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The lichen-derived *Streptomyces* isolated from *Pyxine cocoes* produces the antibiotic with potent antimicrobial and antitumor activities

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ABSTRACT: The actinobacteria strain Lp03 was isolated from the lichen, *Pyxine cocoes*, collected from Lumpini Park, Bangkok, Thailand. Based on the polyphasic approach, strain Lp03 was identified as *Streptomyces parvulus*. Actinomycin D produced by this strain exhibited antibacterial activity against *Bacillus cereus* (MIC 1.56 µg/ml), *Bacillus subtilis* (MIC 5 µg/ml), *Escherichia coli* (MIC 12.5 µg/ml), *Kocuria rhizophila* (MIC 0.076 ng/ml), *Klebsiella pneumoniae* (MIC 0.78 µg/ml), *Staphylococcus aureus* (MIC 9.76 ng/ml), *Staphylococcus epidermidis* (MIC 9.76 ng/ml), and *Mycobacterium tuberculosis* (MIC 12.5 µg/ml). Actinomycin D was inactive against *Acinetobacter baumannii*, but the new finding revealed that it showed activity against *A. baumannii* when combined with β-naphthylamide (PNβN), the efflux inhibitor. Antiparasitic activity was observed against *Plasmodium falciparum* with an IC_{50} value of 14.8 ng/ml. It showed cytotoxicity against Vero cells, NCI-H187, KB, and MCF-7 with IC_{50} values of 125, 0.694, 18.4 ng/ml, and 21.94 µg/ml, respectively. The genome of *S. parvulus* harbors a high diversity of biosynthetic gene clusters for secondary metabolites (BGCs). The actinomycin BGCs could be detected in all publicly available genomes of *S. parvulus* but could be varied among the strains. This study is the first record for the complete taxonomic study and bioactive compound produced by *S. parvulus* isolated from the lichen.

KEYWORDS: actinobacteria, actinomycin, bioactive compounds, drug discovery, lichen

INTRODUCTION

Antibiotics are one of the main approaches to combat infection. Since the discovery of antibiotics, average human life expectancy has increased significantly, and many infectious diseases have become controllable [1]. Nowadays, antibiotic resistance is one of the most severe crises in the world and threatens humans, animals, and environmental health [2]. Actinobacteria, especially *Streptomyces* spp., are an essential source of antibiotics. More than 10,000 bioactive compounds have been produced from these bacteria [3]. For a century, the isolation of actinobacteria from the soil let actinobacteria confront the rediscovery problem of the known compounds. To overcome this problem, new habitats such as marine environments as well as lichens have been used as alternative sources for actinobacterial isolation [4].

Lichens are organisms made up of the symbiotic associations of the photobiont, green algae and/or cyanobacteria, and the mycobiont [5]. In 2020, Hawksworth and Grube re-defined lichen symbiosis as "a lichen is a self-sustaining ecosystem formed by the interaction of exhabitant fungus and an extracellular arrangement of one or more photosynthetic partners as an indeterminate number of other microscopic organisms" [6]. Lichens are distributed worldwide, growing on solid surfaces including rock, soil, and tree barks. To date, much evidence has confirmed the presence of actinobacterial communities associated with lichens [7]. In the past decade, several novel lichen-derived actinobacteria, including *Actinoplanes lichenicola*, *Actinoplanes ovalisporus*, and *Streptomyces lichenis*, were isolated [8, 9]. Although many novel actinobacteria were obtained from lichen, studies of the bioactive secondary metabolites from these actinobacteria associated with lichen are still rare. In this paper, we describe the taxonomic studies of the actinobacteria strain Lp03 isolated from lichen and its bioactive secondary metabolites.

MATERIALS AND METHODS

Isolation of actinobacteria

The lichen sample, *Pyxine cocoes* (Sw.) Nyl. grown on tree bark, was collected from Lumpini Park, Bangkok,

Fig. 1 (a) The lichen, *Pyxine cocoes* (Sw.) Nyl., grown on tree bark; (b) cultural characteristics of Lp03 grown on ISP2 agar at 30 °C for 14 days; (c) the scanning electron micrographs showing the morphology of spore chains and the surface of mature spores. Bar, 1 cm (a); 10 mm (b); 5 µm (c).

Thailand (Fig. 1). The sample was kept in a sterile conical tube prior to laboratory examination. The lichen thallus was ground using a mortar and added into the basic lauryl sulfate solution [0.1 g SDS, 1.75 g KH_2PO_4, and 3.5 g K_2HPO_4 in 1 l distilled water, pH 7.0] to perform standard serial dilutions (10^{-1} to 10^{-3}). Then, 1 ml of each dilution was spread on the surface of a starch casein nitrate agar supplemented with nalidixic (25 µg/ml) and cycloheximide (50 µg/ml) and incubated at 30 °C for 14 days [10]. After incubation, the colony of actinobacteria was selected and purified on ISP2 agar [11] to obtain the pure culture of strain Lp03.

