellobiose	−	+	+	ND	+	ND
Dulcitol	−	+	w	ND	ND	ND
D-Fructose	−	+	w	+	+	−
myo-Inositol	−	−	w	+	+	+
Maltose	−	−	−	+	+	+
Raffinose	−	+	w	+	+	−
D-Sorbitol	−	+	−	+	−	+
Sucrose	−	+	+	+	−	+
D-Xylose	−	+	−	+	−	−

closely related species. Therefore, DNA–DNA hybridization between GKU 164^T and the closest strains, *N. monospora* PT708^T and *N. thailandensis* KC-061^T (Fig. 1) was fluorometrically determined using photobiotin-labelled DNA probes and microplate wells as described by Ezaki *et al.* (1989). The DNA–DNA relatedness values were examined from two independent determinations and revealed values of 40.11 ± 2.33 % and 37.61 ± 0.23 % for *N. monospora* PT708^T and *N. thailandensis* KC-061^T, respectively. The results clearly indicated that strain GKU 164^T was distinguished from *N. monospora* PT708^T and *N. thailandensis* KC-061^T because the values were below the recommended threshold value of 70 % for the definition of bacterial species (Wayne *et al.*, 1987).

On the basis of data from this taxonomic study using a polyphasic approach, strain GKU 164^T differs from closely related species of the genus *Nonomuraea*; therefore, strain GKU 164^T represents a novel species of this genus, for which the name *Nonomuraea syzygii* sp. nov. is proposed.

Description of *Nonomuraea syzygii* sp. nov.

Nonomuraea syzygii (sy.zy′gi.i. N.L. gen n. *syzygii* of *Syzygium cumini* L. Skeels, the jambolan plum tree from which the type strain was isolated).

Aerobic, Gram-stain-positive, non-acid-fast actinomycete that forms branched, non-fragmenting substrate ~~and aerial~~ hyphae. Non-motile spores are borne singly on the aerial mycelia and the spore surface is rough. Sporangia are not detected. No soluble pigment is observed in any of the media tested. The optimal temperature and pH for good growth is 18–32 °C and pH 7–8. The NaCl tolerance range for growth is up to 3 % (w/v). Catalase, oxidase, DNase, gelatin liquefaction and starch hydrolysis are positive, but negative results are observed for urease, milk peptonization and coagulation, nitrate reduction, and production of H_2S and melanin. Casein, L-tyrosine and xanthine are degraded, while hypoxanthine is weakly degraded and citrate is not degraded. D-Glucose, inulin, D-mannitol, D-mannose, L-rhamnose and trehalose are utilized as sole carbon sources, but L-arabinose, cellobiose, dulcitol, D-fructose, *myo*-inositol, β-lactose, maltose, raffinose, D-sorbitol, sucrose, xylitol and D-xylose are not. According to the API ZYM system, *N*-acetyl-β-glucosaminidase, acid phosphatase, alkaline phosphatase, α-chymotrypsin, esterase (C4), esterase lipase (C8), α-galactosidase, β-galactosidase, α-glucosidase, β-glucosidase, leucine arylamidase, α-mannosidase, trypsin and valine arylamidase are positive; while, arylamidase, cysteine, α-fucosidase and naphthol-AS-BI-phosphohydrolase are weakly positive, and β-glucuronidase and lipase (C4) are negative. The diagnostic diamino acid in the cell-wall peptidoglycan is *meso*-diaminopimelic acid. The whole-cell hydrolysates contain madurose, galactose, mannose, ribose, rhamnose and glucose. The *N*-acyl type of muramic acid is acetyl. Mycolic acids are not detected. The predominant menaquinone is MK-9(H_4), while minor amounts of MK-9(H_6), MK-9(H_2) and MK-9(H_0) are also present. The phospholipid profile consists of DPG, PE, OH-PE, PG, PI, PIMs, PME, OH-PME, an unidentified aminophosphoglycolipid and four unknown phospholipids. The major fatty acids are iso-$C_{16:0}$ and 10-methyl $C_{17:0}$.

The type strain, GKU 164^T (=BCC 70457^T=NBRC 110400^T), was isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels) collected at Khao Khitchakut National Park, Chantaburi province, Thailand. The genomic DNA G+C content of the type strain is 70.4 mol%.

Acknowledgements

This research was supported by the Thailand Research Fund (TRF) under grant number DBG5480002 and Kitasato Institute for Life Sciences, Kitasato University.

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International Journal of Systematic and Evolutionary Microbiology

Actinomadura syzygii sp. nov., an endophytic actinomycete isolated from the roots of a jambolan plum tree (Syzygium cumini L. Skeels)

--Manuscript Draft--

Manuscript Number:	IJSEM-D-14-00166R2
Full Title:	Actinomadura syzygii sp. nov., an endophytic actinomycete isolated from the roots of a jambolan plum tree (Syzygium cumini L. Skeels)
Short Title:	Actinomadura syzygii sp. nov.
Article Type:	Note
Section/Category:	New taxa - Actinobacteria
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Manuscript Region of Origin:	THAILAND
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Actinomadura syzygii sp. nov., an endophytic actinomycete isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels)

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Running Title: *Actinomadura syzygii* sp. nov.

Keywords: *Actinomadura syzygii* sp. nov., endophytic actinomycete, jambolan plum

The subject category: Actinobacteria

The GenBank accession number for the 16S rRNA gene sequences of strain GKU 157^T is KF667496.

Abstract

The taxonomic position of an endophytic actinomycete, strain GKU 157^T, isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels) collected at Khao Khitchakut National Park, Chantaburi province, Thailand, was determined using a polyphasic taxonomic approach. 16S rRNA gene analysis revealed that strain GKU 157^T belongs to genus *Actinomadura* and formed distinct phyletic line with *Actinomadura chibensis* NBRC 106107^T (98.6 % sequence similarity). Strain GKU 157^T formed an extensively branched, non-fragmenting substrate mycelium and aerial hyphae that differentiated into hooked to short spiral chains about 20 non-motile spores with warty surface. The cell wall contained *meso*-diaminopimelic acid and the whole-cell sugars were galactose, glucose, madurose, mannose and ribose. The *N*-acyl type of muramic acid was acetyl. Mycolic acids were absent. The phospholipids included phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylinositolmannoside (PIM) and two unknown phospholipids (PLs). The major menaquinone was MK-9(H_6) and the predominant fatty acids were $C_{16:0}$, iso-$C_{16:0}$, $C_{18:1}$ ω9c, $C_{18:0}$, 10-methyl $C_{18:0}$ (tuberculostearic acid). The genomic DNA G+C content was 73.1 mol%. Furthermore, a combination of DNA–DNA hybridization result and significant differences from the related species in cultural, physiological and chemical characteristics indicated that strain GKU 157^T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura syzygii* sp. nov. is proposed. The type strain is GKU 157^T (= BCC 70456^T = NBRC 110399^T).

