IDENTIFICATION AND CHARACTERIZATION OF SOIL-ISOLATED *STREPTOMYCES* SJE177 PRODUCING ACTINOMYCIN

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Abstract. One hundred seventy-seven actinomycetes strains were isolated from soils collected from fruit orchards in Thailand. All were tested for antibacterial activity against seven pathogenic bacteria using co-cultivation methods. Forty strains (22.6%) were active against at least one indicator bacteria. Twenty-seven strains (15.3%) inhibited only gram-positive bacteria, four strains (2.3%) inhibited only gram-negative bacteria, and nine strains (5.1%) showed activity against both. Strain SJE177 had potent activity against all tested bacteria, and was selected for further investigation. A crude ethyl acetate extract of this strain retained inhibitory activity as tested by disk-diffusion method. Analysis of morphological and biochemical characteristics and the 16S rRNA gene sequence indicated this strain belonged to the genus *Streptomyces*. The strain formed a monophyletic line in a phylogenetic tree of 16S rRNA gene sequences with other *Streptomyces* reference strains. High performance liquid chromatography (HPLC) analysis showed SJE177 produced actinomycin. Since many isolates showed inhibitory activity against indicator bacteria, these results suggest Thai soil could be an interesting source to explore for antibacterial substances.

Key words: *Streptomyces*, antibacterial activity, co-cultivation method, actinomycin, Thailand

INTRODUCTION

Streptomyces spp are gram-positive soil-dwelling filamentous bacteria with a complex cycle of morphological differentiation (Claessen *et al*, 2006). Members of the genus *Streptomyces* are potential sources for secondary metabolites possessing a variety of biological activities, including antibacterial activity, which is used for human and animal treatment. It is estimated this bacteria synthesizes more than 7,000 metabolites (Bérdy, 2005). Antibiotic resistant bacteria emerge clinically within months to years following their use (Walsh, 2000; Palumbi, 2001). There is an urgent need to study how to overcome the resistance of pathogenic bacteria and fungi

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to commonly used antimicrobials. Identification of new antimicrobials and new ecological niches are important. This includes discovery of antibiotic-producing bacteria, mining of uncovered natural-product biosynthetic clusters in bacterial genomes, and inhibitors of antibiotic efflux systems (Wright and Sutherland, 2007).

Thailand is an agricultural country located in the tropics and contains great ecological diversity. Relatively few scientific studies have been carried out searching for new antibiotics from microorganisms isolated from Thai soil, where actinomycetes, in particular *Streptomyces* spp, is found abundantly. This study aimed to isolate *Streptomyces* from Thai soil and test for antibacterial activity against multiple drug resistant bacteria. Strain SJE177, showing potent activity against multiple drug resistant bacteria, was selected for biochemical and molecular characterization.

MATERIALS AND METHODS

Soil sample collection and tested microorganisms

Soil samples were collected from 4 provinces in 2006: Chanthaburi, Bangkok, Phetchaburi, and Nong Bua Lamphu. The samples were taken at a depth of 5 centimeters from the surface in shaded areas of fruit orchards.

Two standard strains (*Staphylococcus aureus* ATCC25923 and *Escherichia coli* ATCC 25922) and five multidrug resistant strains (methicillin-resistant *S. aureus*-2, methicillin-resistant *S. aureus*-1302, *E. coli*-7, *Acinetobacter*-1275, and *Pseudomonas aeruginosa*-6) were used for the antibacterial tests. Drug resistant strains were obtained from Siriraj Hospital (Bangkok, Thailand). Their resistance profiles are shown in Table 1. They were maintained on Mueller-Hinton (MH) (Becton, Dickinson, Sparks, MD) plates at 4ºC. Stock cultures were kept in 20% glycerol at -80ºC.