Identification of actinobacteria

The phenotypic characteristics, including starch hydrolysis, liquefaction of gelatin, nitrate reduction, skimmed milk peptonization and coagulation, were determined by conventional method as described by Shirling and Gottlieb [11] and William and Cross [12]. The enzymatic activities of the strain were determined using APIZym (bioMérieux, France). The cultural characteristics were observed after the culture had been grown on ISP agar media (ISP2, ISP3, ISP4, ISP5, and ISP6) [11] at 30 °C for 14 days. Scanning electron microscopy (JSM-IT500HR; JEOL, Japan) was used to observe the morphology of the spore chain. The chemotaxonomic study including the isomers of diaminopimelic acids and whole cell sugar was analyzed using thin layer chromatography [13].

The genomic DNA of actinobacteria was extracted using the PureLink™ genomic DNA kit (Thermo Fisher Scientific, USA). The 16S rRNA gene was amplified using the primers 20F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1500R (5′-GTTACCTTGTTACGACTT-3′) and sequenced by sequencing service (Macrogen, Korea) [14]. BLAST analysis was determined using the EzBioCloud database [15]. The phylogenetic tree, maximum-likelihood (ML), based on the 16S rRNA gene was constructed using MEGA X [16]. The whole genome of strain Lp03 was sequenced using an Illumina HiSeq platform. The low-quality read and adaptors were eliminated using Trim Galore (Babraham Bioinformatics) and assembled using Unicycler [17]. Annotation of the assembled genome was carried out using PATRIC 3.6.7 [18]. The phylogenetic tree based on genome sequences was constructed using the TYGS server [19]. The secondary metabolite biosynthetic gene clusters (BGCs) in the genome were analyzed using antiSMASH (bacterial version) [20].

Fermentation

The strain was cultured on ISP2 agar for 14 days. Then, the culture on the agar plate was cut into small pieces (1 × 1 cm) and used for the inoculum. Each piece of the inoculum was added into the Erlenmeyer flask (500 ml in size × 6 flasks) containing 150 ml of ISP2 broth and incubated in a shaker at 180 rpm, 30 °C for 14 days. After fermentation, the secondary metabolites in the culture broth were extracted with the equal volume of ethyl acetate 3 times. The ethyl acetate layer was evaporated to dryness.

Antimicrobial activity of the crude extract

Antimicrobial activity screening of the crude extract was carried out using the disk diffusion method. The crude extract was dissolved using methanol to obtain the final concentration of 10 mg/ml. Then, 20 µl of the extract was loaded into the sterile 6-mm paper disc and air-dried in the laminar air flow. After drying, the disc was placed on the surface of Mueller-Hinton agar (MHA) which had surface swabbed by the tested microorganisms according to the Kirby-Bauer method [21]. For anticandidal activity, the tested microorganism was cultured on Sabouraud dextrose agar (SDA, Difco, USA) instead of MHA. Then, the screening plates were cultured at 37 °C for 24 h, and the inhibition zone was measured. Commercial antibi-

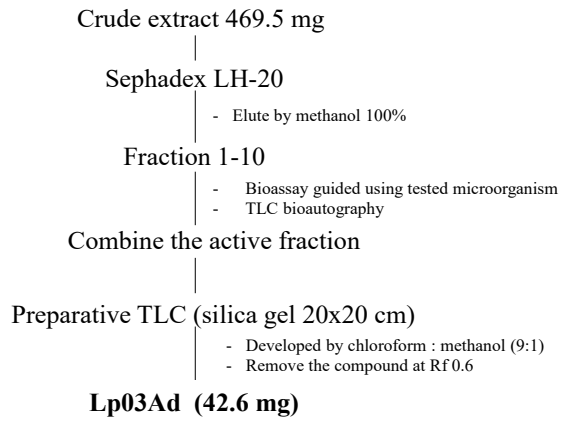

Fig. 2 Isolation scheme of the compound Lp03Ad.

otic discs including chloramphenicol and gentamicin were used as the positive control for testing bacteria. Amphotericin B was used as the positive control for the *Candida* strain. A paper disc loaded with methanol was used as the negative control. In this study, the common human pathogens including *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Candida albicans* ATCC 10231 were used as the tested microorganisms. The experiments were performed in triplicate.

Isolation and identification of bioactive compounds

The chemical composition of the crude extract was analyzed by using high performance liquid chromatography (HPLC, Dionex Ultimate 3000, USA) equipped with a C-18 column (Puropher®Star; Merck, Germany, 5 µm, 2.1×50 mm) with the linear gradient system (0–100% CH_3CN in H_2O + 0.05% formic acid), and flow rate 0.5 ml/min for 15 min. The bioactive compound of the selected strain was purified using the Sephadex LH-20 column (20.5×2.5 cm) eluted by 100% methanol. The bioactive fractions were chosen by bioassay-guided fractionation using the TLC method [22]. The fraction containing bioactive compound(s) was further purified using preparative TLC (20×20 cm, Merck, Germany). TLC and preparative TLC were developed using chloroform: methanol (3:1) for the mobile phase. The isolation chart is shown in Fig. 2. The chemical structure of the pure compound (Lp03Ad) was identified by comparing the HPLC chromatogram of the isolated pure compound with the known compounds (actinomycin D, Sigma-Aldrich, USA) recorded in the in-house database. The ^{1}H-NMR spectrum (Avance 500 NMR, Bruker, Germany) and high-resolution mass spectral data (HRESIMS) (MicrOTOF, Bruker) were used to confirm the identity of the pure compound.