The genus *Actinomadura* was firstly described by Lechevalier and Lechevalier (1970a) belonging to the family *Thermomonosporaceae*, which also includes the genera *Actinoallomurus*, *Actinocorallia*, *Spirillospora* and *Thermomonospora* (Zhang *et al.*, 2001; Tamura *et al.*, 2009). Members of the genus *Actinomadura* form an extensively branched, non-fragmenting substrate mycelium and aerial hyphae that differentiate into various spore chain morphologies. These spore chains are short to long, straight, hooked or spiral (1-4 turns) with either folded, irregular, smooth, spiny or warty surface ornamentation (Trujillo & Goodfellow, 2012). At present, genus *Actinomadura* comprises 50 species with validly published names,, including the recently described species *Actinomadura geliboluensis* (Sazak *et al.*, 2012), *Actinomadura meridiana* (Lee, 2012a), *Actinomadura rupiterrae* (Lee, 2012b), *Actinomadura sediminis* (He *et al.*, 2012) and *Actinomadura xylanilytica* (Zucchi *et al.*, 2013). One further specie, '*Actinomadura rayongensis*' has been recently described but the name is not yet validly published (Phongsopitanun *et al*., 2014). The type species of the genus is *Actinomadura madurae* (Vincent, 1894; Lechevalier & Lechevalier, 1970b).

Most species of the genus *Actinomadura* have been isolated from soil (Ara *et al.*, 2008; Tseng *et al.*, 2009; Lee, 2012a,b; Sazak *et al.*, 2012; Zucchi *et al.*, 2013), a few are found in clinical materials such as *Actinomadura chibensis, Actinomadura latina, Actinomadura madurae, Actinomadura pelletieri* and *Actinomadura sputi* (Trujillo & Goodfellow, 1997; Hanafy *et al.*, 2006; Yassin *et al.*, 2010), and one strain was reported as an endophyte, *Actinomadura flavalba*, from the leaves of a medicinal plant (*Maytenus austroyunnanensis*) (Qin *et al.*, 2009). During the course of a study on endophytic actinomycetes from medicinal plants at Khao Khitchakut National Park, Chantaburi province, Thailand, a total of 16 isolates were identified from a jambolan plum tree (*Syzygium cumini* L. Skeels). They were classified to the genera *Streptomyces* (*n*=12, 75 %), *Actinomadura* (n=2, 12.5 %), *Nonomuraea* (n=1, 6.25 %) and *Streptosporangium* (n=1, 6.25 %). Recently, a novel species of *Nonomuraea* has been proposed from this study (Rachniyom *et al*., 2015). Here, we propose another novel species isolated from the same tree, strain GKU 157^T, belonging to the genus *Actinomadura* by polyphasic taxonomic study.

The excised roots of a jambolan plum tree (*Syzygium cumini* L. Skeels) were surface-sterilized and endophytic actinomycetes were isolated by method described by Rachniyom *et al.* (2015). Colonies of endophytic actinomycetes appeared on starch-casein agar (SCA; Küster & Williams, 1964) supplemented with 100 μg/ml ampicillin, 2.5 U/ml penicillin G, 50 μg/ml amphotericin B and 50 μg/ml cycloheximide after incubation at 28 °C for 3–4 weeks. Colonies of strain GKU 157^T were purified on mannitol-soya (MS) agar (Hobbs *et al.*, 1989). The pure culture was maintained in 20 % (v/v) glycerol suspension at –80 °C and as lyophilized cells for long-term preservation.

Genomic DNA was extracted by the method of Kieser *et al*. (2000). 16S rRNA gene was amplified using primers and conditions described by Rachniyom *et al.* (2015). The PCR product was purified using a Gel/PCR DNA Fragment Extraction kit (Geneaid, Taiwan) and then sent to Macrogen (Korea) for DNA sequencing using primers described by Rachniyom *et al.* (2015). The resulting 16S rRNA gene sequence of the strain GKU 157^T was compared with corresponding sequences of the type strains available in the EzTaxon-e server (Kim *et* *al*., 2012). Sequences of strain GKU 157^T and other related strains obtained from the GenBank/EMBL/DDBI database were aligned by using Clustal X version 2.0 (Larkin *et al.*, 2007). Phylogenetic trees were constructed using neighbour-joining (Saitou & Nei, 1987) and maximum likelihood (Felsenstein, 1981) algorithms by MEGA software version 6.0 (Tamura *et al.*, 2013). Topology of the phylogenetic trees was evaluated with bootstrap analysis based on 1,000 resampling (Felsenstein, 1985). A distance matrix was generated using Kimura's two-parameter model (Kimura, 1980). All positions containing gaps and missing data were eliminated from the dataset. The values of pairwise 16S rRNA gene sequence similarity among strain GKU 157^T and other related species were determined using the EzTaxon-e sever.

The almost complete 16S rRNA gene sequence (1507 nt) of strain GKU 157^T was compared with corresponding sequences in public database by EzTaxon-e server. The results indicated that strain GKU 157^T was a member of the genus *Actinomadura*. The type strains showed the highest similarities were *A. chibensis* NBRC 106107^T (98.6 % 16S rRNA gene sequence similarity;20 nt difference at 1440), *Actinomadura bangladeshensis* 3-46-b3^T (98.5 %; 22 nt difference at 1455), *Actinomadura meyerae* A288^T (98.2 %; 26 nt difference at 1436), *A. geliboluensis* A8036^T (98.2 %; 27 nt difference at 1464), *Actinomadura chokoriensis* 3-45-a/11^T (98 %; 29 nt difference at 1459). However, the neighbour-joining and maximum likelihood trees indicated that strain GKU 157^T fell in a lineage with *A. chibensis* NBRC 106107^T, *A. pelletieri* NBRC 103052^T (97.9 %; 31 nt difference at ,444) and *A. meridiana* JCM 17440^T (96.9 %; 44 nt difference at 1430) (Fig. 1 and S1).

