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Identification and evaluation of biological activity of the terrestrial *Streptomyces* sp. strain G1-3

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Abstract A filamentous actinomycete strain, designed G1-3, was isolated from the soil sample collected in Khao Khichakut, Chanthaburi, Thailand. The microbial identification was performed using phenotypic data that comprised morphological and partial physiological characteristics; the strain G1-3 was classified into the streptomycete group, its produced substrate and aerial hyphae, and presented conidiospores on the aerial hyphae. Genotypic characteristic based on phylogenetic analysis and 16S rRNA gene sequences similarity indicated that strain G1-3 was closely related to type strains of the *Streptomyces* that showed high similar values with *Streptomyces kronopolitis* NEAU-ML8^T (99.66%), *Streptomyces lydicus* ATCC25470^T (99.59%), and *Streptomyces chattanoogensis* NRRL ISP-5002^T (99.59%). Analysis of biological activity, the crude ethyl acetate extract of *Streptomyces* strain G1-3 showed cytotoxic activity against cancerous KB and MCF-7 cells with the IC_{50} values of 3.52 and 18.69 µg/ml, respectively. In addition, this strain also showed cytotoxicity against non-cancerous Vero cell at the IC_{50} value of 12.95 µg/ml.

Keywords: Actinomycetes, Biological activity, Cytotoxicity, Metabolites, *Streptomyces*

Introduction

Filamentous actinobacterial group (actinomycetes) are Gram-stain-positive, generally aerobic, high guanine and cytosine (G + C) in the genome (>50%); this bacterial group is classified into the phylum *Actinomycetota*. Actinomycetes also form asexual spores such as conidiospore, sporangiospore, and a fragment of hypha (arthrospore) that developed on the substrate or aerial hyphae. Based on the population of actinomycete group, they are mainly diverse into two groups consisting of streptomycete (common-actinomycetes) and non-

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streptomycetes (rare actinomycetes). They are distributed in diverse natural sources that are wildly observed in terrestrial habitats (Takizawa *et al.,* 1993; Xu *et al.,* 1996; Malisorn *et al.,* 2020). In addition, they were found in marine sources and some strains associated with plants and insects (Phongsopitanun *et al.,* 2020; Phongsopitanun *et al.,* 2021).

Based on morphology and cell- wall chemotypic characteristic, *Streptomyces* belongs to the family *Streptomycetaceae* within the order *Streptomycetales* (Waksman and Henrici, 1943). Generally, *Streptomyces* present branched substrate and aerial mycelia that differentiated to spore-chains (spiral or long chains). Recently, genome- based taxonomic reclassification, *Streptomyces* was a type genus of the family *Streptomycetaceae*. In addition, this family contained other genera such as *Allostreptomyces*, *Kitasatospora*, and *Streptacidiphilus* and the novel genera *Embleya* and *Yinghuangia* (Nouioui *et al.,* 2018). Most *Streptomyces* species produce a wide range of diffusible pigment colors on the culture media. They showed main chemotype that differentiated from closely related genera using the presence of *LL*-diaminopimelic acid isomer (*LL*-DAP) in cell-wall peptidoglycan and the pattern of whole-cell sugar has no diagnostic form. At present, there are more than 1000 species of this genus *Streptomyces* (Parte, 2018). In the part of microbial natural products study, many *Streptomyces* strains have been well- known as the key producer of various bioactive secondary metabolites including antibacterial macrolide (Supong *et al.,* 2017), antifungal quinone (Han *et al.,* 2021; Wang *et al.,* 2013), and anticancer nathracycline glycoside agents (Gui *et al.,* 2018).

In the present study, strain G1-3 was isolated from terrestrial soil collected in Chanthaburi province, Thailand. The taxonomic position of strain G1-3 was analyzed by morphological, physiological and genotypic data by using the 16S rRNA gene. The strain exhibited potential cytotoxic activity against cancerous KB, and MCF-7 cells. Consequently, this research aimed to investigate the biological activity of the terrestrial *Streptomyces* sp. strain G1-3, and characterize the active strain based on polyphasic data.