Biological activity of the pure compound

Antimicrobial activity of the pure compounds was observed against *S. aureus* ATCC 25923, *Kocuria rhizophila* ATCC 9341, *Staphylococcus epidermidis* ATCC 12228, *Klebsiella pneumoniae* ATCC 13883, *Bacillus cereus* ATCC 11778, *B. subtilis* ATCC 6633, *Enterococcus faecium* ATCC 51559, *E. coli* ATCC 25922, *Acinetobacter baumannii* ATCC19606, *P. aeruginosa* ATCC 15692, and *C. albicans* ATCC 90028 by the broth microdilution method [23]. Vancomycin was used as the positive control for *B. cereus*, *B. subtilis*, *S. aureus*, *S. epidermidis*, and *K. rhizophila*. Erythromycin was used as the positive control for *A. baumannii*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. Tetracycline HCl was used as the positive control for *E. faecium*. In addition, amphotericin B was used as the positive control for anticandidal activity.

The cytotoxicity was tested against KB (oral human epidermoid carcinoma, ATCC CCL-17), MCF-7 (human breast cancer, ATCC HTC-22), and NCI-H187 (human small cell lung cancer, ATCC CRL-5804) cell lines using the resazurin microplate assay (REMS) method [24]. Ellipticine and doxorubicin were used as positive controls for anti-KB and anti-NCI-H187 cell lines, while doxorubicin and tamoxifen were used as positive controls for the anti-MCF-7 cell line. The anti-*Mycobacterium tuberculosis* H37Ra (ATCC 25177), cytotoxic against Vero cells, anti-herpes simplex type-1 (HSV-1), and anti-phytopathogenic fungi, including *Curvularia lunata* and *Magnaporthe grisea* BCC 10261, were determined using the green fluorescent protein microplate assay (GFPMA) [25, 26]. Amphotericin B was used as the positive control for antifungal activity. Isoniazid, ofloxacin, rifampicin, streptomycin, and ethambutol were used as the positive controls for the anti-*M. tuberculosis* test. Acyclovir was used as the positive control for anti-HSV-1. The microculture radioisotope technique was used to determine antimalarial activity against the *Plasmodium falciparum* K1 multidrug resistant strain [27]. Dihydroartemisinin and mefloquine were used as the positive controls for antimalarial activity. Neuraminidase (NA) inhibition assay was determined using fluorometric determination (MUNANA-based enzyme inhibition assay) [28]. Oseltamivir carboxylate was used as the positive control.

RESULTS

In our previous report [29], 13 actinobacterial strains were isolated from *P. cocoes* (Sw.) Nyl. samples collected from Lumpini Park, Bangkok, Thailand. Among them, the isolate with the best antimicrobial activity was selected for this study.

Identification and characterization of strain Lp03

Strain Lp03 grew well on all ISP media. Strain Lp03 had the morphology of spore chains and key chemo-