The morphological and physiological characteristics of strain GKU 157^T were then determined in compared with those of phylogenetically closely related type strains, *A. chibensis* NBRC 106107^T, *A. pelletieri* NBRC 103052^T and *A. meridiana* JCM 17440^T. Cultural characteristics were determined after incubation at 27 °C for 21 days on glucose-yeast extract-malt extract agar (GYM; medium 65 DSMZ Catalogue), modified Bennett's agar (MBA; Jones, 1949), oatmeal-nitrate agar (Prauser & Bergholz, 1974) and various ISP media (International *Streptomyces* Project; Shirling & Gottlieb, 1966) namely, yeast extract-malt extract agar (ISP 2), oatmeal agar (ISP 3), inorganic salt-starch agar (ISP 4), glycerol-asparagine agar (ISP 5), peptone-yeast extract-iron agar (ISP 6) and tyrosine agar (ISP 7). Color of mycelium and soluble pigment were determined by comparison with color chips from the Color Harmony Manual (Jacobson *et al.*, 1958). Spore chain morphology and spore ornamentation of strain GKU 157^T were observed by light and scanning electron microscopy (model JSM-5410; JEOL) using cultures grown on ISP 4 medium at 28 °C for 21 days. The range of temperatures (5–50 °C), pH values (pH 4.0–11.0) and NaCl concentrations (0–7 % w/v) for growth tolerance was examined on nutrient agar (NA; Difco) for 14–21 days. For pH adjustments, medium were amended with 75 mM sodium citrate at the respective pH. Catalase and oxidase activities were observed with 3 % (v/v) hydrogen peroxide solution and 1 % (v/v) tetramethyl-*ρ*-phenylenediamine solution, respectively. Acid production from carbohydrates was determined by using the media and methods described by Gordon *et al.* (1974). Reduction of nitrate was observed using nitrate broth (Difco). Starch hydrolysis was examined on ISP 4 medium. Urease activity was determined based on a color change in Stuart's urea agar (Stuart *et al.*, 1945). Gelatin liquefaction was evaluated on gelatin medium (2.0 % glucose, 0.5 % peptone, 20 % gelatin; pH 7.0). Coagulation and peptonization of milk were observed in 10% (w/v) skim milk broth (Difco). Hydrolysis of DNA was tested on DNase agar (Difco). Production of H_2S and melanin pigment were determined on peptone iron agar (Difco) and ISP 7 medium (Difco), respectively. Citrate utilization was tested on Simmons' citrate agar (Difco). Decomposition of casein, L-tyrosine, xanthine and hypoxanthine were evaluated by using the media described by Gordon *et al.* (1974). Utilization of carbon sources were examined on ISP 9 medium (Shirling & Gottlieb, 1966) supplemented with filter-sterilized carbon source used at a final concentration of 1 % (w/v). Enzyme activity profiles were tested by using the API ZYM system (bioMérieux) according to the manufacturer's instruction.

Strain GKU 157^T grew well on all of the media tested (Table 1 and S1). Substrate mycelia were well developed and the colors were variable depending on the growth medium. Aerial mycelia were observed on ISP 2, ISP 3, ISP 4 and oatmeal-nitrate agar. Pale pink aerial mycelium was found on ISP 2, ISP 3 and ISP 4 media, with abundant sporulation on ISP 4 medium. No soluble pigments were produced on any of the media tested. Strain GKU 157^T produced abundantly branched, non-fragmenting substrate mycelium and aerial hyphae, which differentiated into spore chains. Hooked to short spiral (1 turn) spore chains consisted of about 20 non-motile spores with warty surface (Fig. 2). The temperature and pH range for growth of strain GKU 157^T were observed at 14–40 °C and pH 6–11, with optimum growth at 20–34 °C and pH 8–10. Strain GKU 157^T could be differentiated from its phylogenetically closely related type strains by culture characteristics, pH and NaCl for growth tolerance, acid production from carbohydrates, gelatin liquefaction, coagulation and peptonization of milk,

H_2S production, utilization of carbon sources and enzyme activities (Table 1 and S1). Additional phenotypic properties of strain GKU 157^T are presented in the species description.

Freeze-dried cells of strain GKU 157^T were used for chemotaxonomic studies that were obtained from culture grown in yeast extract-glucose broth (1 % yeast extract, 1 % glucose; pH 7.0) on a rotary shaker at 27 °C for 7 days. The isomer of diaminopimelic acid and reducing sugars in the cell-wall were determined by TLC using whole-cell hydrolysates according to the method of Hasegawa *et al.* (1983) and Becker *et al.* (1965), respectively. *N*-acyl type of muramic acid in peptidoglycan was examined by the method of Uchida & Aida (1984). The presence of mycolic acids were monitored by TLC following the procedure of Tomiyasu (1982). Isoprenoid quinones were extracted and purified by using the method of Collins *et al.* (1977) and were determined by LC/MS (JSM-T100LP; JEOL). Analyses of phospholipids and whole-cell fatty acids were carried out by the service at Faculty of Science, King Mongkut's Institute of Technology Ladkrabang (KMITL), Thailand. Phospholipids were extracted and identified by two-dimensional TLC as described by Minnikin *et al.* (1984). Cellular fatty acids were extracted, methylated and analysed by using the Microbial Identification System (MIDI) according to the method of Sasser (1990) and the manufacturer's instructions. Methyl esters of cellular fatty acids were identified by GLC and using the Microbial Identification software package (Sherlock version 6.1; MIDI database RTSBA6). The G+C content of genomic DNA was extracted according to the method of Marmur (1961) and determined by HPLC according to the procedure of Tamaoka & Komagata (1984).

Chemotaxonomic analyses revealed that strain GKU 157^T exhibited characteristics which were typical of members of the genus *Actinomadura*. The strain contained *meso*-diaminopimelic acid as the dignostic diamino acid and whole-cell sugars were galactose, glucose, madurose, mannose and ribose showing that its possessed cell-wall type III (Lechevalier & Lechevalier, 1970b) and whole-cell sugar pattern B (Lechevalier, 1968). The *N*-acyl type of muramic acid in peptidoglycan was acetyl. Mycolic acids were absent. The predominant menaquinone was MK-9(H_6) (69 %), while the minor amount of MK-9(H_4) (14 %), MK-9(H_8) (11 %), MK-9(H_2) (5 %) and MK-9(H_0) (1 %) were also detected. The diagnostic phospholipids were phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylinositolmannoside (PIM) and two unknown phospholipids (PLs) (Fig. S2), corresponded to phospholipid type I (Lechevalier *et al.*, 1977).

