Agr. Nat. Resour. 57 (2023) 271–280

AGRICULTURE AND NATURAL RESOURCES

Journal homepage: http://anres.kasetsart.org

Research article

Investigation of biological activity and identification of culturable insect-derived *Streptomyces* strains from *Cossus chloratus*

Khomsan Supong[a,*], Sukritta Anutrakunchai[b], Titi Thongkamngam[b], Nantiya Bunbamrung[c], Pattama Pittayakhajonwut[c], Somboon Tanasupawat[d]

[a] *Department of Applied Science and Biotechnology, Faculty of Agro-Industrial Technology, Rajamangala University of Technology Tawan-ok, Chanthaburi campus, Chanthaburi 22210, Thailand*

[b] *Department of Plant Production and Landscape Technology, Faculty of Agro-Industrial Technology, Rajamangala University of Technology Tawan-ok, Chanthaburi campus, Chanthaburi 22210, Thailand*

[c] *National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand Science Park, Pathum Thani 12120, Thailand*

[d] *Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok 10330, Thailand*

Article Info

Article history:
Received 25 November 2022
Revised 26 February 2023
Accepted 4 March 2023
Available online 30 April 2023

Keywords:
Actinobacteria,
Biological activity,
Cossus chloratus,
Insect,
Streptomyces

Abstract

Importance of the work: Insects are interesting sources of various bioactive-producing actinomycetes that guide new metabolite discovery.

Objectives: To investigate the biological activity and identify the insect-derived actinomycete strains isolated from *Cossus chloratus*.

Materials & Methods: In total, 20 actinomycete strains were isolated from *C. chloratus* collected from Chanthaburi province, Thailand. Taxonomic studies used both phenotypic and genotypic data. Crude ethyl acetate extracts were assayed for biological activity that comprised antibacterial, antifungal, antimalarial and anticancer activities.

Results: Based on the phenotypic and genotypic characteristics, insect-derived actinomycetes were identified to belong to the genus *Streptomyces* and categorized into nine groups (I–IX) closest to *S. drozdowiczii*, *S. xylanilyticus*, *S. kunmingensis*, *S. daghestanicus*, *S. cavourensis*, *S. badius*, *S. parvulus*, *S. flavofungini* and *S. diacarni*. The insect-derived *Streptomyces* strains CoC1-5 and CoC1-10 inhibited *Bacillus cereus* with a MIC of 12.50 µg/mL. The strains CoC2-2, CoC2-4 and CoC2-13 exhibited moderate anti-*Mycobacterium tuberculosis* activity, with MICs of 12.50 µg/mL, 6.25 µg/mL and 25.00 µg/mL, respectively. The strains CoC1-5 and CoC1-21 displayed anti-phytopathogenic *Colletotrichum acutatum* activity, with MICs of 25.00 µg/mL and 50.00 µg/mL, respectively. The strain CoC1-21 showed strong anti-malarial activity with a MIC of 0.18 µg/mL, while the strains CoC1-5, CoC1-11, CoC1-21 and CoC2-2 exhibited cytotoxicity (Vero, NCI-H187 and McF7) with IC_{50} values in the range 0.32–17.93 µg/mL.

Main finding: This was the first report detailing the biological activities of *Streptomyces* strains isolated from the longkong bark-eating caterpillar *C. chloratus*.

* Corresponding author.
E-mail address: khomsan_su@rmutto.ac.th (K. Supong)

online 2452-316X print 2468-1458/Copyright © 2023. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/), production and hosting by Kasetsart University Research and Development Institute on behalf of Kasetsart University.

https://doi.org/10.34044/j.anres.2023.57.2.07

Introduction

Streptomyces, as the type genus within the family *Streptomycetaceae* that belongs to the phylum *Actinobacteria*, are Gram-positive, aerobic, filamentous, non-acid fast that lack mycolic acid and are non-motile actinomycetes. The general chemotype, peptidoglycan-cell wall contains the *LL*-isomer of diaminopimelic acid and glycine (cell-wall type I) with no diagnostic sugar of bacterial whole-cell hydrolysates (Nouioui et al., 2018). The genus *Streptomyces* is the most numerous in the phylum, currently consisting of more than 1,000 published species (https://lpsn.dsmz.de/genus/streptomyces; Parte et al., 2020). *Streptomyces* species are mainly found in terrestrial soils, while some have mutualistic associations with other organisms, such as plant endophytes, and insect-derived *Streptomyces* (Watanabe et al., 2003; Matarrita-Carranza et al., 2017; Chevrette et al., 2019; Wang et al., 2020). Recently, three new species, namely *Streptomyces fractus*, *S. lasiicapitis* and *S. smaragdinus* have been proposed based on strains isolated from insects (Rohland et al., 2015; Ye et al., 2017; Schwitalla et al., 2020). Bioactive-producing actinomycete strains are mostly found in the genus *Streptomyces*, which is widespread in terrestrial soils (Labeda et al. 2012). *Streptomyces* are symbiotically associated with insects, displaying interesting data as new compound producers, such as anti-cancer metabolites, strepantibins A and C produced by *Streptomyces* sp. isolated from the mud dauber wasp *Sceliphron madraspatanum* (Song et al., 2019) and hamuramicin C from *Streptomyces* sp. associated with *Vespa crabro flavofasciata* (An et al., 2022).

During investigation of the bioactive-producing actinomycetes, the insect-derived actinomycetes were isolated from the longkong bark-eating caterpillar (*Cossus chloratus* Swinhoe) samples collected from Chanthaburi province, Thailand. The current study reported the identification of the isolated culturable actinomycetes using some phenotypic characteristics and 16S rRNA gene sequences analyses, as well as reporting the biological activities of crude ethyl acetate extracts of the actinomycetes.