Isolation of actinomycetes

One gram of each soil sample was 10-fold serially diluted in saline solution, 0.1 ml of each solution, at dilutions of 10^{-4}-10^{-6}, then spread on selective media: Pridham medium [per 1 liter: 10 g glucose, 10 g starch, 2 g $(NH_4)_2SO_4$, 2 g $CaCO_3$, 1 g K_2HPO_4, 1 g $MgSO_4$, 1 g NaCl, and 12 g agar, pH 7.0] and water proline medium (per 1 liter: 10 g L-proline, 12 g agar, pH 7.0), then incubated at 28ºC for 7-14 days. Both screening media were supplemented with 25 µg/µl nalidixic and 50 µg/µl cyclohexamide to prevent growth of other bacteria and fungi, respectively. Typical *Streptomyces* colonies were picked and purified by streaking onto an agar plate of Waksman medium (per 1 liter: 10 g glucose, 5 g meat extract, 5 g peptone, 3 g NaCl, and 12 g agar, pH 7.0) and incubated at 28ºC for 7-14 days. The obtained isolates were maintained on a preservation medium: Seino's agar slant (per 1 liter: 10 g starch, 3 g N-Z amine type A, 1 g meat extract, 1 g yeast extract, 3 g $CaCO_3$, and 12 g agar).

Antibacterial bioassay by co-culture method

The seven indicator bacteria were grown in MH broth for 5 hours. A sterile cotton swab was dipped into a solution of properly adjusted cell density and swabbed on the MH agar surface. Isolated actinomycetes strains were previously grown on 301 seed medium (1 liter: 24 g starch, 10 g glucose, 3 g peptone, 3 g meat extract, 5 g yeast extract, 4 g $CaCO_3$, and 12 g agar) for 7 days. Colonies of actinomycetes were cut using a cork borer (8 mm in diameter) and placed on the MH plate

Table 1
Drug resistance profiles of indicator bacteria.

Indicator bacteria	Resistance profile
Gram-positive bacteria	
S. aureus ATCC 25923	-
MRSA-1302	Cefoxitin, Oxacillin Gentamicin Erythromycin,Ofloxacin, Levofloxacin Clindamycin, Cotrimoxazole
MRSA-2	Cefoxitin, Oxacillin, Gentamicin Erythromycin, Ciprofloxacin Clindamycin, Cotrimoxazole
Gram-negative bacteria	
E. coli ATCC 25922	-
P. aeruginosa-6	Cefepime, Cefoperazone/sulbactam, Cefoxitin, Ceftazidime, Ceftriaxone, Imipenem, Meropenem, Piperacillin/tazobactam Amikacin, Gentamicin, Netilmicin Ciprofloxacin
E. coli-7	Amoxicillin/clavulanic acid, Cefepime, Cefoperazone/sulbactam, Ceftriaxone, Ceftazidime, Piperacillin/tazobactam Gentamicin Ciprofloxacin
Acinetobacter-1275	Cefepime, Cefoperazone/sulbactam, Ceftriaxone, Ceftazidime, Piperacillin/Tazobactam Imipenem, Meropenem, Amikacin, Gentamicin Ciprofloxacin, Levofloxacin

previously swabbed with indicator bacteria. After overnight incubation at 37ºC, growth inhibition of the indicator bacterium was observed by measuring the diameters of the inhibition zones (including the diameter of the cork borer) and recorded (in mm).

Organic solvent extraction and chemical characterization

SJE177 was precultured in 5 ml of 301 seed medium for 2 days. The 5 ml inoculum was transferred to a 250 ml baffled flask containing 100 ml of production medium, and cultivated at 28ºC in a rotating shaker at 150 rpm for 6 days. The whole culture was mixed with an equal volume of ethanol and further incubated at 28ºC with rotation at 150 rpm for 30 minutes. The cell debris was removed by centrifugation at 3,000 rpm at 4ºC for 10 minutes. The collected supernatant was evaporated

at 40ºC to half the initial volume. The resulting extract was mixed with an equal volume of ethyl acetate and shaken vigorously. After centrifugation at 3,000 rpm at 4ºC for 10 minutes, the ethyl acetate extract (upper layer) was collected and evaporated at 40ºC until dry, then the residue was redissolved in ethanol (at 1 ml/ 100 ml culture).

The crude extract was analyzed with high pressure liquid chromatography (HPLC) using a Rainin Microsorb C18 column (4.6 x 75 mm) on an Agilent Hp1100 system with a photodiode array detector (200-600 nm) using a flow rate of 1.2 ml/ minute, and additional UV detection at 254 nm. The mobile phase used was a stepwise gradient of CH_3CN-0.15% KH_2PO_4 (pH 3.5) (15-85% v/v). Chemical library analysis was performed by comparing the UV spectrum and retention time of the respective peak to those of known compounds in the library.