The major cellular fatty acids were $C_{16:0}$ (29.5 %), iso-$C_{16:0}$ (19.7 %), $C_{18:1}$ ω9c (9.5 %), $C_{18:0}$ (7.4 %), 10-methyl $C_{18:0}$ (tuberculostearic acid; 6.9 %), corresponded to fatty acid type 3a (Kroppenstedt *et al.*, 1985); while the minor amount of a summed feature 3 ($C_{16:0}$ ω7c and/or $C_{16:1}$ ω6c; 4.5 %), $C_{17:1}$ ω8c (4.1 %), $C_{17:0}$ (3.9 %), a summed feature 9 (10-methyl $C_{16:0}$ and/or iso-$C_{17:1}$ ω9c; 2.4 %), iso-$C_{18:0}$ (2.4 %), a summed feature 8 ($C_{18:1}$ ω7c and/or $C_{18:1}$ ω6c; 1.9 %), $C_{17:1}$ ω6c (1.7 %), $C_{18:3}$ ω6c (1.7 %) and $C_{14:0}$ (1.6 %) were also presented. The genomic DNA G+C content was 73.1 mol%.

Comparison of genotypical, morphological and phenotypical characteristics of strain GKU 157^T to its closest phylogenetically neighbors evidently distinguished strain GKU 157^T from the others (Table 1 and S1). Strain GKU 157^T harbored long spore chain with warty surface similar to those of *A. chibensis* NBRC 106107^T and *A. meridiana* JCM 17440^T, while *A. pelletieri* NBRC 103052^T carried short spore chain. However, similarity of 16S rRNA gene sequences between strain GKU 157^T and *A. meridiana* JCM 17440^T and *A. pelletieri* NBRC 103052^T were lower (97.9 % and 96.9 %, respectively) than that of *A. chibensis* NBRC 106107^T (98.6 %). Taken together of 16S rRNA gene sequencing and phylogenetic analyses, strain GKU 157^T was mostly related to *A. chibensis* NBRC 106107^T with 98 % and 95 % boothstrap values of neighbour-joining and maximum likelihood algorithms supported, respectively (Fig. 1 and S1). Therefore, the validity of a novel species status of strain GKU 157^T was confirmed by DNA–DNA hybridization by fluorometrically determined using photobiotin-labelled DNA probes and microplate wells (Ezaki *et al.*, 1989). Level of DNA–DNA relatedness value between strain GKU 157^T and *A. chibensis* NBRC 106107^T was examined from two independent determinations and revealed 48.8±2.1% which was clearly below the 70 % value considered to be the threshold for the definition of bacterial species (Wayne *et al.*, 1987). Based on all evidences, strain GKU 157^T represents a novel species of the genus *Actinomadura*, for which the name *Actinomadura syzygii* sp. nov. is proposed.

Description of *Actinomadura syzygii* sp. nov.

Actinomadura syzygii (sy.zy'gi.i. N.L. gen. n. syzygii of *Syzygium cumini* L. Skeels, the jambolan plum tree from which the type strain was isolated).

Aerobic, Gram-stain-positive, non-acid-alcohol-fast, non-motile actinomycete that forms an extensively branched, non-fragmenting substrate and aerial mycelium. Abundant aerial mycelium is present on ISP 4 medium, which differentiated into hooked to short spiral chains about 20 non-motile spores with warty surface. No soluble pigment is produced on any of the media tested. The optimal temperature for good growth occurs at 20–34 °C and pH at 8–10. The NaCl tolerance range for growth is up to 4 % (w/v). Oxidase, DNase and nitrate reduction are positive, while catalase is weakly positive, but urease, citrate utilization, gelatin liquefaction, milk coagulation and peptonization, H_2S and melanin production are negative. Acid is produced from D-glucose, but not from adonitol, D-cellobiose, D-galactose, D-mannose, D-sorbitol, L-arabinose, L-rhamnose and maltose. Starch, casein and L-tyrosine are degraded, but xanthine and hypoxanthine are not. D-cellobiose, D-glucose and D-trehalose are utilized as the sole carbon sources, while maltose is weakly utilized, but D-mannose, D-raffinose, D-sorbitol, D-xylose, dulcitol, inulin, L-arabinose, L-rhamnose, myo-inositol and sucrose are not. According to the API ZYM system, acid phosphatase, alkaline phosphatase, cysteine arylamidase, esterase (C4), esterase lipase (C8), leucine arylamidase, lipase (C4), *N*-acetyl-*β*-glucosaminidase, naphthol-AS-BI-phosphohydrolase, trypsin, valine arylamidase, *α*-chymotrypsin and *α*-glucosidase are positive, but *α*-fucosidase, *α*-galactosidase, *α*-mannosidase, *β*-galactosidase, *β*-glucosidase and *β*-glucuronidase are negative. The cell-wall contains *meso*-diaminopimelic acid and the whole-cell sugars are galactose, glucose. madurose, mannose and ribose. The *N*-acyl type of muramic acid is acetyl. Mycolic acids are absent. The predominant menaquinone is MK-9(H_6) with the minor amounts of MK-9(H_4), MK-9(H_8), MK-9(H_2) and MK-9(H_0). The phospholipid profile contains phosphatidylglycerol (PG), diphosphatidylglycerol (DPG), phosphatidylinositol (PI), phosphatidylinositolmannoside (PIM) and two unknown phospholipids (PLs). The major cellular fatty acids are $C_{16:0}$, iso-$C_{16:0}$, $C_{18:1}$ ω9c, $C_{18:0}$, 10-methyl $C_{18:0}$ (tuberculostearic acid). The genomic DNA G+C content of the type strain is 73.1 mol%.

The type strain, GKU 157^T (= BCC 70456^T = NBRC 110399^T), was isolated from the roots of a jambolan plum tree (*Syzygium cumini* L. Skeels) collected at Khao Khitchakut National Park, Chantaburi province, Thailand.

Acknowledgements

This research was supported by the Thailand Research Fund (TRF) under grant number DBG5480002 and Kitasato Institute for Life Sciences, Kitasato University.

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Table 1. Comparison of the phenotypic properties of strain GKU 157^{T} and closely related type species of the genus *Actinomadura* with validly published names. Species: 1, GKU 157^{T}; 2, *A. chibensis* NBRC 106107^{T}; 3, *A. pelletieri* NBRC 103052^{T}; 4, *A. meridiana* JCM 17440^{T}. All data were obtained in this study. +, positive; -, negative; w, weakly positive.