Materials and Methods

Sample preparation and isolation of insect-derived actinomycetes

The actinomycetes were isolated from three samples of *Cossus chloratus* collected from Chanthaburi province (12.8397023°N, 102.1167445°E), Thailand. The preparation and isolation methods of insect-derived actinomycetes were modified from Hanshew et al. (2015) and Maimone et al. (2021). The insect samples were washed with sterile distilled water and surface-sterilized in 70% ethanol for 3 min, before rinsing in 3% sodium hypochlorite (NaOCl) for 2 min, followed by three repeated washes using sterile distilled water and then grinding with a mortar and pestle under sterile conditions. A 10-fold serial dilution prepared using 1 g of ground sample was suspended in 9 mL of 0.85% (w/v) NaCl. The dilution samples (0.1 mL) were spread on three isolation agar media, namely modified half-ISP 2 agar (w/v: 0.2% glucose, 0.5% malt extract, 0.2% yeast extract), chitin agar (CA; w/v, 0.53% chitin, 0.077% K_2HPO_4, 0.037% KH_2PO_4, 0.024% $MgSO_4$, 0.001% $FeSO_4{:}7H_2O$, 0.0001% $ZnSO_4{:}7H_2O$, 0.0001% $MnCl_2{:}7H_2O$) and starch casein agar (SCA; w/v, 1% soluble starch, 0.1% sodium-caseinate, 0.2% KNO_3, 0.05% K_2HPO_4, 0.05% $MgSO_4$). In addition, the isolation media were supplemented with anti-Gram negative bacterial nalidixic acid (30 µg/mL) and anti-fungal cyclohexamide (30 µg/mL). The isolation plate was incubated at 30 °C. After incubation for 2−3 weeks, the single colony of actinomycete was purified and sub-cultured onto ISP 2 agar (w/v, 0.4% glucose, 0.4% yeast extract, 1% malt extract). The pure culture of actinomycete strains was preserved on ISP 2 agar slants and 10% glycerol.

Characterization and identification of actinomycetes

Phenotypic characteristics

Phenotypic characteristics, such as cell morphological and cultural properties, were characterized based on 14-day cultures grown on ISP 2 agar at 30 °C (Shirling and Gottlieb, 1966). The mycelial colors of the actinomycetes and diffusible pigments were compared with the standard NBS/IBCC color system (Kelly, 1946). Utilization of carbons was determined using a carbon utilization agar medium (ISP 9) supplemented with 1% (v/w) carbon sources (Shirling and Gottlieb, 1966). Physiological characteristics (the effects of sodium chloride (0−8% w/v), pH (3−13), and temperature for growth) were determined using the culture on ISP 2 agar. Starch hydrolysis, gelatin liquefaction, coagulation and peptonization of milk were determined using the standard culture media (Williams and Cross, 1971; Arai, 1975).

Genotypic characteristics

Genomic DNA of the actinomycete strains was extracted from the cells grown in the ISP 2 broth for 4 d at 30 °C

following the method described by Tamaoka (1994). The 16S rRNA gene was amplified using universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGY TAC CTT GTT ACG ACT T-3′) (Inahashi et al., 2010). The amplification products were direct-sequenced using primers 785F (5'-GGA TTA GAT ACC CTG GTA-3') and 907R (5'-CCG TCA ATT CMT TTR AGTT T-3') by Macrogen (Seoul, Republic of Korea). The obtained 16S rRNA gene sequences were aligned (using the BioEdit software; Ibis Biosciences) and compared with reference sequences of validly published *Streptomyces* type strains using the EzBioCloud database (www.ezbiocloud.net/identify; Yoon et al., 2017). After gaps and ambiguous nucleotide bases were removed, phylogenetic trees based on 16S rRNA sequences were analyzed based on the neighbour-joining method (Saitou and Nei, 1987) with the genetic distances calculated using a Kimura's 2-parameter model (Kimura, 1980). The tree was reconstructed using the MEGA software version 11 (Tamura et al., 2021). Reliability of the tree topology was examined by bootstrapping the original data for1,000 replicates (Felsenstein, 1985). The 16S rRNA gene sequences of the examined strains were submitted to GenBank database (https://www.ncbi.nlm.nih.gov/nuccore).

Fermentation and extraction

Cultures of actinomycetes (20 isolates) were activated on ISP 2 agar at 30 °C for 7 d and then inoculated into 500 mL Erlenmeyer flasks containing 200 mL of ISP 2 broth and fermented at 30 °C on rotary shakers (200 revolutions per minute) for 14 d. After fermentation, the culture was extracted three times with an equal volume of ethyl acetate (EtOAc) solvent and then the extract was dried over sodium sulfate anhydrous. The EtOAc extract solution was dried in an evaporator to produce the crude EtOAc extract.

Biological activity of crude extracts

Anti-bacterial activity

Crude extracts from insect-derived *Streptomyces* (20 isolates) were tested for anti-bacterial activity against *Bacillus cereus* (ATCC 11778), *Staphylococcus aureus* (ATCC 29213) and *Acinetobacter baumannii* (ATCC 19606). The method for anti-bacterial activity was determined using the standard broth microdilution assay described by the Clinical and Laboratory Standards Institute (2006). A 384-well plate was filled with crude extract and bacterial cell suspension and incubated at 37 °C. After 5 hr of incubation, the microbial density was measured using a microplate reader at an optical density of 600 nm (OD_{600}). Inhibition of less than 90% was reported as an inactive result. Percentage inhibition values were calculated following the formula [1 - [(FUT / FUC)] × 100, where FUT and FUC are the mean fluorescent units from cells tested with the test sample and the negative control (0.5% dimethyl sulfoxide, DMSO), respectively. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of compound that exhibited inhibition values of more than 90%. The maximum concentration of crude extract used was 50 µg/mL, with 0.5% DMSO as the negative control. The positive control antibiotics rifampicin and vancomycin were used to inhibit the Gram-positive bacteria (*B. cereus* and *S. aureus*), while erythromycin and rifampicin were used as positive controls for the Gram-negative *A. baumannii*.

Anti-phytopathogenic fungal activity

Anti-phytopathogenic fungal activity that comprised anti-leaf spot *Alternaria brassicicola* (BCC42724), and anti-anthracnose *Colletotrichum acutatum* (BCC58146) was determined based on carboxy-fluorescein diacetate (CFDA) fluorometric detection (Guarro et al., 1998; Haugland et al., 2002; Aremu et al., 2003). The fungal strains were prepared for sport suspension by flooding the surface of the agar slants with sterile distilled water and scraping the sporulated aerial hyphae before filtering through sterile gauze. For spore germination, the spore suspension (25 mL) in a 384-well plate was activated at 30 °C for 3 hr. Then, the spore suspension plates were mixed with crude extract and incubated at 25 °C for 16–18 hr. Subsequently, a cocktail (25 mL) consisting of 2 µL of 0.9 mg/mL CFDA in DMSO (70%) and glycerol (40%), was added and the mixture was incubated at room temperature in the dark. After 5−10 min, the incubated plates were washed and filled with sterile distilled water. The well-plate was determined for absorption data using a fluorometer on the bottom-reading mode that observed wavelengths at 485 nm and 535 nm. The inhibition percentage was calculated following the formula [1 - [(FUT / FUC)] × 100, where FUT and FUC are the mean fluorescent units from cells tested with the test sample and the negative control (0.5% DMSO), respectively, with inhibition less than 90% reported as inactive result. The MIC value represents the lowest concentration of the tested crude sample that displayed more than 90% inhibition. Maximum concentration of crude extract was performed at 50 µg/mL, with amphotericin B used as positive control.