Disk-diffusion susceptibility assay

The assay was performed using the Kirby-Bauer method. The extract (20 µl) from each isolate was loaded into blank sterile disks. The completely dried disks were transferred using sterile forceps onto the surface of the MH agar plates previously swabbed with an indicator bacterium. After overnight incubation at 37ºC, growth inhibition was recorded (in mm).

Identification by cultural and physiological characteristics

The taxonomic properties of isolate SJE177 were evaluated following the methods given in the international *Streptomyces* project (ISP)(Shirling and Gottlieb, 1966). The sporophores were studied under a light microscope. The morphology of the mature culture and color was observed on yeast extract-malt extract agar (ISP2), inorganic salts-starch agar (ISP4) and glycerol-asparagine agar (ISP5). Melanin production was determined on peptone-yeast extract-iron agar (ISP6), tyrosine agar (ISP7), and tryptone-yeast extract broth (ISP1), while the carbon utilization test was carried out in carbon utilizing medium (ISP9) with the addition of one of the following sugars: D-glucose (positive control), L-arabinose, sucrose, D-xylose, I-inositol, D-mannitol, D-fructose, D-sorbitol, cellubiose, and in the absence of a carbon source (negative control) as described in the ISP project (Shirling and Gottlieb, 1966). For other biological properties, milk coagulation and peptonization, hydrolysis of starch, and gelatin liquefaction were performed using the methods adapted from Cappuccino and Sherman (2005).

Molecular characterization of SJE177 and phylogenic classification

SJE177 was grown on Waksman agar plates for 7 days at 28ºC. The mycelia at the edge of the colonies were taken by sterile loop and resuspended in 200 µl of Tris-EDTA (TE) buffer (10 mM Tris-HCl and 1 mM EDTA) in an Eppendorf tube. The mixture was frozen at -70ºC and thawed by boiling at 100ºC for 10 minutes. After 10 minutes of centrifugation at 8,000 rpm, the DNA in the supernatant was collected and used as the DNA template for PCR. A 16S ribosomal DNA sequence of isolate SJE177 was amplified using universal primers (Nilsson and Strom, 2002): UFUL (5'-GCC TAA CAC ATG CAA GTC GA-3') and 1500R (5'-TTC AGC ATT GTT CCA TTG G-3'). Sequencing of the PCR-amplified product was conducted using primers: 350F (5'-TAC GGG AGG CAG CAG-3'), 785F (5'-GGA TTA GAT ACC CTG GTA GTC-3'), 1099F (5'-GCA ACG AGC GCA ACC C-3') on an automated 3100-AVANT GENETIC ANALYZER (Applied Biosystems, Foster City, CA). The obtained 16S rDNA

Table 2
Soil samples used for actinomycetes isolation.

Sources	Sample number	Trees under which soil was collected	Number of isolates	Designated number
Chanthaburi	1	Siam cardamom (*Amomum testaceum*)	2	SJE001 – SJE002
	2	Sala (*Salacca edulis*)	0	
	3	Dragon fruit (*Hylocereus undutus*)	2	SJE003 – SJE004
	4	Pisang mas (*Musa* AA group)	8	SJE005 – SJE012
	5	Durian (*Durio zibethenus*)	0	
	6	Tamarind (*Tamarindus indica*)	4	SJE013 – SJE016
	7	Sapodilla (*Manilkara kauki*)	2	SJE017 – SJE018
	8	Variegatum (*Piper sarmentosum*)	2	SJE019 – SJE020
	9	Santol (*Sandoricum koetjape*)	0	
	10	Spiny bamboo (*Bambusa arundinacea*)	3	SJE021 – SJE023
	11	Cassumunar (*Zingiber cassumunar*)	5	SJE024 – SJE028
	12	Mango (*Mangifera indica*)	6	SJE029 – SJE034
Bangkok	13	Fiji fan palm (*Pritchardia pacifica*)	10	SJE053 – SJE062
	14	Ixora (*Ixora chinensis*)	7	SJE063 – SJE069
	15	Hara-Champa (*Artabotrys siamensis* Miq.)	22	SJE070 – SJE091
	16	Eagle Wood (*Aquilaria crassna*)	30	SJE095 – SJE124
	17	Rain Tree (*Samanea saman)*	34	SJE125 – SJE158
	18	Cork Tree (*Millingtonia hortensis*)	19	SJE159 – SJE177
Phetchaburi	19	Rambutan (*Nephelium lappaceum*)	3	SJE092 – SJE094
	20	Jackfruit (*Artocarpus heterophyllus*)	0	
	21	Pomelo (*Citrus maxima)*	0	
Nong Bua Lamphu	22	Mango (*Mangifera indica* Linn.)	18	SJE035 – SJE053
		Total isolates	177	