Materials and methods

Sample preparation and isolation of actinomycete

The terrestrial soil sample was collected from Khao Khichakut area in Chanthaburi, Thailand. Preparation of sample, the dried soil was ground under sterile conditions and was suspended into 0.85% (w/v) NaCl followed by a standard serial dilution method. The diluted samples were spread on modified half-ISP 2 agar (glucose 2 g, malt extract 5 g, yeast extract 2 g, in 1 L of distilled

water, at pH 7.8). The half-ISP 2 isolation agar medium supplemented with the antibiotics that comprised nalidixic acid (30 µg/ml), and cycloheximide (30 µg/ml), which were used to inhibit Gram-negative bacteria and fungal group, respectively. After incubating at 30°C for 21 days, strain G1-3 was isolated and selected from the isolated plate, then the bacterial colony was sub-cultured onto ISP 2 agar (glucose 4 g, yeast extract 4 g, and malt extract 10 g in 1 L of distilled water). An actinomycete strain was cultivated on ISP 2 slants and preserved the mycelium into 10% glycerol at -20°C for long-term storage.

Characterization of actinomycete strain G1-3

The morphological characteristic that comprised of vegetative mycelium and pattern of spore production was determined using a light microscope that the strain was aerobically grown on ISP 2 agar at 30°C for 14 days. Cultural characteristics using the international *Streptomyces* project media was determined by Shirling and Gottlieb (1966). After the strain G1-3 grew on ISP 2 agar for 14 days, the standard ISCC–NBS Color Charts sample number 2106 (Kelly, 1964) was used to determine the color of bacterial colony (substrate and aerial mycelium) and diffusible pigment. The physiological characteristic consisting of starch hydrolysis, gelatin liquefaction, coagulation and peptonization of milk, and nitrate reduction test was determined using the modified methods that described by Arai (1975) and Williams and Cross (1971). Utilization of carbon sources (arabinose, fructose, galactose, glucose, lactose, raffinose, sucrose and xylose) was determined by the method previously described by Shirling and Gottlieb *(*1966*)*. The effect of temperature for growth, pH range of 3–13, and tolerance of 0–8% (v/w) NaCl were determined by culture on ISP 2 agar.

Genotypic characteristic analysis, the genomic DNA was used for PCR amplification of 16S rRNA gene of strain G1-3 that was obtained from the cells grown in ISP 2 broth at 30°C (200 rpm) for 4 days. The DNA extraction method was described by Tamaoka (1994). The 16S rRNA gene of the strain was amplified using the universal primers (Inahashi *et al.,* 2010). The nucleotide sequencing of 16S rRNA gene was sequenced by universal primers that comprised 785F (5′- GGATTAGATACCCTGGTA- 3′) and 907R (5′- CCGTCAATTCMTTTRAGTTT-3′) that determined by Macrogen from Seoul, Republic of Korea. Multiple aliments of the sequences were analysed using BioEdit software (Hall, 1999) that compared with the selected top high similarity values of *Streptomyces* species. Analysis of 16S rRNA gene, the sequences of the *Streptomyces* type strains were selected and obtained from the nucleotide database (GenBank/EMBL/DDBJ/EzBioCloud) (Yoon *et al.,* 2017). Before

phylogenetic analysis, gaps and ambiguity in the sequences were compared and eliminated. The relationship between strain G1-3 and type strains of *Streptomyces* species was compared using neighbor-joining phylogenetic tree that evaluated by Kimura's 2-parameter model (Saitou and Nei, 1987; Kimura, 1980). The tree was reconstructed with the bootstrap values based on 1000 replicates that analyzed by MEGA software version 11 (Tamura *et al.,* 2021).