Anti-malarial activity

Anti-malarial activity against *Plasmodium falciparum* K1 (multidrug-resistant strain) was analyzed based on the microculture radioisotope technique (Desjardins et al., 1979). Crude EtOAc extracts (25 mL) were added into a 96-well plate that contained 200 µL of parasite mixture (1% parasitemia and 1.5% erythrocytes). After incubating, the medium containing 0.5 µL [^{3}H] hypoxanthine was added and incubated for 18–20 hr. Parasitic growth was indicated by the in vitro uptake of labeled hypoxanthine by *P. falciparum*, determined using a microplate scintillation and luminescence counter (TopCount NXT; PerkinElmer; Packard Instrument Company). Percentage inhibition was calculated using [1 - (CPMT / CPMC)] × 100, where CPMT and CPMC values represent the results of parasites treated with crude samples and negative solvent (0.1% DMSO), respectively. The half maximal inhibitory concentration (IC_{50}) was the concentration of the sample that inhibited 50% malarial cell growth. Less than 50% parasite inhibition was reported as inactive, while 10 µg/mL of crude sample was used as the maximum concentration. Dihydroartemisinine and chloroquine were used as the positive controls.

Antituberculosis and cytotoxic activities

Anti-tuberculosis activity against *Mycobacterium tuberculosis* H37Ra (ATCC 25177) and cytotoxic activity against non-cancerous Vero cells (African green monkey kidney fibroblasts, ATCC CCL-81) were assessed using green fluorescent protein microplate assay (GFPMA) according to Changsen et al. (2003). The method was conducted in a 384-well plate in quadruplicate, with each well added with 5 µL of crude extract followed by 45 µL of cell suspension. The well-plate was incubated at 37°C for 4 d in a humidified incubator that contained 5% carbon dioxide. Fluorescence analysis was observed using the bottom-reading mode, with wavelength of excitation at 485 nm and emission at 535 nm. Percentage cytotoxicity was calculated using the formula [1 - [(FUT / FUC)] × 100, where FUT and FUC are the fluorescent units observed from testing the crude extract and the negative control (0.5% DMSO), respectively. The IC_{50} value was observed from the dose-response curve between cytotoxicity values compared with crude concentrations using the SOFTMax Pro software (Molecular Devices, USA).

Cytotoxic activities against cancerous MCF-7 (human breast cancer, ATCC HTB-22) and NCI-H187 (human small cell lung cancer, ATCC CRL-5804) cells were determined based on resazurin microplate assay (O'Brien et al., 2000). Cytotoxic values less than 50% were reported as inactive results. Rifampicin, ofloxacin, streptomycin, isoniazid and ethambutol were used as the positive controls for anti-tuberculosis. The positive control for cytotoxic Vero cells was ellipticine. Ellipticine and doxorubicin were used as cytotoxic positive controls with NCI-H187 cells, while doxorubicin and tamoxifen were used as cytotoxic positive controls with MCF-7 cells.

Results and Discussion

Isolation and identification of insect-derived actinomycetes

In total, 20 actinomycete strains were isolated from 3 samples of longkong bark-eating caterpillar *Cossus chloratus* Swinhoe collected from Chanthaburi province, Thailand. The isolated actinomycetes were selected from isolation media that comprised chitin agar (10 isolates), half-ISP 2 agar (8 isolates) and starch-casein agar (2 isolates), as shown in Table 1. Based on the genotypic characteristics from 16S rRNA gene sequence analysis, the actinomycetes (20 strains) were identified as being in the genus *Streptomyces*. These strains were classified into nine groups (I–IX) using genotypic and phenotypic characteristics that comprised cultural characteristics including colony morphology (Fig. 1) and other characteristics (Tables 1 and 2).

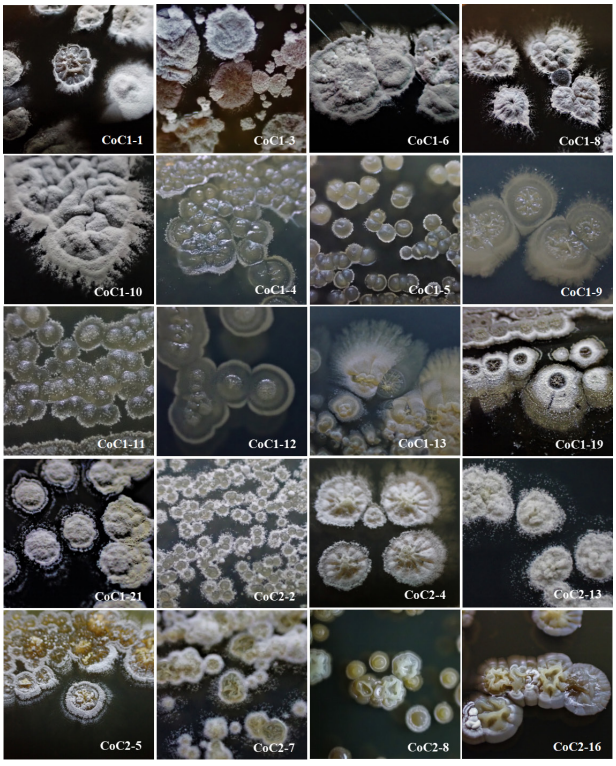

Fig. 1 Colony morphology of insect-derived actinomycetes strains with culture grown on ISP 2 agar at 30 °C for 14 d