nucleotide sequence (1,402 bp) was deposited at the National Center for Biotechnology Information (NCBI) GenBank database under accession number GU130178 . Closely related homologs were identified by comparing the partial 16S rDNA sequence with sequences deposited in the GenBank database by BLAST analysis (www.ncbi.nlm.nih.gov).

The 16S rRNA gene sequence of strain SJE177 (1,402 bp) was aligned with other nucleotide sequences, and the phylogenic tree of the 16S rRNA gene sequences was constructed by the neighbor-joining method using MEGA 4.0 software package (Tamura *et al*, 2007).

RESULTS

Isolation of actinomycetes from Thai soil

One hundred fifty-nine actinomycetes isolates were obtained from 21 soil samples collected from rhizospheric soil from fruit orchards in Chanthaburi, Bangkok, and Phetchaburi, while 18 other isolates were obtained from Nong Bua Lamphu. The numbers of isolates and their origins are shown in Table 2.

Screening of actinomycetes for antibacterial activity

Using the co-culture method it was determined that of the 177 isolates, 40 (22.6%) produced inhibitory substances

Table 3
Antibacterial activity of *Streptomyces* strain SJE177.

Indicator organisms	Inhibition zone (mm)	
	Agar block	Crude extract
S.aureus ATCC 25923	16	18.5
E. coli ATCC 25922	2	-
MRSA-1302	19	17
MRSA-2	17	18.5
P. aeruginosa-6	2(uc)	-
E. coli-7	0.5	-
Acinetobacter-1275	5	-

uc, unclear inhibition; -, no inhibition
Diameter of the inhibition zone using the co-culture method excluding the diameter of *Streptomyces* agar block (8 mm).
Diameter of the inhibition zone using crude extract excluding the diameter of the disk (6 mm).

against at least one indicator bacterium. Among the active isolates, 27 inhibited only gram-positive bacteria, 4 strains inhibited gram-negative bacteria, and 9 other strains had activity against both gram-positive and gram-negative bacteria (data not shown). The active isolates suppressed or inhibited bacterial growth with inhibition zones ranging from 0.5 to 19 mm. From primary screening, several isolates were found to inhibit multi-drug resistant pathogenic bacteria. SJE177 possessed activity against both gram-positive and gram-negative bacteria, especially MRSA, as shown in Table 3. Strain SJE177 was selected for disk-diffusion testing using an organic solvent extract. The results show the extract from SJE177 retained activity against gram-positive bacteria but lost activity against gram-negative bacteria (Table 3).

Compound identification of crude extract

Extract from SJE177 was further analyzed by HPLC to identify compounds. The antibacterial activity was found to have a retention time of 22.8 minutes (Fig 1A). The UV spectrum of the compound in this peak, when compared to a database along with considering its retention time from the HPLC, indicates the compound is actinomycin (Fig 1B).

Characteristics of SJE177

Colonies, mycelia, and conidia of strain SJE177 are shown in Fig 2A. Strain SJE177 produced a rectiflexibile spore chain containing 10-50 or more spores per chain (Fig 2B). This strain is generally round with a convex elevation and a ciliate margin. Aerial mycelium proliferated well on most of the ISP media; Waksman medium (WM) and Seino medium (SN). The color of the aerial mycelium on most media was white-yellow, while that of the substrate mycelium was yellow-brown or orange-brown (Table 4). Yellow diffusible pigment was present in all tested media (Table 4).

The other physiological and biochemical properties of this strain are shown in Table 4. Melanoid pigment was not produced in any of the three tested media (ISP1, ISP6, and ISP7). No growth or only

Table 4
Morphological, physiological and biochemical characteristics of strain SJE177 and phylogenetically related *Streptomyces* species.