Characteristic	**1**	**2**	**3**	**4**
Spore morphology				
Arrangement	Hooked to spiral	Hooked to spiral	Hooked to spiral	Hooked
Number of spore (per chain)	About 20	About 20	2-6	About 20
Colony characteristics on ISP 2 medium				
Growth	Good	Good	Good	Moderate
Aerial mycelium	Pale pink	White	None	None
Substrate mycelium	Light coral red	Mustard	Light coral rose	Bamboo
Diffusible pigment	None	Amber	None	Golden brown
Growth at pH 11	+	+	+	-
Growth at 5 % Na-Cl (w/v)	-	+	-	-
Catalase activity	w	+	+	+
Oxidase activity	+	+	w	+
Acid production from:				
Adonitol	-	-	+	-
D-cellobiose	-	+	-	-
D-glucose	+	+	-	+
D-mannose	-	-	-	+
L-rhamnose	-	+	-	-
Gelatin liquefaction	-	+	-	-
Milk coagulation	-	w	+	+
Milk peptonization	-	w	+	+
H_2S production	-	-	-	+
Hypoxanthine decomposition	-	w	-	-
Utilization of:				
D-cellobiose	+	+	-	-
D-mannose	-	-	-	+
D-sorbitol	-	+	-	-
D-trehalose	+	w	w	+
Dulcitol	-	w	-	-
L-rhamnose	-	+	-	-
Maltose	w	-	-	-
Enzyme activities of:				
α-glucosidase	+	+	+	-
β-glucosidase	-	+	-	-

Figure legend

Fig. 1. Neighbour-joining tree based on almost complete 16S rRNA gene sequences (1,260 nt) showing the relationship between strain GKU 157^T and all recognized species of the genus *Actinomadura*. *Nonomuraea pusilla* ATCC 27296^T (GenBank acession no. D85491) was used as an outgroup. Asterisks denote branches that were also recovered from maximum-likelihood tree. Numbers at branch points indicate bootstrap percentage (based on 1,000 replications); only values > 50 % are showed. Bar, 0.005 substitutions per nucleotide position.

Fig. 2. Scanning electron micrograph of hooked to short spiral spore chains with warty surface of strain GKU 157^T grown on ISP 4 medium at 28 °C for 21 days. Bar, 1 μm.