Table 1 Cultural characteristics of *Streptomyces* group grown on ISP 2 agar

Group	Strain	Isolation medium	Colony color		Diffusible pigment color
			Substrate mycelium	Aerial mycelium	
I	CoC1-1	Half-ISP 2	Light brown	Gray	Brown
	CoC1-3	Half-ISP 2	Olive brown	Reddish gray	Grayish brown
	CoC1-6	Half-ISP 2	Brown	Reddish gray	Grayish brown
	CoC1-8	CA	Olive brown	Reddish gray	Brown
	CoC1-10	Half-ISP 2	Olive brown	Reddish gray	Brown
II	CoC1-4	CA	Yellowish gray	Yellowish white	-
	CoC1-5	Half-ISP 2	Yellowish gray	Yellowish white	-
	CoC1-9	Half-ISP 2	Yellowish gray	Yellowish white	-
	CoC1-11	Half-ISP 2	Yellowish gray	Yellowish white	-
	CoC1-12	Half-ISP 2	Yellowish gray	White	-
III	CoC1-13	SCA	Grayish pink	Pinkish white	-
IV	CoC1-19	CA	Grayish brown	White	Gray
V	CoC1-21	CA	Dark yellow	Yellowish white	-
VI	CoC2-2	CA	Yellowish white	White	-
	CoC2-4	CA	Yellowish white	White	-
	CoC2-13	CA	Yellowish white	White	-
VII	CoC2-5	CA	Pale orange yellow	Moderate orange yellow	Orange yellow
VIII	CoC2-7	CA	Grayish yellow	Yellowish white	-
IX	CoC2-8	SCA	Brown	Yellowish gray	Yellowish brown
	CoC2-16	CA	Brown	Yellowish gray	Yellowish brown

Table 2 Phenotypic characteristics of *Streptomyces* groups I–IX

Characteristic	Group								
	I	II	III	IV	V	VI	VII	VIII	IX
NaCl tolerance (%, w/v)	0−7	0−6	0−6	0−5	0−5	0−6	0−6	0−7	0−7
pH range	5−12	5−10	5−10	512	5−12	5−12	5−12	5−12	5−12
Temp. (°C)	25−35	25−35	25−35	2535	25−35	25−35	25−35	25−35	25−35
Gelatin liquefactiom	+	+	+	+	+	-	+	+	+
Milk peptonization	+	-	+	+	+	-	+	-	+
Milk coagulation	+	+	+	+	+	+	+	-	-
Starch hydrolysis	+	-	+	+	+	-	+	+	+
Utilization of:									
D-glucose	+	+	+	+	+	+	+	+	+
D-fructose	+	+	+	+	+	+	+	+	+
D-galactose	+	+	+	+	+	+	+	+	+
D-xylose	+	+	-	+	+	+	+	+	+
L-arabinose	+	-	+	+	+	+	+	+	+
Lactose	-	-	+	+	+	+	+	+	+
Sucrose	-	-	-	-	-	+	+	+	-
D-raffinose	+	-	-	-	-	+	-	+	+
Salicin	+	-	-	-	-	-	-	-	-

Group I consisted of five strains (CoC1-1, CoC1-3, Coc1-6, CoC1-8 and CoC1-10) that grew well on ISP 2 agar and a diffusible brown pigment appeared in the culture medium (Table 1). The physiological characteristics of actinomycete group I are shown in Table 2. Based on the 16S rRNA gene sequence analyses, these strains were closely related to *Streptomyces* and showed similar values with *S. drozdowiczii* NBRC 101007^T in the range 98.96−99.86% (Table 3). Phylogenetic analysis of these sequences revealed that these strains were clustered within group I (Fig. 2). The polyphasic characteristics that comprised partial phenotypic data and the 16S rRNA gene sequences revealed that these strains were similar to cellulolytic *S. drozdowiczii*, which produced gray-colored aerial hyphae and a brown-colored diffusible pigment on ISP 2 agar (Semêdo et al., 2004).

Group II (five isolates; CoC1-4, CoC1-5, CoC1-9, CoC1-11, CoC1-12) showed yellowish gray-to-yellowish white color mycelia on ISP 2 medium agar (Fig. 1). Variable characteristics were found in growth at pH 5–10 and on 0–6% NaCl, gelatinization, milk coagulation and starch hydrolysis. No growth was observed at 40 °C, with other physiological properties are listed in Table 2. The 16S rRNA gene sequence pairwise alignment showed that the strains CoC1-4, CoC1-5, CoC1-9, CoC1-11 and CoC1-12 belonged to the genus *Streptomyces*, which displayed the highest similarity values with *S. xylanilyticus* SR2-123^T in the range 99.03−99.17% based on Eztaxon analysis of 16S rRNA gene sequences (Table 3). The phylogenetic analysis showed that *S. xylanilyticus* SR2-123^T was the closest phylogenetic neighbor of the strains in group II, forming a cluster supported by high bootstrap values (91−93%), as shown in Fig. 2. For different characteristics of similar species, *S. xylanilyticus* SR2-123^T produced yellow-to-white aerial hyphae on ISP 2 agar, with maximum temperature and pH values for growth of 37 °C and pH 9, respectively (Moonmangmee et al., 2017).

The strain CoC1-13 (group III) produced grayish-pink substrate mycelia and pinkish-white aerial mycelia that appeared on ISP 2 agar. This strain belonged to the genus *Streptomyces*, related to *S. kunmingensis* NBRC 14463^T at 99.51% similarity, based on 16S rRNA gene analysis. The neighbour-joining phylogenetic analysis showed that the strain was clustered within *S. kunmingensis* NBRC 14463^T. Some physiological differences were found between the strain CoC1-3 and *S. kunmingensis* NBRC 14463^T; the latter could not degrade starch nor grow in the presence of 10% (w/v) NaCl (Rohland and Meyers, 2015). The strain CoC1-13 utilized carbon sources, such as D-glucose, D-fructose, D-galactose, D-arabinose and lactose. The maximum NaCl tolerance for growth was 6% with a pH range of 5−10. The results of gelatin liquefaction, starch hydrolysis and milk peptonization are shown in Table 2.

Group IV consisted of the strain CoC1-19, which was identified as *Streptomyces* and was very similar to *S. daghestanicus* NRRL B-5418^T (100%), *S. albidoflavus* DSM 40455^T (100%) and *S. violascens* SP 5183^T (100%) based on 16S rRNA gene analyses. However, the phylogenetic tree using the neighbor-joining method revealed that strain CoC1-19 was closely related within a phyletic line with *S. daghestanicus* NRRL B-5418^T (Fig. 2). Species level identification between the strain and type strain, NRRL B-5418^T which may be compared with other phenotypic characteristics, suggested that the strain CoC1-19 was *S. daghestanicus*. Genomic analysis, a DNA−DNA homology study and other analyses not using the 16S rRNA gene should also be performed to confirm this opinion (Song et al., 2004). In the current study, the strain CoC1-19 had grayish brown mycelia, with gray, diffusible pigment produced on ISP 2 agar (Table 1). The physiological characteristics for *Streptomyces* strain CoC1-19 grown on 0−5% NaCl, pH 5−12, at 25−35°C and other properties are shown in Table 2.