Fermentation and extraction of crude extract

The stock culture of strain G1-3 was prepared by culture on ISP 2 agar and incubated at 30°C for 4 days. Preparation of seed culture, the stock culture was inoculated into Erlenmeyer flasks that contained 150 ml of ISP 2 broth medium. After incubating at 30°C for 4 days, the seed culture was inoculated into 500 ml Erlenmeyer flask (200 ml of ISP 2/flask). The inoculated flasks were fermented on rotary shakers at 30°C for 14 days. The fermented flasks of strain G1-3 were extracted three times with ethyl acetate (EtOAc) to give EtOAc extract solution. Then the extract solution was dehydrated with the sodium sulfate (Na_2SO_4) powder and evaporated to give the crude extract (180 mg).

Bioassays of crude extract

Cytotoxic activity

The crude EtOAc extract of strain G1-3 was evaluated cytotoxic activity against the African green monkey kidney fibroblasts ATCC CCL-81 (Vero cells) was determined using green fluorescent protein microplate assay (GFPMA) that described by Changsen *et al.* (2003). The 384-well plate contained the crude extract (5 µl) and Vero cell line suspension (45 µl). Then the prepared well plate was incubated at 37°C for 4 days. For incubator condition contained 5% carbon dioxide (CO_2) gas. The plate was analyzed with fluorescence that measured in bottom-reading mode on wavelengths 485 and 535 nm for excitation and emission, respectively. The formula [1-[(FUT/FUC)] × 100 were used for calculated the values (%) that FUT and FUC were the mean fluorescent unit from cells treated with the crude extract and 0.5% DMSO (dissolved solvent), respectively. Evaluation of cytotoxic activity against cancer cells that comprised MCF-7 (human breast cancer ATCC HTB-22), and KB (oral human epidermoid carcinoma, ATCC CCL-17) were determined using the resazurin microplate assay (REMA) that was described by O'Brien *et al.* (2000). The IC_{50} value was calculated from dose-response curve of cytotoxicity values (%) compared with concentration of crude sample that analyzed by SOFTMax Pro software. For cytotoxicity result, the value less than 50% inhibition was indicated that it was

inactive result. The maximum concentration of test crud extract was done at 50 μg/ml. The IC_{50} value displays the concentration that reduced the cell growth by 50%. For cytotoxicity against non-cancerous Vero cells, ellipticine (1.58 μg/ml) was used as positive control. Ellipticine (1.58 μg/ml) and doxorubicin (8.07 μg/ml) were used as the positive controls with cytotoxicity against KB, while doxorubicin (8.07 μg/ml) and tamoxifen (6.96 μg/ml) were used as the positive controls for cytotoxicity against cancerous MCF-7 cells.

Anti-bacterial activity

The antibacterial activity of crude extract was tested with Gram-positive (*Bacillus cereus*) and Gram-negative (*Escherichia coli*) that was determined by using the optical density microplate assay (Clinical and Laboratory Standard Institute, 2006). The solution of crude extract (5 μl) and bacterial cell suspension (4 ul) was added into of each well (384-well plate). After incubating at 37°C for 5–6 h, the optical density of bacterial cells was determined using microplate reader on wavelength 600 nm. The percentage of inhibition (%), which is less than 90%, was reported as inactive calculated following the equation n [1-[(FUT/FUC)] × 100. The value of minimal inhibitory concentration (MIC) was defined by the lowest concentration of the tested crude showing more than 90% inhibition. For anti-bacterial activity, the maximum concentration of crude extract was used at 50 μg/ml. The positive controls with anti-*B. cereus* contained rifampicin (3.13 μg/ml) and vancomycin (2.00 μg/ml). Rifampicin (3.13 μg/ml) was used as a positive control with Gram-negative *E. coli*.