Figure 1

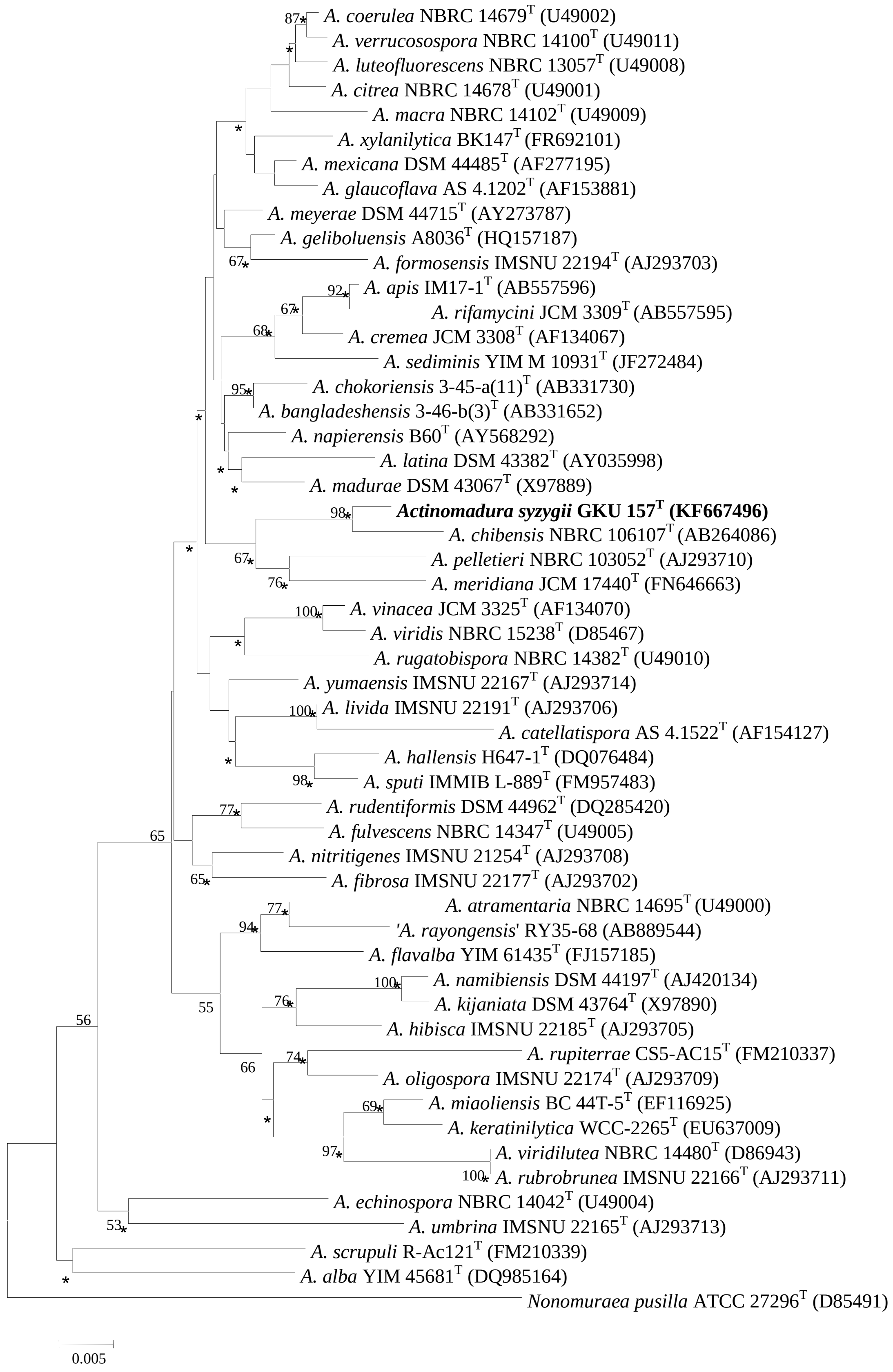

Fig. 1

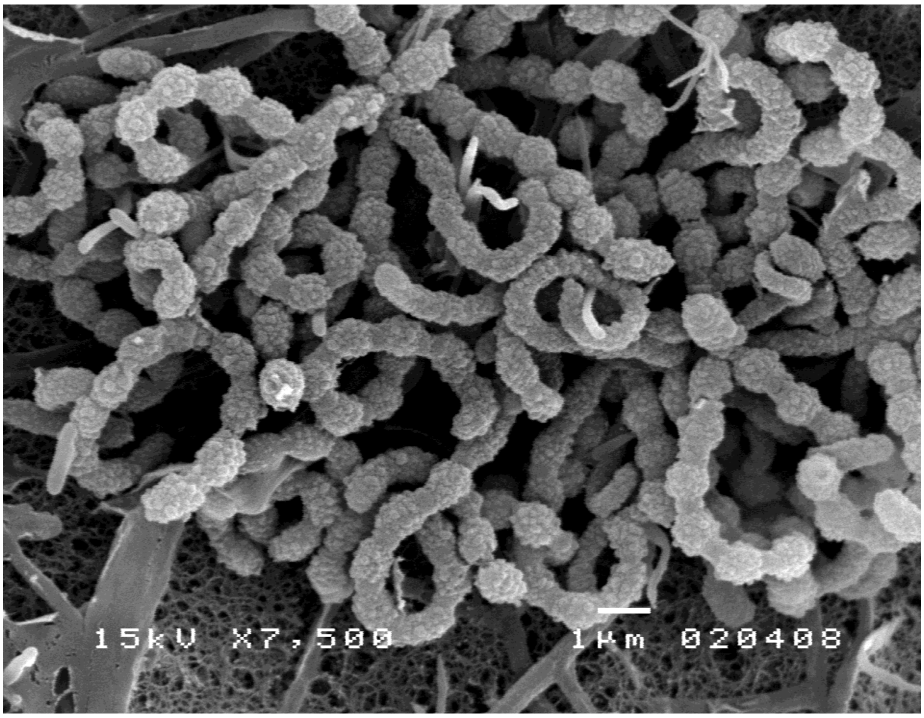

Fig. 2.

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Actinomadura syzygii sp. nov., an endophytic actinomycete isolated from the roots of a jambolan plum tree (Syzygium cumini L. Skeels) Hathairat Rachniyom; Atsuko Matsumoto; Chantra Indananda; Kannika Duangmal; Yoko Takahashi; Arinthip Thamchaipenet, Ph.D.

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บทคัดย่องานประชุมวิชาการระดับนานาชาติ

Endophytic actinomycetes: novel taxa, bioactive compounds and plant growth enhancement

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Abstract

Endophytic actinomycetes isolated from Thai tropical plants are rich sources for microbial diversity and new secondary metabolites. Several new genus and species as well as novel bioactive compounds have been discovered. Moreover, these actinomycetes exhibit plant growth promoting (PGP) traits (i.e. phosphate solubilization, siderophore production, IAA production, ACC deaminase and chitinase activities, and anti-phytopathogenic activities) and mutually associated with plants. They help significantly promote plant growth by increase of root and shoot biomass in rice, mungbean, maize and sugarcane plants. In addition, co-inoculation of endophytic bacteria including actinomycetes efficiently enhance growth of sugarcane plants compared to single inoculation. We also demonstrated that a chitinase-producing endophytic actinomycete could secure maize plants from foot rot and wilting diseases infected by *Fusarium moniliforme*. Recent investigation on plant-actinomycete interaction has been approached by generating of some single PGP-trait mutants (e.g. siderophore-deficient and ACC deaminase-deficient mutants). The siderophore-deficient mutant clearly indicated the effects of this important trait involved in growth promotion of rice and mungbean plants. The ACC deaminase-deficient mutant evidently implied that ACC deaminase trait helped plants resisting to stresses including salinity and flooding. Our results promptly suggest that these endophytic actinomycetes can be applied as added value biofertilizers and biocontrol agents in the future.

Biography

Arinthip Thamchaipenet is an Associate Professor of Genetics, Faculty of Science, Kasetsart University, Thailand. She received a Ph.D. (Genetics) from University of Glasgow, UK in 1994 and joined a post-doctoral fellow in combinatorial biosynthesis at Kosan Biosciences, California, USA in 1998. Her researches focus on molecular interaction of endophytic actinomycetes and plants, genome and biosynthetic genes of actinomycetes.

Endophytic actinomycetes as plant growth enhancers, stress reducers and pathogen protectors

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Abstract

Endophytic actinomycetes isolated from Thai tropical plants are rich sources for microbial diversity and new drug discovery. Several new genus and species as well as novel bioactive compounds have been discovered. Moreover, these plant-living actinomycetes exhibit PGPB properties (i.e. phosphate solubilization, siderophore production, IAA production, ACC deaminase and chitinase activities) as free-living microorganisms. We have demonstrated that they help significantly promote plant growth by increase of root and shoot biomass in rice, mungbean, maize and sugarcane plants. Recent investigation on plant-actinomycete interaction was approached by generating of some single PGPB-trait mutants (e.g. siderophore-deficient and ACC deaminase-deficient mutants). The siderophore-deficient mutant clearly demonstrated the effects of this important trait involved in growth promotion of rice and mungbean plants. The ACC deaminase-deficient mutant evidently implied that ACC deaminase trait helped plants resisting to stress conditions including salinity and flooding. Furthermore, a chitinase-producing endophytic actinomycete could secure maize plants from foot rot and wilting diseases infected by *Fusarium moniliforme*. Our results highlight the value of a substantial understanding of the relationship of the endophytic actinomycetes towards the plants and suggest high possibility to apply these endophytes as biofertilizers and biocontrol agents.

Keywords: endophytes, actinomycetes, plant growth promotion

Endophytic actinomycetes act as plant growth enhancers, pathogen protectors and stress reducers.

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Endophytic actinomycetes were found diversely in various plant species. Several new genus and species as well as novel bioactive compounds have been discovered suggesting that they are rich sources for microbial diversity and new drug discovery. Moreover, these plant dwelling actinomycetes exhibit PGPB properties (i.e. phosphate solubilization, siderophore production, IAA production, ACC deaminase and chitinase activities) as free-living microorganisms. We have demonstrated that they help significantly promote plant growth by increase of root and shoot biomass in rice, mungbean and maize plants. Furthermore, a chitinase-producing endophytic actinomycete could secure maize plants from foot rot and wilting diseases infected by *Fusarium moniliforme*. Recent investigation on plant-actinomycete interaction was approached by generating of some single PGPB-trait mutants (e.g. siderophore-deficient and ACC deaminase-deficient mutants). The siderophore-deficient mutant clearly demonstrated the effects of this important trait involved in growth enhancement of rice and mungbean plants with and without iron. The ACC deaminase-deficient mutant evidently indicated that ACC deaminase could improve plant resistance to stress conditions including salinity and flooding. Our results highlight the value of a substantial understanding of the relationship of the endophytic actinomycetes towards the plants.