Table 3 16S rRNA gene similarity (%) of isolated actinomycetes and closest relative *Streptomyces* species

Group	Strain	Accession no.	Closest species	Similarity (%)
I	CoC1-1	OP648161	*S. drozdowiczii* NBRC 101007^T	98.96
	CoC1-3	OP648168	*S. drozdowiczii* NBRC 101007^T	99.79
	CoC1-6	OP648189	*S. drozdowiczii* NBRC 101007^T	99.86
	CoC1-8	OP648192	*S. drozdowiczii* NBRC 101007^T	99.31
	CoC1-10	OP683533	*S. drozdowiczii* NBRC 101007^T	99.79
II	CoC1-4	OP658848	*S. xylanilyticus* SR2-123^T	99.03
	CoC1-5	OP658849	*S. xylanilyticus* SR2-123^T	99.03
	CoC1-9	OP658850	*S. xylanilyticus* SR2-123^T	99.10
	CoC1-11	OP658851	*S. xylanilyticus* SR2-123^T	99.16
	CoC1-12	OP658867	*S. xylanilyticus* SR2-123^T	99.17
III	CoC1-13	OP658847	*S. kunmingensis* NBRC 14463^T	99.51
IV	CoC1-19	OP658846	*S. daghestanicus* NRRL B-5418^T	100
V	CoC1-21	OP658845	*S. cavourensis* NBRC 13026^T	100
VI	CoC2-2	OP648264	*S. badius* NRRL B-2567^T	98.41
	CoC2-4	OP648279	*S. badius* NRRL B-2567^T	98.97
	CoC2-13	OP648280	*S. badius* NRRL B-2567^T	98.69
VII	CoC2-5	OP648263	*S. parvulus* NBRC 13193^T	100
VIII	CoC2-7	OP648260	*S. flavofungini* NBRC 13371^T	97.72
IX	CoC2-8	OP648245	*S. diacarni* LHW51701^T	99.31
	CoC2-16	OP648248	*S. diacarni* LHW51701^T	99.31

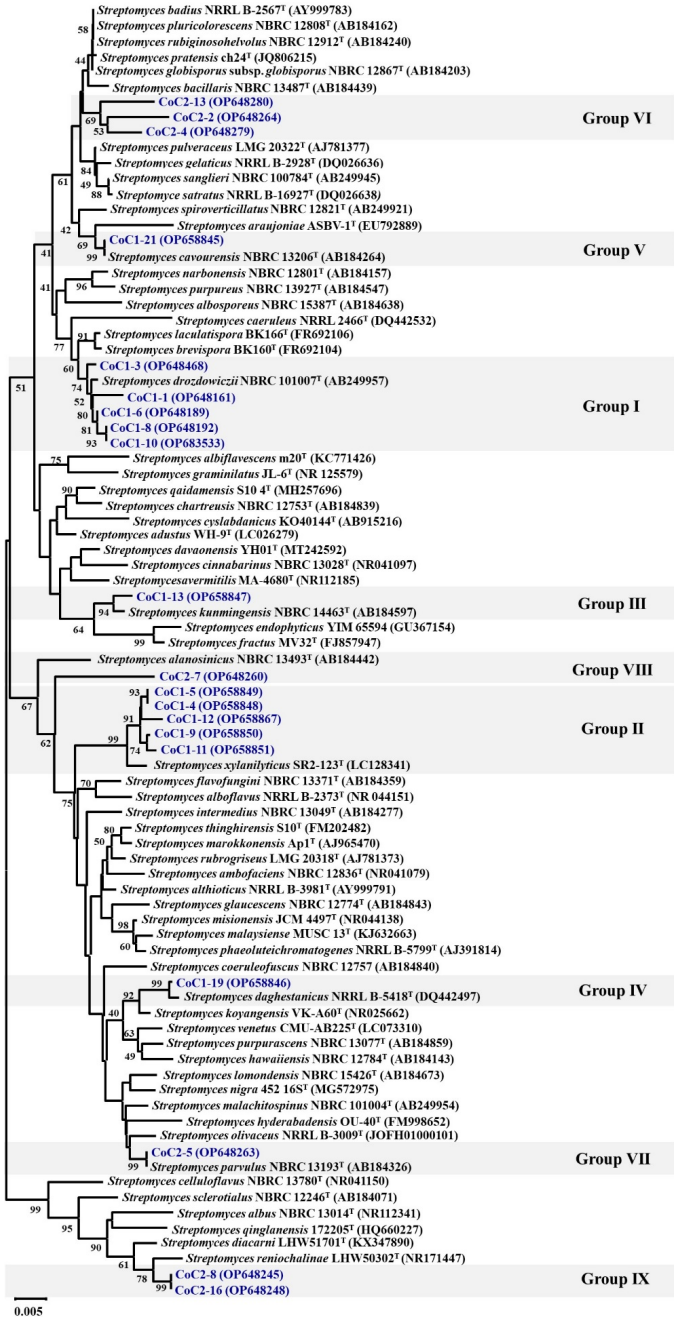

Fig. 2 Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences comparing isolated actinomycetes and closely related *Streptomyces* species, where numbers on branches indicate percentage bootstrap values of 1,000 replicates, with only values of more than 40% shown

Group V contained the strain CoC1-21 that grew well on ISP 2 agar, with dark yellow-to-yellowish white mycelia observed at 14 d of culture and general characteristics similar to the genus *Streptomyces* (Table 1). The BLAST results of 16S rRNA gene sequences showed that the strain displayed highest similarity (100%) with *S. cavourensis* NBRC 13206^{T}. Neighbor-joining phylogenetic analysis confirmed that CoC1-21 was closely related within a phyletic line with *S. cavourensis*. *Streptomyces* CoC1-21 utilized D-glucose, D-fructose, D-galactose, D-xylose, D-arabinose and disaccharide lactose as carbon sources. The strain CoC1-21 grew on ISP 2 agar in the presence of 0−5% NaCl, at pH 5−12 (optimum 7−8) and in the range 25−35 °C. The results of soluble starch degradation, milk peptonization and gelatin liquefaction are shown in Table 2.

The insect-derived actinomycete strains CoC2-2, CoC2-4 and CoC2-13 in group VI grew well on ISP 2 agar at pH 5−12 in the presence of maximum 6% NaCl, utilizing D-glucose, D-fructose, D-galactose, D-xylose, D-arabinose, lactose and D-raffinose as carbon sources (Table 2). Their morphological characteristics revealed that these strains produced yellowish white and white substrate and aerial mycelia, respectively. Diffusible pigments were not found in this group (Table 1). Phylogenetic analysis using 16S rRNA gene sequences indicated that the strains CoC2-2, CoC2-4 and CoC2-13 were classified into the genus *Streptomyces* (Fig. 2). However, these strains differed from previously described *Streptomyces* spp. available in public databases and showed similarity values at 98.41%, 98.97% and 98.69%, respectively, with *S. badius* NRRL B-2567^{T} (Table 3).