Anti-phytopathogenic fungal activity

Determination of anti-phytopathogenic fungal *Fusarium oxysporum* and *Colletotrichum acutatum* was analyzed by carboxy- fluorescein diacetate (CFDA) fluorometric detection (Aremu *et al.,* 2003; Guarro *et al.,* 1998; Haugland, 2002). Preparation of spore adhesion and germination, the 384-well plate was added with spore suspension (25 μl) and incubated at 30°C for 2-3 h. Then, 25 μl of the crude extract was added and incubated at 25°C for 16–18 h. And 25 μl of cocktail, containing 2 μl of 0.9 mg/ml CFDA dissolved in 70% DMSO and 25 μl of 40% glycerol, which was added and incubated at 30°C on the dark condition for 5-10 min. The well plate was washed with sterile distilled water, blotted dry with paper towels, and each well was filled with sterile distilled water (25 μl). Then, the well plate was detected using the fluorometer at wavelengths 485 nm for excitation and 535 nm for emission with the bottom-reading mode. The inhibition value was calculated following the formula [1-[(FUT/FUC)] × 100, where was reported to be as inactive for the value less than 90% inhibition. The activity results based on MIC value were indicated as the

lowest concentration of the tested sample exhibiting more than 90% inhibition of fungal test after exposure incubation. The maximum concentration of the sample was 50 μg/ml. The positive control for anti-phytopathogenic fungal test was amphotericin B at 4.00 μg/ml.

Results

Isolation and characterization of actinomycete strain G1-3

The terrestrial actinomycete strain G1-3 was isolated from a soil sample that was collected from Chanthaburi province. The phenotypic results based on morphological, cultural, and physiological characteristics showed that strain G1-3 presented grayish brown, and yellowish gray color of substrate, and aerial mycelia on ISP 2 agar. Strain G1-3 displayed spiral chain spore that developed on aerial hypha (Figure 1). Based on the cultural characteristic on the standard ISP media indicated that strain G1-3 grew well on ISP 2, ISP 3, ISP 4, and nutrient agars. For the physiological characteristics, strain G1-3 grew at pH 5–10 and on 0–6% NaCl. Strain G1-3 can be grown at 25–40°C (optimum, 30°C). In addition, starch hydrolysis, gelatin liquid fraction, and coagulation of milk had positive results. Carbon utilization results, the strain G1-3 utilized arabinose, fructose, galactose, glucose, lactose, raffinose, and xylose but sucrose was negative (Table 1.). The basic study by partial phenotype indicated that strain G1-3 was classified as the streptomycete group. For the EzBiocould determination, the 16S rRNA gene sequence of actinomycetes strain G1-3 (1459 nucleotides) revealed that the strain was identified to *Streptomyces* that showed the highest similarity 99.66% with *Streptomyces kronopolitis* NEAU-ML8^{T}. In addition, the strain displayed similarity with *Streptomyces lydicus* ATCC25470^{T} (99.59%), *Streptomyces chattanoogensis* NRRL ISP-5002^{T} (99.59%), which showed 16S rRNA gene similarity lower than 99.50% with other species. Neighbor-joining phylogenetic analysis based on 16S rRNA gene sequences of strain G1-3 with related *Streptomyces* species confirmed that strain G1-3 was closely related with the group of *Streptomyces kronopolitis* NEAU-ML8^{T} (Figure 2).

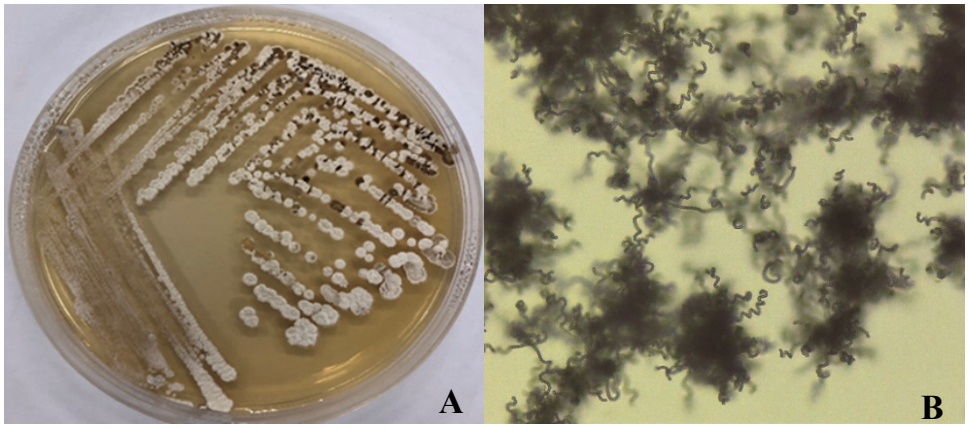

Figure 1. Colony characteristic (**A**), and light micrograph of spore chain (**B**) of *Streptomyces* strain G1-3