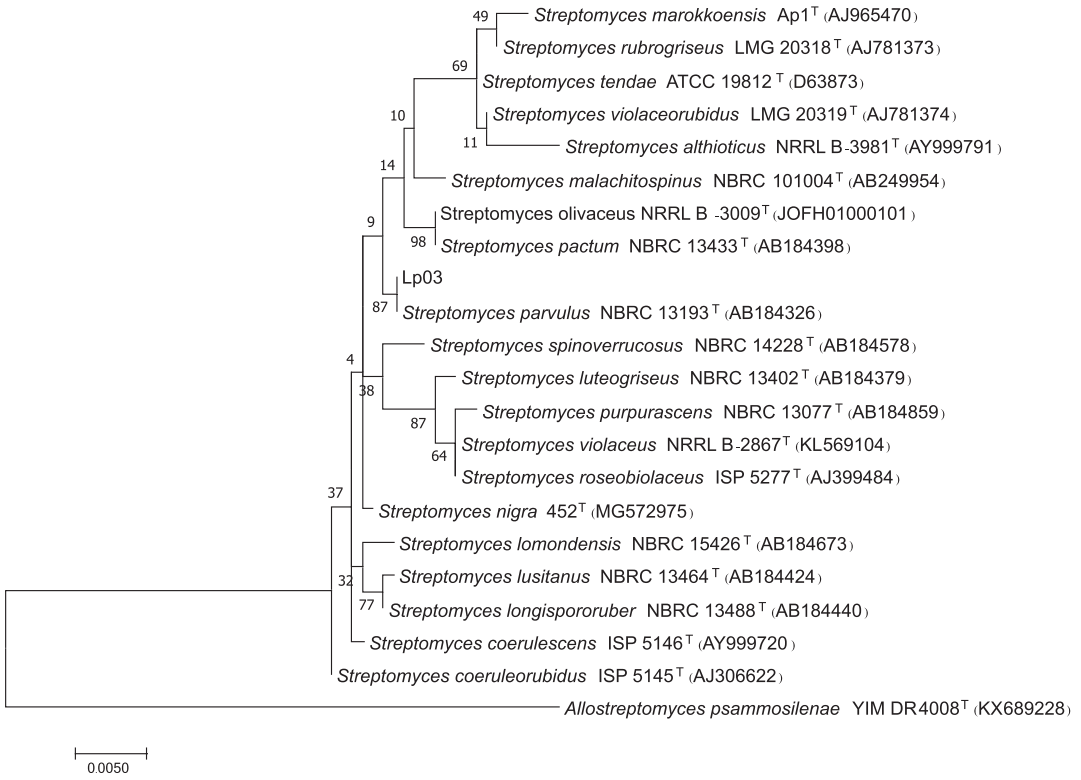

Fig. 3 Phylogenetic tree based on the 16S rRNA gene of strain Lp03 and closely related *Streptomyces* species. *Allostreptomyces psammosilenae* YIM DR4008^{T} was used as the out group. Bar, 0.005 substitutions per nucleotide position.

Table 1 Phenotypic characteristics of strain Lp03 compared with the type strain of *S. parvulus* NBRC 13193^{T}.

Characteristic	Lp03	$^{\alpha}$*S. parvulus* NBRC 13193^{T}
Spore chain morphology	Spiral	Spiral
Spore surface	Smooth	Smooth
Diffusible pigment	+	+
Milk peptonization	+	+
Carbon utilization		
Glucose	+	+
Lactose	+	+
Fructose	+	+
Raffinose	−	−
Xylose	+	−
Sucrose	−	+
Nitrogen utilization		
L-Cysteine	+	−
Growth at 7% NaCl	+	+

$^{\alpha}$ Data were obtained from Reddy et al [40].

taxonomic characteristics similar to the members of the genus *Streptomyces*. It produced a yellow pigment in all ISP media (Fig. 1b). White to gray aerial mass was observed on the agar plates. The cultural characteristics of strain Lp03 are summarized in Table S1 and Fig. S1. The young spore chain was of a spiral type, which differentiates to the mature rectiflexible to spiral spore chains (Fig. 1c). These spores could be observed on all ISP media. The mature spores had smooth surfaces (Fig. 1d). LL-diaminopimelic acid, glucose, and ribose were detected in the whole-cell hydrolysate.

Strain Lp03 showed positive results for the liquefaction of gelatin, starch hydrolysis, milk coagulation, and nitrate reduction. The strain grew well at 30–37 °C with the maximum NaCl tolerance up to 9% (w/v), but growth was not observed at 45 °C. Strain Lp03 used L-asparagine, L-arginine, L-valine, and L-proline for nitrogen sources but did not use ammonium sulfate. It also utilized glucose, mannitol, glycerol, *myo*-inositol, mannose, lactose, maltose, fructose, rhamnose, xylose, melibiose, cellobiose, and galactose as sole carbon sources but did not utilize melezitose, raffinose, or sucrose. It showed enzymatic activity of alkaline phosphomonoesterase, leucine arylamidase, acid phosphatase, napththol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, *N*-acetyl-β-glucosaminidase, and α-mannosidase. It also showed weak activity of esterase C4, cystine arylamidase, and trypsin.

The almost complete 16S rRNA gene sequence (1526 nt) of strain Lp03 showed the highest 16S rRNA gene similarity (100%) with *Streptomyces parvulus*

NBRC 13193^T. The phylogenetic tree based on the 16S rRNA gene revealed that strain Lp03 shared the same node with *S. parvulus* NBRC 13193^T (Fig. 3).

Based on the result mentioned above, strain Lp03 showed phenotypic properties similar to the type strain of *S. parvulus*. However, some characteristics may vary such as utilization of xylose and cysteine (Table 1). According to the phenotypic properties and 16S rRNA gene analysis, strain Lp03 was identified as *S. parvulus* Lp03.

Identification of bioactive compounds

In this study, 469.5 mg of the deep red crude ethyl acetate extract was obtained. The crude extract of strain Lp03 showed antimicrobial activity against *S. aureus* and *B. subtilis*, but no activities were observed against *P. aeruginosa*, *E. coli*, and *C. albicans*. The TLC-bioautography using $CHCl_3$:CH_3OH (9:1) revealed that the yellow spot at the R_f value of 0.6 showed antibacterial activity (Fig. S2). Therefore, the compound at this R_f value was designated as the bioactive compound. The bioactive compound in the extract was consecutively fractionated by Sephadex LH-20 column, and final purification was accomplished by preparative TLC to give a pure compound, designated the code as Lp03Ad.

The HPLC chromatogram of the Lp03Ad showed a peak at a retention time of 7.90 minutes with maximum absorption at λ 201.1, 241.7, and 442.3 nm in the UV spectrum. After comparison with the compounds in the in-house database, Lp03Ad showed an identical retention time and UV spectrum to those of actinomycin D (Fig. 4). The HRESIMS showed a sodium adduct mass ion peak at *m/z* 1277.6177 $[M+Na]^+$, suggesting a molecular formula of $C_{62}H_{86}N_{12}NaO_{16}$ (Fig. S3). The ^{1}H-NMR spectrum of Lp03Ad (in $CDCl_3$) showed an identical spectrum to actinomycin D (Fig. S4) [30]. Based on the evidence from the HPLC chromatogram, mass spectral data, and ^{1}H-NMR spectrum, the compound Lp03Ad could be identified as actinomycin D.