Group VII, with the strain CoC2-5 presented substrate and aerial mycelia that were pale orange yellow and moderate orange yellow, respectively (Table 1). Analysis of the 16S rRNA gene sequence of CoC2-5 showed 100% similarity with *S. parvulus* NBRC 13193^{T}. The strain formed a clade within the 16S rRNA gene sequence phylogenetic tree, indicating that it was closely related with *S. parvulus* NBRC 13193^{T}. For physiological characteristics, the strain CoC2-5 grew on 1−6% NaCl at pH 5−12 and 25−35°C (optimum 30 °C) and showed positive data for gelatin liquefaction, starch hydrolysis, coagulation and peptonization of milk. Utilization of carbon sources and other properties are shown in Table 2.

Group VIII, consisting of the strain CoC2-7was isolated using CA. The strain grew well on ISP 2 agar and had grayish yellow, yellowish white substrate and aerial hyphae (Fig. 1). For cultural characteristics based on carbon utilization, sugars were mostly used but salicin was not utilized. The strain CoC2-7 hydrolyzed starch and gelatin, while its NaCl tolerance and pH growth range were 1−7% and 5−12, respectively (Table 2). For 16S rRNA gene analysis, the strain showed low similarity values with *S. flavofungini* NBRC 13371^{T} (98.05%), *S. alboflavus* NRRL B-2373 (97.58%) and *S. alanosinicus* NBRC 13493^{T} (97.55%). The neighbor-joining phylogenetic tree revealed that the strain CoC2-7 was clearly

separated from similar species and might be a novel species (Fig. 2). However, a new species must be confirmed based on complete taxonomic data comprising the chemotypic, phenotypic and genome sequences analyses.

Group IX contained CoC2-8 and CoC2-16 that exhibited good growth in ISP 2 agar, with brown and yellowish gray mycelia on substrate and aerial mycelia, respectively. They also produced yellow-brown diffusible pigment on ISP 2 agar (Table 1). These strains grew on 5% NaCl, pH 4−12, at 20−45 °C and showed positive results for starch, gelatin and protein hydrolysis, while sucrose and salicin were not utilized (Table 2). The strains CoC2-8 and CoC2-16 were classified to the genus *Streptomyces* that showed highest similarity values with *S. diacarni* (99.31%) based on 16S rRNA gene analysis (Table 3.). Close relationships between these strains and *S. diacarni* were confirmed by the phylogenetic analysis (Fig. 2). *S. diacarni* had been reported from marine sponge (Li et al., 2019).

Biological activity

Crude EtOAc extracts of insect-derived *Streptomyces* spp. (20 strains) were assessed for anti-microbial (anti-bacterial, anti-TB, anti-malarial), and anti-cancer (MCF-7 and NCI-H187) activities as well as cytotoxicity against non-cancerous Vero cells. For anti-bacterial activity against Gram-positive bacteria, the active *Streptomyces* strains CoC1-5 (group II) and CoC1-10 (group I) inhibited *B. cereus* with MIC value of 12.50 µg/mL, while anti-*S. aureus* was only found in crude extract of the strain CoC1-11 with a MIC value of 25.0 µg/mL. None of the crude EtOAc extracts of the test strains showed activity against Gram-negative *A. baumannii* at the maximum tested concentration (50 µg/mL), as shown in Table 4. The strains CoC2-4, CoC2-2, CoC2-13 and CoC1-11 showed moderate anti-*M. tuberculosis* activity with MIC values of 6.25 µg/mL, 12.50 µg/mL, 25.00 µg/mL and 50.00 µg/mL, respectively. For analysis of anti-phytophathogenic fungi, the strains CoC1-5 and CoC1-21 exhibited weak activity against *C. acutatum* with MIC values of 25.00 µg/mL and 50.00 µg/mL, respectively. For anti-cancer activity against McF7 cancer cells, the cytotoxic *Streptomyces* strains CoC1-5 and CoC1-11 (group II) showed strong activities with IC_{50} values of 1.82 µg/mL and 1.76 µg/mL, respectively. Cytotoxicity against Vero cells was found in the crude extract of *Streptomyces* strains CoC1-5 and CoC1-11, with IC_{50} values 17.93 µg/mL and 5.77 µg/mL, respectively. Of the 20 *Streptomyces* strains tested for antimalarial activity, only the strain CoC1-21 revealed strong activity against *P. falciparum*, with an IC_{50} value of 0.18 µg/mL, while the strain CoC1-21 had cytotoxicity against Vero, McF7 and NCI-H187 cells, with IC_{50} values of 0.32 µg/mL, 11.72 µg/mL and 1.79 µg/mL, respectively (Table 5).

Table 4 Anti-bacterial and anti-fungal activities of crude EtOAc extracts of actinomycete strains

Strain no.	Anti-bacterial (MIC, µg/mL)				Anti-fungal (MIC, µg/mL)	
	B. cereus	*S. aureus*	*A. baumannii*	*M. tuberculosis*	*C. acutatum*	*A. brassicicola*
CoC1-1	>50	>50	>50	>50	>50	>50
CoC1-3	>50	>50	>50	>50	>50	>50
CoC1-6	>50	>50	>50	>50	>50	>50
CoC1-8	>50	>50	>50	>50	>50	>50
CoC1-10	12.50	>50	>50	>50	>50	>50
CoC1-4	>50	>50	>50	>50	>50	>50
CoC1-5	12.50	>50	>50	>50	25.00	>50
CoC1-9	>50	>50	>50	>50	>50	>50
CoC1-11	>50	25.0	>50	50.00	>50	>50
CoC1-12	>50	>50	>50	>50	>50	>50
CoC1-13	>50	>50	>50	>50	>50	>50
CoC1-19	>50	>50	>50	>50	>50	>50
CoC1-21	>50	>50	>50	>50	50.00	>50
CoC2-2	>50	>50	>50	12.50	>50	>50
CoC2-4	>50	>50	>50	6.25	>50	>50
CoC2-13	>50	>50	>50	25.00	>50	>50
CoC2-5	>50	>50	>50	>50	>50	>50
CoC2-7	>50	>50	>50	>50	>50	>50
CoC2-8	>50	>50	>50	>50	>50	>50
CoC2-16	>50	>50	>50	>50	>50	>50
Vancomycin	4.00	1.00				
Rifampicin	0.16	0.31	3.13	0.00625		
Erythromycin			50.00			
Ofloxacin				0.391		
Streptomycin				0.625		
Isoniazid				0.0938		
Ethambutol				1.88		
Amphotericin B					3.13	0.78