Characteriatics	*Streptomyces* strains							
	1	2	3	4	5	6	7	8
Spore chain	RF	RF	RF	RF or S	RF	RF	RF	RF
Spores per chain (*n*)	10-50 or more	10-50 or more	10-50 or more	10-50 or more	ND	10-50 or more	3-10 or more	10-50
Spore surface	ND	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Aerial mass color on								
ISP2	W/Y	Y	Y	G	Y	Y/G	Y/R	Y
ISP4	W/Y	Y	Y	G	Y	Y	Y/R	Y
ISP5	W/Y	Y	Y	G	Y	Y	Y/R	Y
Reverse side color on								
ISP2	Y	Y/G	Y	G/Y	Y	Y/B	Y/B	Y
ISP4	Y	Y/G	Y	G/Y	Y	Y/B	Y/B	Y
ISP5	Y	Y/G	Y	G/Y	Y	Y/B	Y/B	Y
Melanin production	-	-	-	-	ND	-	-	-
Soluble color	Y	Y	Y	-	ND	Y/B	-	-
Growth on sole carbon sources:								
D-glucose	+	+	+	+	ND	+	+	+
L-arabinose	+	+	+	+	ND	+	-	+
Cellubiose	+	ND	ND	ND	ND	ND	ND	ND
Fructose	+	+	+	+	ND	+	+	+
I-inositol	-	-	-	ND	ND	-	-	-
D-mannitol	+	+	+	ND	ND	+	+	+
D-sorbitol	-	ND	ND	ND	ND	ND	ND	ND
Sucrose	-	-	-	ND	ND	-	-	-
D-xylose	+	+	+	+	ND	+	+	+

Strains/species: 1, *Streptomyces* sp SJE177; 2, *S. rubiginosohelvolus*; 3, *S. parvus*; 4, *S. olivaceus*; 5, *S. mediolani*; 6, *S. griseinus*; 7, *S. pluricolorescens*; 8, *S. badius*. Data for reference species were taken from Arcamone *et al* (1969) and Shirling and Gottlieb (1968a,b; 1972).
RF, Rectiflexibiles; S, Spiralis; +, Positive; -, Negative; ND, No data available; W, White; Y, Yellow; B, Brown; G, Gray; R, Red

trace growth occurred with some carbon sources tested, namely D-sorbitol, sucrose, and I-inositol in Pridham and Gottlieb's basal medium. It could utilize D-glucose, L-arabinose, D-fructose, D-xylose, D-mannitol, and cellubiose for growth (Table 4). Gelatin liquefaction, milk peptonization and starch hydrolysis were positive, while milk coagulation was negative (data not shown).

16s rDNA sequence analysis of SJE177 and its phylogenetic analysis

Comparison of the 16S rDNA sequences of SJE177 (Genbank accession number GU130178) with the GenBank database showed this isolate belongs to the