Biological activity of the actinomycin D from *S. parvulus*

Based on this study, actinomycin D showed potent antimicrobial activity against all tested Gram-positive bacteria including *B. subtilis* (MIC 1.56 µg/ml), *B. cereus* (MIC 5.0 µg/ml), *K. rhizophila* (MIC 76 µg/ml), *S. aureus* (MIC 9.76 ng/ml), and *S. epidermidis* (MIC 9.76 ng/ml) (Table 2). Actinomycin D had no antibacterial activity against *A. baumannii* and *P. aeruginosa* at the maximum tested concentration of 50 µg/ml. Among these Gram-negative bacteria, actinomycin D showed activity against *K. pneumoniae* and *E. coli* with the MIC values of 0.78 and 12.5 µg/ml, respectively. Based on this study, the MIC values of erythromycin against *K. pneumoniae* and *E. coli* were 0.85 and 57.34 µg/ml, respectively. Interestingly, although actinomycin D was inactive against *A. baumannii*, the activity could be observed against *A. baumannii* (MIC value of 6.25 µg/ml) when adding phenylalanine-arginine β-naphthylamide (PNβN), the efflux inhibitor, in the assay. Actinomycin D also showed activity against *M. tuberculosis* with a MIC value of 12.5 µg/ml. This MIC value was higher than drugs used for tuberculosis treatment, including rifampicin, streptomycin, isoniazid, ofloxacin, and ethambutol, which showed MIC values of 0.025, 0.625, 0.047, 0.781, and 1.88 µg/ml, respectively. Moreover, actinomycin D also showed antimalarial activity against the *P. falciparum* K1 strain with the IC_{50} value of 0.0148 µg/ml. Actinomycin D was inactive for anti-HSV-1 activity and for all yeast and fungal activities in this study. In addition, no neuraminidase inhibitory activity was detected.

Actinomycin D showed cytotoxicity against tested cell lines, including Vero cells, NCI-H187, KB, and MCF-7. It had potent activity against NCI-H187 (IC_{50} 0.69 ng/ml) and KB (IC_{50} 0.18 µg/ml) cell lines, compared with positive controls, ellipticine (IC_{50} 2.95 and 2.81 µg/ml), and doxorubicin (IC_{50} 0.13 and 1.35 µg/ml). It also showed activity against MCF-7 with the IC_{50} value of 21.94 µg/ml (Table 3).

Genomic features of strain Lp03

The genome of strain Lp03 showed good quality according to the PATRIC pipeline. The draft assemblies of the strain has been submitted to GenBank under the accession number JAIWPL010000000. The genome of strain Lp03 was 7,737,917 bp in size with 71 contigs, 72.71% of G+C content, and 5930 coding sequences. Based on the antiSMASH pipeline, 27 BGCs were detected in the genome of strain Lp03, most of which are represented by RiPPs-like, terpenoid, PKS, and NRPs-type clusters (Table S1). Among these BGCs, some showed an identity exceeding 60% to the known molecules which included melanin, desferrioxamin B/E, coelichelin, albaflavenone, spore pigment, isorenieratene, genosmin, ectoine, actinomycin D, hopene, SapB, and rhizomides A, B, and C.

The phylogenomic tree showed that strain Lp03 shared the node with the *S. parvulus* JCM 4068^T (Fig. S5). The digital DNA-DNA hybridization (dDDH) and average nucleotide identity (ANI) between the genomes of strain Lp03 and *S. parvulus* JCM 4068^T were 95.5% and 99.17%, respectively. This value is much higher than the 70% (dDDH) and 95% (ANI), which are the thresholds used to delineate separated species status. Therefore, it can be concluded that strain Lp03 is *S. parvulus*.

DISCUSSION AND CONCLUSION

The use of lichens as an alternative source for actinobacterial isolation was demonstrated in 2005 by