RECENT INVESTIGATION OF ENDOPHYTIC ACTINOMYCETES ISOLATED FROM THAI PLANTS REVEALS PLANT GROWTH ENHANCEMENT

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Endophytic actinomycetes were isolated from several tropical plant species in Thailand including those of agriculturally and medicinally important species. Some of those isolates were tagged by eGFP and proved that they localized within the plant tissues. Within this collection, one new genus, *Actinophytocola*, and four new species of genera *Actinoallomurus*, *Kineococcus* and *Pseudonocardia* have been currently proposed which indicated that endophytic actinomycetes are relatively diverse. In addition, three novel bioactive compounds have been identified which suggested that endophytic actinomycetes are high potential sources for new drug discovery. Moreover, these endophytes exhibited plant growth promoting properties such as production of siderophores, synthesis of phytohormone IAA, solubilization of rock phosphate, production of ACC deaminase and chitinase and also carried anti-phytopathogenic activities. We have demonstrated the effects of plant growth using rice, mungbean and maize plants inoculated with selective endophytic actinomycetes in pot experiments. The results showed that the plants inoculated with the endophytes gave better enhancement of plant growth and significantly increased root and shoot biomass than untreated controls. Furthermore, the chitinase-producing endophytes could secure plants from phytopathogenic fungi. To investigate plant-actinomycete interaction, some mutants such as siderophore-deficient and ACC deaminase-deficient strains of the endophytes were generated by gene disruption. These mutants clearly showed the effects of these important traits involved in plant-microbe interaction in enhancement of growth and reduction of plant stress in pot experiments. Our results highlight the value of a substantial understanding of the relationship of the plant growth promoting properties of endophytic actinomycetes to the plants. Endophytic actinomycetes, therefore, can be applied as potentially safe and environmentally-friendly biocontrol/biofertilizer agents in agriculture.

Diversity of Endophytic Actinomycetes from Thai Tropical Plants and Their Properties of Plant Growth Enhancement

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A collection of endophytic actinomycetes was established under the isolation program of endemic tropical plants in Thailand. More than 60 Thai plants, including those of agriculturally, economically and medicinally important species, were explored. Within the collection, *Streptomyces* was found as a dominant genus following by *Microbispora*, *Micromonospora* and *Actinomadura*. One new genus and four new species were proposed and more promising novel species are underway. In addition, two novel bioactive compounds have been characterized which revealed that these endophytes are high potential sources for new drug discovery. Furthermore, these endophytes show characteristics of plant growth promoting bacteria such as solubilization of rock phosphate, synthesis of phytohormones, production of siderophores, ACC deaminase, anti-phytopathogenic compounds and chitinase. Investigation of plant-actinomycete interaction on localization of the endophytes in plant tissues by eGFP tagging, visualization of chitinase producing strains against phytopathogenic fungi and relationship of siderophore producing/mutant strains on plant growth enhancement, indicated that these endophytic actinomycetes can be further developed as biocontrol/biofertilizer agents. It now appears that these enormous, relatively untapped sources of plant diversity in Thailand could be rich sources of endophytic actinomycetes that are full of genetic diversity and are precious resources for novel taxa, bioactive compounds and plant growth-promoting agents.

Keywords: actinomycete; endophyte; diversity; plant growth

ABSTRACT
International Conference on Microbial, Plant and Animal Research (ICMPAR2012). Mody Institute of Technology and Science, Lakshmangarh, India. 29-31 March 2012.

Investigation of Plant Growth Promoting Properties from Endophytic Actinomycetes Isolated from Thai Tropical Plants

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The collection of endophytic actinomycetes isolated from tropical plants in Thailand including those of agriculturally and medicinally important species have been investigated. Within this collection, one new genus and four new species were proposed and two novel bioactive compounds have been characterised which indicated that endophytic actinomycetes isolated from Thai tropical plants are rich sources for diversity and new drug discovery. Moreover, these endophytes carry anti-phytopathogenic activities of both bacteria and fungi and display characteristics of plant growth promoting bacteria such as production of siderophores, synthesis of phytohormone IAA, solubilization of rock phosphate, production of ACC deaminase and chitinase. It is, thus, very promising that these endophytes can be further developed as biocontrol/ biofertilizer agents for agricultural applications. To investigate plant-actinomycete interaction, current studies such as localization of the endophytes in plant tissues by eGFP tagging, relationship of siderophore producing/mutant strains on seed germination and plant growth enhancement, and visualization of chitinase producing strains against phytopathogenic fungi will be intensively discussed.

ABSTRACT
The 16th International Symposium on the Biology of Actinomycetes (ISBA16). Puerto Vallarta, Mexico. 11-15 December 2011.

Recent Investigation and Future Prospects of Endophytic Actinomycetes from Thai Tropical Plants

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More than 60 tropical plant species in Thailand including those of agriculturally and medicinally important species have been explored for endophytic actinomycetes. Amongst one hundred isolates, *Streptomyces* were found about half, whereas the other half was identified as rare actinomycetes including *Microbispora*, *Micromonospora* and *Actinomadura*. Within this collection, one new genus and four new species were proposed which indicated that Thai tropical plants are rich sources for endophytic actinobacterial diversity. Furthermore, two novel bioactive compounds have been identified from these endophytes which revealed that they are high potential sources for new secondary metabolites. Moreover, these endophytes carry anti-phytopathogenic activities of both bacteria and fungi and display characteristics of plant growth promoting bacteria such as production of siderophores, synthesis of phytohormone IAA, solubilization of rock phosphate, production of ACC deaminase and chitinase. It is, thus, very promising that these endophytes can be further developed as biocontrol/biofertilizer agents and applied to agriculture in Thailand. To investigate plant-actinomycete interaction, some current studies such as localization of the endophytes in plant tissues by eGFP tagging, relationship of siderophore producing/mutant strains and iron utilization on plant growth enhancement, and visualization of chitinase producing strains against phytopathogenic fungi will be discussed.

บทคัดย่อนำเสนองานประชุมวิชาการระดับนานาชาติ แบบปากเปล่าและโปสเตอร์ของนิสิตปริญญาเอก

Endophytic bacteria and actinomycetes promote growth of sugarcane plants

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Abstract

This research aimed to isolate and select plant growth-promoting bacterial endophytes (PGPE) including actinomycetes from sugarcane plants. Eighty-three endophytic actinomycetes represented four genera of *Streptomyces* (56.63%), *Microbispora* (34.94%), *Actinomadura* (6.02%) and *Micromonospora* (2.41%) were identified. Fifty-two endophytic bacteria represented at least 10 genera including *Enterobacter* (50.0%), *Bacillus* (26.29%), *Pantoea* (5.77%) and *Acinetobacter* (3.85%) were obtained. All of them exhibited at least one trait of plant growth promoting properties including indole-3-acetic acid (IAA) and siderophore production, phosphate solubilization and antagonistic activity against phytopathogenic fungi. Based on PGP traits, individual- and co-inoculation of endophytic actinomycetes namely, *Streptomyces* sp. GKU 895 and *Microbispora* sp. GKU 823, and bacteria namely, *Enterobacter* sp. EN-21 and *Bacillus* sp. EN-24 were evaluated for improving growth of sugarcane plants under pot culture experiments. Individual strain enhanced the plant height when compared to the uninoculated control. Interestingly, co-inoculation of *Microbispora* sp. GKU 823 with *Enterobacter* sp. EN-21 and *Bacillus* sp. EN-24 led to significant increase in both plant height and leaf area of sugarcane plants. This study suggested that these endophytes from sugarcane are high potential PGPE that could be used to promote sugarcane growth and may help protect phytopathogenic fungi. Further experiments under field conditions are along the way.