Table 1. Physiological characteristic of *Streptomyces* strain G1-3

Characteristics	***Streptomyces* strain G1-3**
Substate mycelium	Grayish brown
Aerial mycelium	Yellowish gray
Diffusible pigment	Brown
Growth temperature (°C)	25–40
pH range	5–10
NaCl (%) tolerance	0–6
Starch hydrolysis	+
Milk coagulation	+
Milk peptonization	-
Gelatin liquidfaction	+
Utilization of:	
Arabinose	+
Fructose	+
Galactose	+
Glucose	+
Lactose	+
Raffinose	W
Sucrose	-
Xylose	+

+: Positive, w: weakly positive, ⁻: Negative

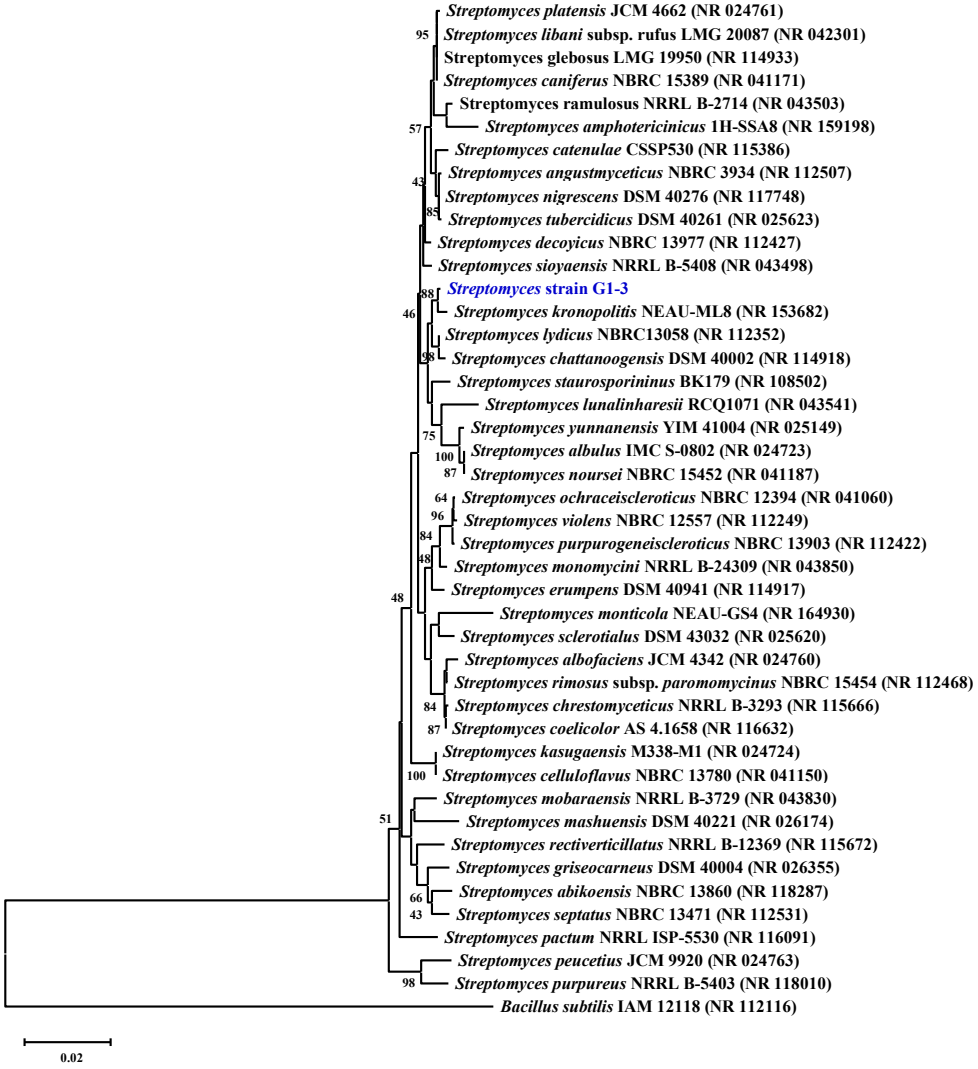

Figure 2. Neighbor- joining phylogenetic tree based on 16S rRNA gene sequences showing the relationship between strain G1- 3 and related *Streptomyces* species. The bootstrap values derived from 1,000 replication, only values >40% are showed. Bar 0.02 substitutions per nucleotide position.

Biological activity

Crude ethyl acetate extract of *Streptomyces* strain G1-3 was tested for biological activities that comprised anti-bacterial, anti-fungal, and cytotoxic activities. For investigation of cytotoxicity, crude extract of strain G1-3 displayed moderate activity against cancerous KB, and MCF-7 cells with the IC_{50} values of 3.52, and 18.69 µg/ml, respectively. The cytotoxicity against Vero (African green monkey kidney fibroblasts) cells, the crude extract exhibited moderate activity with an IC_{50} value of 1295 µg/ml. Antibacterial activity against *B. cereus* and *E. coli* indicated that crude extract of G1-3 was inactive substant that presented the MICs more than 50 µg/ml. In addition, *Streptomyces* strain G1-3 could not inhibit the phytopathogenic fungal test (*F. oxysporam* and *C. acutatum),* MIC values more than 50 µg/ml (Table 2.).

Table 2. Biological activities of crude ethyl acetate from strain G1-3

Sample	Anti-bacterial (MIC, ug/ml)		Anti-fungal (MIC, µg/ml)		IC_{50} (µg/ml)		
	B. cereus	*E. coli*	*F. oxysporum*	*C. acutatum*	KB	MCF-7	Vero
G1-3 -Crude extract	>50	>50	>50	>50	3.52	18.69	12.95