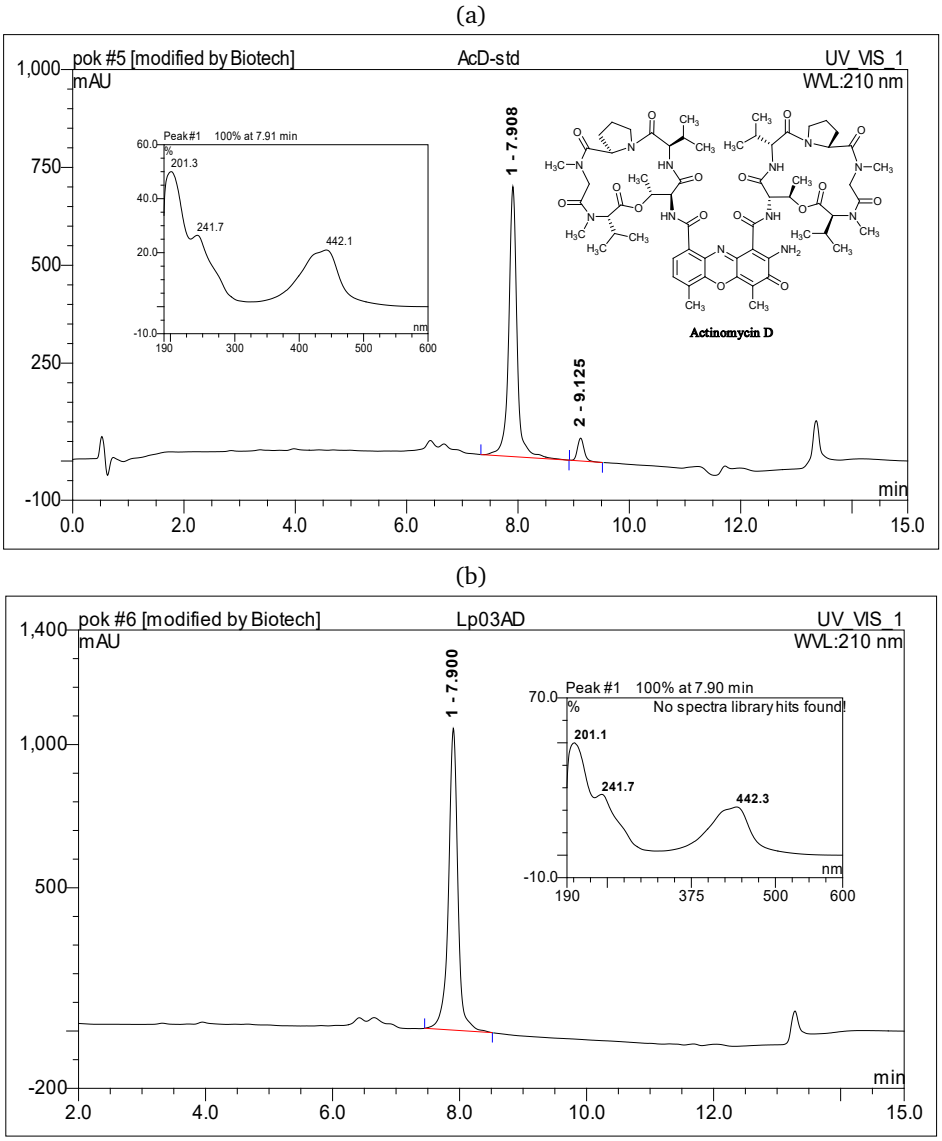

Fig. 4 HPLC chromatograms and UV spectrum of (a) actinomycin D standard (b) compound Lp03Ad.

González et al [7]. They collected the lichens from tropical areas of Hawaii and Reunion islands and from cold areas in Alaska. The pioneering study found that lichens represented an extremely rich reservoir of actinobacteria with a wide variety of species [7]. In Thailand, we have been attempting to isolate actinobacteria from lichen since 2016. To date, 4 novel actinobacterial species, including *Actinoplanes lichenis*, *A. lichenicola*, *A. ovalisporus*, and *S. lichenis* have been proposed.

S. parvulus is widely distributed in various habitats, including terrestrial soil, marine sediment, and symbioses with plants. In 2015, our colleague reported the isolation of *S. parvulus* from the foliose lichens *Flavoparmelia* sp. and *Lecanora helva* [31]. However, in that study, only the 16S rRNA gene was used to identify the strain. Therefore, this present study is the first report for the complete taxonomic study of *S. parvulus* derived from *P. cocoes* (Sw.) Nyl. lichen. This evidence showed that lichens might be the habitat of *S. parvulus*. The relationship between *S. parvulus* and lichens is still obscure and needs further investigation to answer this question.

Several species of *Streptomyces* produce actinomycin D, but *S. parvulus* is the most well-known as the commercial producer of this compound. Actinomycin is the first anticancer and antibiotic peptide discovered by Waksman and Woodruff in 1940, exhibiting

Table 2 Antimicrobial activity of the actinomycin D against tested microorganisms. All data were shown in the MIC (µg/ml) except for *P. aeruginosa* and *P. falciparum* that shown in the IC_{50} (µg/ml).

Compound	Antimicrobial activity											
	M. tuberculosis	*B. cereus*	*B. subtilis*	*S. aureus*	*S. epidermidis*	*K. rhizophila*	*K. pneumoniae*	*E. coli*	*A. baumannii*	*A. baumannii* +PAβN	*P. aeruginosa*	*Pl. falciparum*
Actinomycin D	12.5	1.56	5	0.00976	0.00976	0.076	0.78	12.5	>50	6.25	>50	0.0148
Rifampicin	0.025	nd	nd	nd	nd	nd	nd	nd	3.13	0.19	nd	nd
Streptomycin	0.625	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Isoniazid	0.047	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ofloxacin	0.781	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ethambutol	1.88	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Vancomycin	nd	2.00	1.5316	0.7658	1.5316	0.7658	nd	nd	nd	nd	nd	nd
Erythromycin	nd	nd	nd	nd	nd	nd	0.8539	57.34	12.5	0.78	>32.0	nd
Dihydroartemisinin	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3.46 nM
Mefloquine	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	70.68 nM
Chloroamphenicol	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	>8	nd

Table 3 Cytotoxicity of the actinomycin D against tested cell lines.