Table 5 Anti-malarial and anti-cancer activities of crude EtOAc extracts of actinomycete strains

Strain no.	Anti-malarial (IC_{50}, µg/mL)	Cytotoxicity (IC_{50}, µg/mL)		
		Vero	McF7	NCI-H187
CoC1-1	>10	>50	>50	>50
CoC1-3	>10	>50	>50	>50
CoC1-6	>10	>50	>50	>50
CoC1-8	>10	>50	>50	>50
CoC1-10	>10	>50	>50	>50
CoC1-4	>10	>50	>50	>50
CoC1-5	>10	17.93	1.82	>50
CoC1-9	>10	>50	>50	>50
CoC1-11	>10	5.77	1.76	>50
CoC1-12	>10	>50	>50	>50
CoC1-13	>10	>50	>50	>50
CoC1-19	>10	>50	>50	>50
C0C1-21	0.18	0.32	11.72	1.79
CoC2-2	>10	>50	36.58	>50
CoC2-4	>10	>50	>50	>50
CoC2-13	>10	>50	>50	>50
CoC2-5	>10	>50	>50	>50
CoC2-7	>10	>50	>50	>50
CoC2-8	>10	>50	>50	>50
CoC2-16	>10	>50	>50	>50
Dihydroartemisinine	0.0085			
Chloroquine	0.072			
Ellipticine		1.33		3.19
Doxorubicin			8.26	0.17
Tamoxifen			10.44	

The *Streptomyces* strain CoC1-21 was closely related with *S. cavourensis*, which had a source of bioactive secondary metabolites, such as cytotoxic 1-monolinolein and bafilomycin D from the plant-endophytic *S. cavourensis* strain YBQ59 (Vu et al., 2018). The inactive strain CoC2-5 was identified as *S. parvulus*. Chandrakar and Gupta (2019) revealed that some production conditions of the bioactive metabolite-producing *S. parvulus* strain Av-R5 displayed anti-microbial activity against multidrug-resistant *S. aureus* JNMC-3. This study reveals that the interesting biological activities of insect-associated *Streptomyces* species, such as the highly active strains CoC1-5, CoC1-11 and CoC1-21 could have potential as new sources of bioactive secondary metabolites.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgement

This study was financially supported by the Office of the Permanent Secretary, Ministry of Higher Education, Science Research, and Innovation (RGNS63-098).

References

An, J.S., Lim, H.-J., Lee, J.Y., Jang, Y.-J., Nam, S.-J., Lee, S.K., Oh, D.-C. 2022. Hamuramicin C, a cytotoxic bicyclic macrolide isolated from a wasp gut bacterium. J. Nat. Prod. 85: 936−942. doi.org/10.1021/acs.jnatprod.1c01075

Arai, T. 1975. *Culture Media for Actinomycetes*. The Society for Actinomycetes. Tokyo, Japan.

Aremu, E.A., Furumai, T., Igarashi, Y., Sato, Y., Akamatsu, H., Kodama, M., Otani, H. 2003. Specific inhibition of spore germination of *Alternaria brassicicola* by fistupyrone from *Streptomyces* sp. TP-A0569. J. Gen. Plant Pathol. 69: 211–217. doi.org/10.1007/s10327-003-0038-y

Changsen, C., Franzblau, S.G., Palittapongarnpim, P. 2003. Improved green fluorescent protein reporter gene-based microplate screening for antituberculosis compounds by utilizing an acetamidase promoter. Antimicrob. Agents Chemother. 47: 3682–3687. doi.org/10.1128/AAC.47.12.3682-3687.2003

Chandrakar, S., Gupta, A.K. 2019. Actinomycin-producing endophytic *Streptomyces parvulus* associated with root of *Aloe vera* and optimization of conditions for antibiotic production. Probiotics Antimicrob. Proteins 11: 1055–1069. doi.org/10.1007/s12602-018-9451-6

Chevrette, M.G., Carlson, C.M., Ortega, H.E., et al. 2019. The antimicrobial potential of *Streptomyces* from insect microbiomes. Nat. Commun. 10: 516. doi.org/10.1038/s41467-019-08438-0

Clinical and Laboratory Standards Institute. 2006. M2-A9. Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved standard, 9th ed. *Clinical and Laboratory Standards Institute*. Wayne, PA, USA.

Desjardins, R.E., Canfield, C.J., Haynes, J.D., Chulay, J.D. 1979. Quantitative assessment of antimalarial activity in vitro by a semiautomated

microdilution technique. Antimicrob. Agents Chemother. 16: 710–718. doi.org/10.1128/AAC.16.6.710

Felsenstein, J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783−791. doi.org/10.1111/j.1558-5646.1985.tb00420.x

Guarro, J., Pujol, I., Aguilar, C., Llop, C., Fernandez-Ballart, J. 1998. Inoculum preparation for in-vitro susceptibility testing of filamentous fungi. J. Antimicrob. Chemother. 42: 385–387. doi.org/10.1093/jac/42.3.385

Hanshew, A.S., McDonald, B.R., Díaz, C.D., Djiéto-Lordon, C., Blatrix, R., Currie, C.R. 2015. Characterization of actinobacteria associated with three ant-plant mutualisms. Microb. Ecol. 69: 192–203. doi.org/10.1007/s00248-014-0469-3

Haugland, R.P. 2002. Handbook of Fluorescent Probes and Research Products. Molecular Probes. Eugene, OR, USA.