Keywords: PGPE, actinomycetes, sugarcane, co-inoculation

BIOLOGICAL CONTROL OF CHITINASE-PRODUCING ENDOPHYTIC STREPTOMYCETES AGAINST *FUSARIUM MONILIFORME* IN MAIZE PLANTS (*ZEA MAYS* L. CV. NAKHONSAWAN 3)

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Fifth-five strains of endophytic actinomycetes were isolated from healthy medicinal plants of Thailand. Forty-nine of them (89%) exhibited chitinase activity on shrimp shells chitin agar. Forty-seven strains were able to produce cell-wall degrading enzymes on mycelia (*Fusarium moniliforme*) fragment agar. Family 19 chitinase genes were amplified using specific primers from thirty-two strains and were partially sequenced. The PCR positive strains belong to members of genera *Actinoallomurus*, *Actinomadura*, *Microbispora*, *Nonomuraea*, *Promicromonospora* and *Streptomyces*. Within twenty-five strains of genus *Streptomyces*, strains GMKU 301 and GMKU 322 showed the highest level of chitinase and fungal cell-wall degrading activity without antifungal activity against plant pathogenic fungi, *F. moniliforme*. *F. moniliforme* cell-wall degrading activity on glass slide by dual culture method was observed under scanning electron microscope (SEM). Extensive hyphal plasmolysis and cell wall lysis of the fungal hyphae occurring at the area of interaction between *F. moniliforme* and both endophytic streptomycetes strains were obviously visualized. Moreover, *Streptomyces* sp. GMKU 301 and GMKU 322 exhibited biocontrol ability against *F. moniliforme* causing foot rot and wilting in maize plants (*Zea mays* L. cv. Nakhonsawan 3) in pot experiments. Application of individual strains GMKU 301 and GMKU 322 significantly reduced incidence of disease caused b*y F. moniliforme* in maize plants when compared to the un-inoculated treatment. This study demonstrated effective biological control by endophytic streptomycetes that capable of producing chitinase for management of *Fusarium* diseases.

1-AMINOCYCLOPROPANE-1-CARBOXYLATE (ACC) DEAMINASE PRODUCTION FROM ENDOPHYTIC ACTINOMYCETES AND ITS GROWTH-PROMOTING EFFECT ON MUNGBEAN PLANTS

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Sixty-seven strains of endophytic actinomycetes from GMKU culture collection were screened for their ACC deaminase ability to utilize 1-aminocyclopropane-1-carboxylate (ACC), the precursor of ethylene. Sixteen strains (24%) could grow on minimum medium supplemented with ACC as nitrogen source. PCR amplification with specific primers and sequencing of partial ACC deaminase (*acdS*) gene from those positive strains confirmed that they in fact harboring the *acdS* genes. The partial *acdS* gene of *Streptomyces* sp. GMKU 336 was then cloned into an integrative vector, pIJ8671. The recombinant plasmid was then transferred and integrated into chromosome of *Streptomyces* sp. GMKU 336 by intergeneric conjugation to achieve gene disruption. The *acdS* mutants could not grow on minimum medium supplemented with ACC and were confirmed the integration by PCR of thiostrepton resistance (*tsr*) gene located on pIJ8671. The wildtype strain GMKU 336 and the *acdS* mutant strain were then individually inoculated into mungbean plant grown under stress conditions (i.e. salinity and flooding) to evaluate effect of ACC deaminase. Results from pot experiments showed that salinity stress (100mM NaCl) retarded plant growth in un-inoculated and the mutant treatments but the plants inoculated with the wildtype could survive. Furthermore, the mungbean plants inoculated with the wildtype showed some increased tolerance to flooding stress. Root/shoot growth and leaf chlorophyll content decreased in flooded plants treated with *acdS* mutant and un-inoculated control, while the plants treated with the wildtype appeared to be taller and greener. The results suggested that endophytic actinomycetes producing ACC deaminase could result in improvement of plant resistance to stress condition by conversion of ACC to ammonia and α-ketobutyrate and subsequently reduced ethylene in the host plant. These endophytic actinomycetes are therefore acting as plant growth promoting agents to enhance plant growth by decreasing ethylene levels. Understanding the interactions between these bacteria and the host plants will enable further applications of them as potential biofertilizer agents.

CO-INOCULATION EFFECTS OF ENDOPHYTIC BACTERIA AND ACTINOMYCETES ISOLATED FROM SUGARCANE ON PROMOTE GROWTH OF MAIZEPLANTS (*ZEA MAYS* L. CV. NAKHONSAWAN 3)

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Sixty-three isolates of endophytic actinomycetes belonging to genera *Microbispora* (58.73%), *Streptomyces* (23.81%), *Actinomadura* (1.58%) and other genera (15.88%) and 52 isolates of endophytic bacteria belonging to genera *Bacillus* (25.0%), *Enterobacter* (25.0%), *Pentoae* (3.84%) and other genera (46.16%) were isolated from healthy roots of sugarcane plants (*Saccharum officinarum* L.). They were tested for their plant growth-promoting (PGP) traits and effects on plant growth. Thirty-six isolates (57.14%) of endophytic actinomycetes produced siderophores, 30 isolates (47.62%) showed phosphate solubilization and 30 isolates (47.62%) produced indole-3-acetic acid (IAA); whereas 17 isolates (32.69%) of endophytic bacteria produced siderophores, 12 isolates (23.07%) exhibited phosphate solubilization and 20 isolates (38.46%) produced IAA. Based on their PGP traits, individual and co-inoculation of endophytic bacteria and actinomycetes, *Enterobacter* sp. EN-16, *Pentoae* sp. EN-29*, Microbispora* sp. GKU 827 and *Streptomyces* sp. GKU 833 were tested for effects of plant growth on maize plants (*Zea mays* L. cv. Nakhonsawan 3). Pot culture experiments revealed that maize plants with the co-inoculation between *Microbispora* sp. GKU827, *Enterobacter* sp. EN-16 and *Pentoae* sp. EN-29 gave the best enhancement of plant growth and significantly increased root and shoot biomass compared with individual inoculated and un-inoculated treatments. This experiment will be further investigated in sugarcane plants in order to apply these endophytes as active biofertilizer agents.

รางวัลที่ได้รับ

SARC Distinguished Scientist Award-2011, Scientific and Applied Research Center, India