Compound	Cytotoxicity IC_{50} (µg/ml)			
	Vero cell	NCI-H187	KB	MCF-7
Actinomycin D	0.125	0.000694	0.0184	21.94
Ellipticine	0.959	2.95	2.81	nd
Doxorubicin	nd	0.1333	1.35	8.98
Tamoxifen	nd	nd	nd	7.91

potent antibacterial and antitumor activities [32]. It has been used for the treatment of Wilms tumor (a type of renal tumor), Ewing's sarcoma, testicular cancer, NPM1-mutated acute myeloid leukemia, and choriocarcinoma [33]. The antiproliferative activity of actinomycin D against cancerous cell lines has been reported for HeLa (cervical cancer), PC-3 (prostate cancer), TH-1 (leukemia), and Caco-2 (colon cancer) with IC_{50} values of 4.9–7.3 µm [34]. Although actinomycin D has long been reported for its anticancer activity, the cytotoxicity against the NCI-H187 cell line was limited. Based on this study, actinomycin D showed potent cytotoxicity against the NCI-H187 cell line with IC_{50} value of 0.694 ng/ml. This value was significantly lower than the positive controls, ellipticine and doxorubicin, that showed IC_{50} values of 2.95 and 0.13 µg/ml, respectively.

The antibacterial and antimalarial activities of actinomycin D have been confirmed by many studies [35, 36]. Chandrakar and Gupta [35] reported the antifungal activity of actinomycin D against *C. albicans* MTCC-183 and *Aspergillus niger* MTCC-872 at MIC and MBC of 1 and >1 mg/ml, respectively. In contrast, actinomycin D showed no activity against all tested fungi at 50 µg/ml concentrations. This negative result was not surprising because the maximum concentration used in this study was much lower than that previously reported [35]. The antimicrobial activity of actinomycin D (unknown concentration) against *A. baumannii* was recorded by Wong et al [37]. Actinomycin D was inactive against *A. baumannii* at a 50 µg/ml concentration in this study. The differences in the strain of microorganisms and the tested concentration might be the cause of this distinction. Interestingly, the activity of actinomycin D against *A. baumannii* could be observed with the MIC value of 6.25 µg/ml after we added an efflux inhibitor, PNβN, to the assay system. This result showed that the efflux pump might be involved in the resistance mechanism of *A. baumannii* to actinomycin D. In this study, the MIC value of actinomycin D against *M. tuberculosis* was 12.5 µg/ml. This value was close to that previously reported by Chen et al [38], who reported the activity of actinomycin D against *M. tuberculosis* H37Rv with the MIC value of 8 µg/ml. The antiviral activity against HSV-1 virus and the neuraminidase inhibitory activity of actinomycin D have not been reported. In this study, we recorded that actinomycin D was inactive for both neuraminidase inhibitory activity and anti-HSV-1.

The predicted actinomycin D biosynthetic gene cluster of strain Lp03 using antiSMASH was shown to be 64% identical to the known actinomycin D biosynthetic gene cluster of *Streptomyces alulatus* (BGC0000296) (Fig. S6). The detection of BGCs in the genome and isolation of the pure compounds strongly supported that strain Lp03 could produce actinomycin D. We analyzed the genomes of 4 different *S. parvulus* strains that were available on Genbank, including strain 2297 (ASM166004v1), JCM4068^T (ASM1464885v1), SX6 (ASM2090726v1), and VCCM 22513 (ASM2139447v1). The genome of *S. parvu-*

lus had a high diversity of BGCs. All genomes of *S. parvulus* strains harbored biosynthetic gene clusters of actinomycin biosynthesis. However, these actinomycin BCGs varied between strains. The common BGCs in the genome of all *S. parvulus* strains are non-ribosomal peptide synthetase (NRP), T1PKS, ectoine, and terpene. Moreover, the predicted BGCs for ectoine, geosmin, albaflavenone, desferrioxamin B (siderophore), and coelichelin are generally found in all genomes of *S. parvulus*. The BGCs in the genome of *S. parvulus* could vary between strains. For example, a piece of the predicted BGC of icosaline was detected in the genome of strain SX6 but was not found in the genome of other strains. In addition, some BGCs were detected, but it was not possible to predict the products. This result supported the previous study by Belknap et al [39] who investigated 1100 publicly available *Streptomyces* genomes and found that the same species can vary in the BGCs they carry and could potentially produce derivatives of any compound.

In our previous report, 13 actinobacteria were isolated from *Pyxine cocoes* lichen [29]. Eight of those 13 isolates showed antimicrobial activity, but strain Lp03 showed the most potent activity and was selected to be used in this study. Based on this selection by the best antimicrobial activity, the study then repeated the isolation of actinomycin D. In the genomic era, we know that *Streptomyces* harbored a huge diversity of BGCs in their genome. Therefore, in further studies, the strain should be selected based on the genomic evidence support. Eventually, based on this study, lichen could be a promising source for actinobacterial isolation. This study demonstrates that the actinobacteria isolated from lichen could produce bioactive secondary metabolites that can be used for pharmaceutical applications.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.025.