Inahashi, Y., Matsumoto, A., Danbara, H., Omura, S., Takahashi, Y. 2010. *Phytohabitans suffuscus* gen. nov., sp. nov., an actinomycete of the family *Micromonosporaceae* isolated from plant roots. Int. J. Syst. Evol. Microbiol. 60: 2652−2658. doi.org/10.1099/ijs.0.016477-0

Kelly, K. 1964. Inter-Society Color Council–National Bureau of Standard Color Name Charts Illustrated with Centroid Colors. US Government Printing Office. Washington, DC, USA.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 6: 111−120. doi.org/10.1007/BF01731581

Labeda, D.P., Goodfellow, M., Brown, R., et al. 2012. Phylogenetic study of the species within the family *Streptomycetaceae*. Antonie van Leeuwenhoek. 101: 73−104. doi.10.1007/s10482-011-9656-0

Li, L., Wang, J., Zhou, Y.-J., Lin, H.-W., Lu, Y.-H. 2019. *Streptomyces reniochalinae* sp. nov. and *Streptomyces diacarni* sp. nov., from marine sponges. Int. J. Syst. Evol. Microbiol. 69: 99−104. doi.org/10.1099/ijsem.0.003109

Maimone, N.M., Oliveira, L.F.P., Santos, S.N., Lira, S.P. 2021. Elicitation of *Streptomyces lunalinharesii* secondary metabolism through co-cultivation with *Rhizoctonia solani*. Microbiol. Res. 251: 126836. doi.org/10.1016/j.micres.2021.126836

Matarrita-Carranza, B., Moreira-Soto, R.D., Murillo-Cruz, C., Mora, M., Currie, C.R., Pinto-Tomas, A.A. 2017. Evidence for widespread associations between Neotropical Hymenopteran insects and actinobacteria. Front. Microbiol. 8: 2016. doi.org/10.3389/fmicb.2017.02016

Moonmangmee, D., Kanchanasin, P., Phongsopitanun, W., Tanasupawat, S., Moonmangmee, S. 2017. *Streptomyces xylanilyticus* sp. nov., isolated from soil. Int. J. Syst. Evol. Microbiol. 67: 4189−4194. doi.org/10.1099/ijsem.0.002282

Nouioui, I., Carro, L., García-López, M., et al. 2018. Genome-based taxonomic classification of the phylum *Actinobacteria*. Front. Microbiol. 9: 2007. doi.org/10.3389/fmicb.2018.02007

O'Brien, J., Wilson, I., Orton, T., Pognan, F. 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. Eur. J. Biochem. 267: 5421–5426. doi.org/10.1046/j.1432-1327.2000.01606.x

Parte, A.C., Carbasse J.S., Meier-Kolthoff, J.P., Reimer, L.C., Göker, M. 2020. List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. Int. J. Syst. Evol. Microbiol. 70: 5607−5612. doi.org/10.1099/ijsem.0.004332

Rohland, J., Meyers, P.R. 2015. *Streptomyces fractus* sp. nov., a novel streptomycete isolated from the gut of a South African termite. Antonie van Leeuwenhoek 107: 1127–1134. doi.org/10.1007/s10482-015-0404-8

Saitou, N., Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4: 406−425. doi.org/10.1093/oxfordjournals.molbev.a040454

Schwitalla, J.W., Benndorf, R., Martin, K., Vollmers, J., Kaster, A-K., Beer, Z.W., Poulsen, M., Beemelmanns, C. 2020. *Streptomyces smaragdinus* sp. nov., isolated from the gut of the fungus growing-termite *Macrotermes natalensis*. Int. J. Syst. Evol. Microbiol. 70: 5806−8511. doi.org/10.1099/ijsem.0.004478

Semêdo, L.T.A.S., Gomes, R.C., Linhares, A.A., et al. 2004. *Streptomyces drozdowiczii* sp. nov., a novel cellulolytic streptomycete from soil in Brazil. Int. J. Syst. Evol. Microbiol. 54: 1323–1328. doi.org/10.1099/ijs.0.02844-0

Shirling, E.B., Gottlieb, D. 1966. Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313−340. doi.org/10.1099/00207713-16-3-313

Song, J., Lee, S.-C., Kang, J.-W., Baek, H.-J., Suh, J.-W. 2004. Phylogenetic analysis of *Streptomyces* spp. isolated from potato scab lesions in Korea on the basis of 16S rRNA gene and 16S–23S rDNA internally transcribed spacer sequences. Int. J. Syst. Evol. Microbiol. 54: 203209. doi.org/10.1099/ijs.0.02624-0

Song, Y.-J., Zheng, H.-B., Peng, A.-H., Ma, J.-H., Lu, D.-D., Li, X., Zhang, H.-Y., Xie, W.-D. 2019. Strepantibins A−C: Hexokinase II Inhibitors from a mud dauber wasp associated *Streptomyces* sp. J. Nat. Prod. 82: 1114– 1119. doi.org/10.1021/acs.jnatprod.8b00821

Tamaoka, J. 1994. Determination of DNA base composition. In: Goodfellow, M., O'Donnell, A.G. (Eds.). Chemical Methods in Prokaryotic Systematics. John Wiley and Sons. Chichester, UK, pp. 463−470.

Tamura, K., Stecher, G., Kumar, S. 2021. MEGA11: Molecular evolutionary genetics analysis version 11. *Mol. Biol. Evol. 38*: *3022−3027*. doi.org/10.1093/molbev/msab120

Vu, H.-N.T., Nguyen, D.T., Nguyen, H.Q., Chu, H.H., Chu, S.K., Chau, M.V., Phi, Q.-T. 2018. Antimicrobial and cytotoxic properties of bioactive metabolites produced by *Streptomyces cavourensis* YBQ59 isolated from *Cinnamomum cassia* Prels in Yen Bai province of Vietnam. Curr. Microbiol. 75: 1247−1255. doi.org/10.1007/s00284-018-1517-x

Watanabe, Y., Shinzato, N., Fukatsu, T. 2003. Isolation of actinomycetes from termites' guts. Biosci. Biotechnol. Biochem. 67: 1797–1801. doi.org/10.1271/bbb.67.1797

Wang, Z., Yu, Z., Zhao, J., Zhuang, X., Cao, P., Guo, X., Liu, C., Xiang, W. 2020. Community composition, antifungal activity and chemical analyses of ant-derived actinobacteria. Front. Microbiol. 11: 201. doi.org/10.3389/fmicb.2020.00201

Williams, S.T., Cross, T. 1971. Chapter XI actinomycetes. Methods Microbiol. 4: 295−334. doi.org/10.1016/S0580-9517(09)70016-9

Ye, L., Zhao, s., Li, Y., et al. 2017. *Streptomyces lasiicapitis* sp. nov., an actinomycete that produces kanchanamycin, isolated from the head of an ant (*Lasius fuliginosus* L.). Int. J. Syst. Evol. Microbiol. 67: 1529−1534. doi.org/10.1099/ijsem.0.001756

Yoon, S.-H., Ha, S.-M., Kwon, S., Lim, J., Kim, Y., Seo, H., Chun, J. 2017. Introducing EzBioCloud: A taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int. J. Syst. Evol. Microbiol. 67: 1613–1617. doi.org/10.1099/ijsem.0.001755