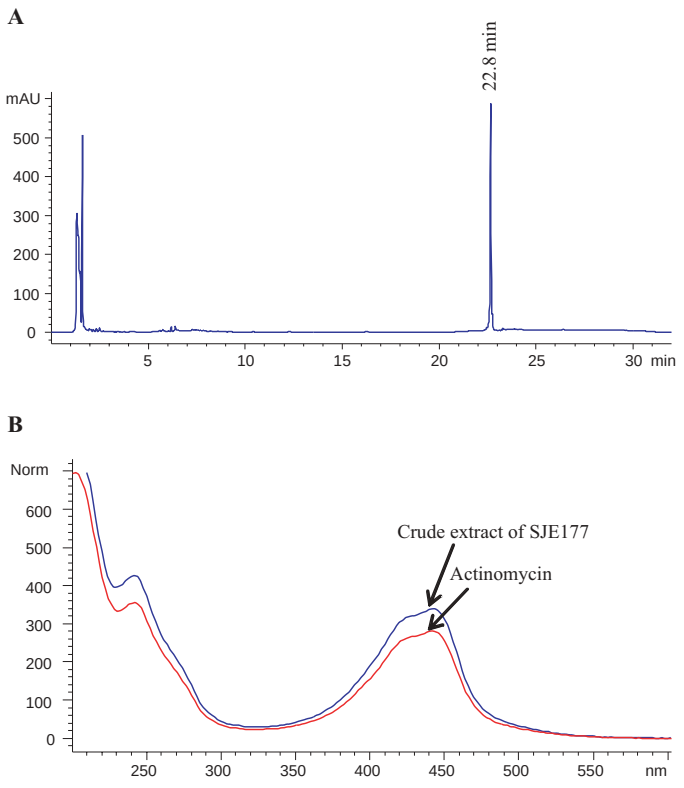

Fig 1–Reversed-phase HPLC analysis of crude extract from strain SJE177. (A) Chromatogram of crude extract of strain SJE177, (B) UV spectrum of the 22.8 min-peak in the crude extract of SJE177 and actinomycin.

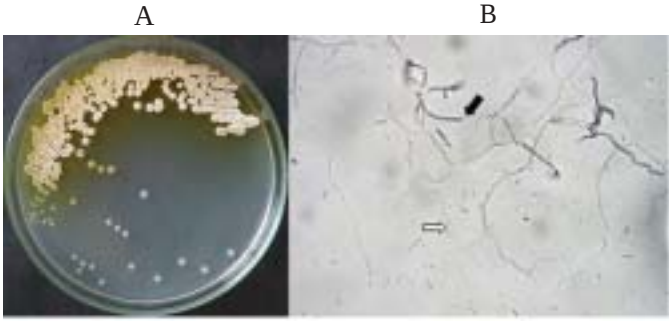

Fig 2–*Streptomyces* strain SJE177. (A) Colony morphology of SJE177 grown for 7 days at 28ºC on yeast extract-malt extract agar (ISP-2). (B) Sporulating mycelia (1,000x) on ISP-2 medium. White arrow indicates mycelia, black arrow indicates conidia.

genus *Streptomyces* with > 99% certainty. The strain is similar to several *Streptomyces* strains, such as *S. rubiginosohelvolus* strain NBRC 12912 and *S. parvus* NBRC 3388 (data not shown). The phylogenetic tree derived from neighbor-joining analysis showed a correlation between the 16S rDNA sequence of SJE177 and twenty-four other actnomycetes strains (Fig 3) in the NCBI database. It can be divided into two main clusters by the *Streptomyces* and non-*Streptomyces* branches, which are bacteria in the same family. SJE177 belongs to cluster I as *Streptomyces*, with a close relationship to seven *Streptomyces* strains: *S. rubiginosohelvolus* NBRC 12912 (AB184240), *S. parvus* NBRC 3388 (AB184756), *S. olivaceus* NBRC 3724 (AB184793), *S. mediolani* NBRC 15427 (AB184674), *S. griseinus* NBRC 12869 (AB184205), *S. pluricolorescens* NBRC 12808 (AB184162), and *S. badius* NBRC 12745 (AB184114). Based on several characteristics, the 16S rRNA gene sequence and soluble pigment formation, SJE177 could be assigned to either *S. rubiginosohelvolus* or *S. parvus*.

DISCUSSION

Soil is an ecological niche with many orgainisms living together, some producing useful natural products, including therapeutically important antibiotics. Microorganisms, especially, those belonging to the ge-