Vancomycin*	2.00						
Rifampicin *		3.13					
Amphotericin B*			4.0	4.0			
Ellipticine*					1.81		1.58
Doxorubicin*					0.655	8.07	
Tamoxifen*						6.96	

*: positive control for activity test

Discussion

In this study, biological activity was evaluated and polyphasic taxonomy of the potential actinomycete strain G1-3 obtained from a soil sample collected in Chanthaburi, Thailand was examined. Taxonomic study base on morphological clearly showed that the strain displayed general properties within the actinobacterial group and was classified into the genus *Streptomyces*, family *Streptomycetaceae* (Waksman, 1967) that its formed branched hyphae and developed conidiospore con the aerial hyphae. In addition, the streptomycete group could be confirmed by chemotaxonomic data using the pattern of the *LL*-isomer form of DAP in the peptidoglycan. The result from the genotypic

characteristic based on phylogenetic relationship of 16S rRNA gene cleared that strain G1-3 presented the highest similarity level (99.66%), closely related with the insect-derived *Streptomyces kronopolitis* that previously isolated from millepede *Kronopolites svenhedind* Verhoeff (Liu *et al.,* 2016). *Streptomyces kronopolitis* is a producer of antifungal phoslactomycin derivatives that could inhibit *Pyricularia oryzae and Septoria tritici* with ED_{50} values in a range of 7-16 uM (Fotso *et al.,* 2013). The phoslactomycin series (PLMs) are found in various terrestrial *Streptomyces* species, some derivatives exhibited cytotoxicity against cancer cells (Chen *et al.,* 2012).

The results of phenotypic, genotypic characterization, and bioactive natural products of the soil-associated *Streptomyces* strain G1-3 showed a potential source of bioactive metabolites that can be useful in the future also including exploring the determination of chemical compounds from the strain.

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