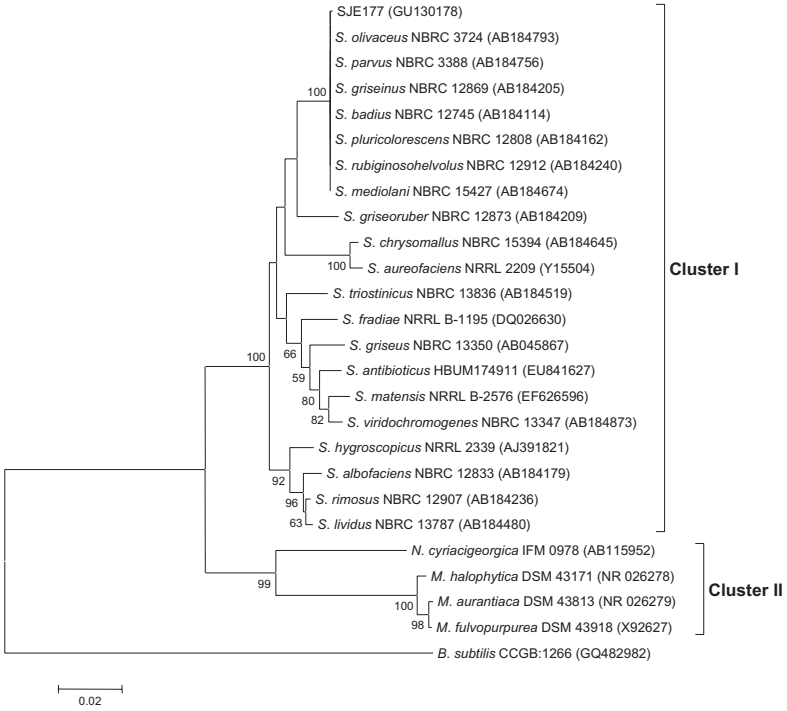

Fig 3–Phylogenetic tree of the 16S rDNA sequence of strain SJE177. Bootstrap values calculated from 1,000 re-samples with values over 50% shown at the respective node. The phylogenetic analysis was carried out using MEGA 4.0 software package. The cluster I and cluster II were indicated. The GenBank accession numbers of the strains were indicated. The scale bar indicates 2% estimated sequence divergence.

nus *Streptomyces*, are abundant in the soil. *Streptomyces* grows relatively slower than other bacteria. Serial dilution and plating on selective media supplemented with antibiotics to inhibit growth of other bacteria and fungi is important to allow preferential growth of *Streptomyces* spp over other species and reduces contamination. Most actinomycetes isolates had the typical morphology of streptomycetes, as described in The Bergey's Manual of Determinative Bacteriology (Holt *et al*, 1994). They were slow-growing, aerobic, chalky, and contain both aerial and substrate mycelia with a variety of colors.

Of the 40 isolates with activity against the seven indicator bacteria, 27 inhibited gram-positive bacteria, 4 inhibited gram-negative bacteria, and 9 had activity against both groups. These results agree with a previous study, which showed most isolated *Streptomyces* spp had activity against gram-positive bacteria (Thakur *et al*, 2007). The inhibition zone against gram-positive bacteria was relatively larger and clearer compared to the gram-negative bacteria (data not shown) indicating gram-negative bacteria are less susceptible to active substances produced by these isolates. The SJE177 strain with inhibitory activity against all tested bacteria was subjected to secondary screening by disk-diffusion. The inhibition pattern from the disk-diffusion method was different from the co-culture method in which activity against all tested gram-negative bacteria was lost. There are two possible explanations for this: first, cultivation on solid media and liquid media may lead to production of different secondary metabolites (Robinson *et al*, 2001); second, some compounds may be lost during the organic solvent extraction method: active components may become inactivated during the extraction step or may be excluded from the solvent phase due to their hydrophilic nature.

The 16S rRNA gene sequence of the SJE177 isolate had high homology (>99%)

with the various *Streptomyces* species recorded in the GenBank database. This result was confirmed by phylogenetic analysis. The phylogenetic relationship showed the strain SJE177 lies within the same subclade in the tree with seven other *Streptomyces* reference strains. The attempt made to classify SJE177 was conducted by comparing all available data from the seven other strains to this strain as shown in Table 4. The similar properties were: a rectiflexibile spore chain, smooth spore surface, negative melanin production and negative utilization of I-inositol and sucrose. A variety of aerial mass color, reverse side pigment, and soluble color among strains were observed. From our data, SJE177 is closest to *S. rubiginosohelvolus* or *S. parvus*. There was a previous report stating *S. parvus* produced actinomycin (Benedict, 1953). However, different species reported by different groups of researchers may be the same species due to a lack of standard strain and not enough information regarding the characteristics of each strain (Waksman, 1957; Krasil'nikov, 1960; Shirling and Gottlieb, 1966).

In conclusion, Thai rhizospheric soils provided a rich source of streptomycetes. We retrieved some actinomycetes isolates with high antibacterial activity, including strain SJE177, which produced actinomycin. From multi-disciplinary characterization, it was shown that this strain belongs to the genus *Streptomyces*. In the future, antibiotic production from this strain needs to be further analyzed in order to discover the antibacterial compound and understand the genetic regulation of antibiotic production.

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