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Appendix A. Supplementary data

Table S1 Cultural characteristics of *S. parvulus* Lp03 on ISP media.

Media	Growth	Color		
		Aerial mass	Substrate mycelia	Soluble pigment
ISP2	Good	Greenish gray	Light olive brown to Greenish yellow	Yellow
ISP3	Very good	Light greenish gray	Light olive brown	Yellow
ISP4	Very good	Greenish gray	Strong greenish yellow	Yellow
ISP5	Moderate	Very pale green	Light yellow green to moderate yellow green	Yellow
ISP6	Good	Cream white to very pale green	Vivid yellow	Yellow

Table S2 The distribution of secondary metabolite biosynthetic gene clusters found in the genome of *S. parvulus* Lp03.

Cluster	Type[†]	Most similar known cluster (% similarity)
1	Melanin	Melanin (60 %)
2	Siderophore	Desferrioxamins B/E (83%)
3	NRPS	Coelichelin (90%)
4	T3PKS	Kanamycin (1%)
5	Terpene	Albaflavenone (100%)
6	T2PKS	Spore pigment (66%)
7	NRPS	Arylomycin (22%)
8	Siderophore	Grincamycin (5%)
9	Indole	5-isoprenylindole-3-carboxylate β-D-glycosyl ester (23%)
10	Terpene	Isoreneiratene (100%)
11	Terpene	Ebelactone (5%)
12	Terpene	Abyssomicin C (10%)
13	RiPP-like	Informatipeptin (42%)
14	Terpene	Geosmin (100%)
15	Ectoine	Ectoine (100%)
16	NRP	Ulleungmycin (16%)
17	NRP	Actinomycin D (64%)
18	Terpene	Hopene (69%)
19	RiPP:Lanthipeptide	SapB (100%)
20	T1PKS, NRPS	Aurantimycin A (51%)
21	T2PKS, T1PKS, butyrolactone	Rifamycin (29%)
22	NRPS	Vazabitide A (30%)
23	NRPS-like, T1PKS	Streptovaricin (21%)
24	T1PKS	Rifamycin (7%)
25	T1PKS	Rifamorpholine (21%)
26	NRPS	Rhizomide (100%)
27	NRPS	–

[†] NRPS, nonribosomal synthesized peptide; NRPS-like, NRPS-like fragment; T1PKS, Type I polyketide synthase; T2PKS, Type II polyketide synthase; and T3PKS, Type III polyketide synthase.

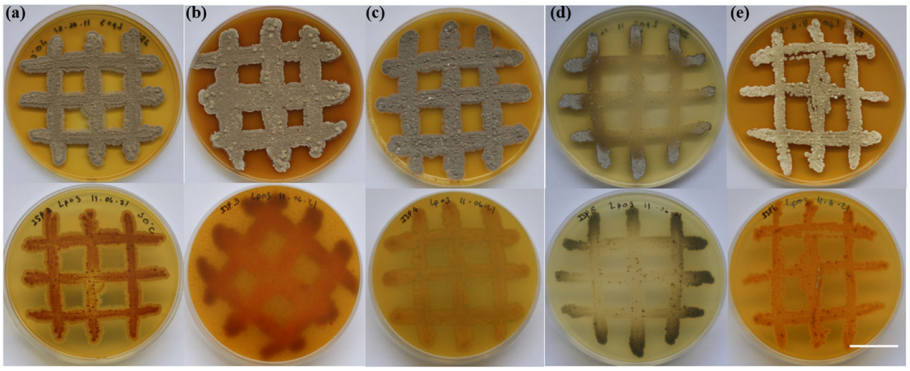

Fig. S1 Cultural characteristics of *S. parvulus* Lp03 grown on (a) ISP2, (b) ISP3, (c) ISP4, (d) ISP5, and (e) ISP6. All pictures were taken after the strain grown at 30 °C for 14 days. Bar 20 mm.

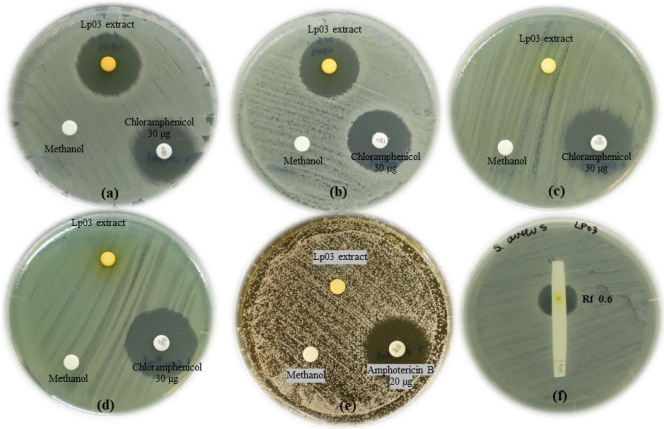

Fig. S2 Disk diffusion assay of the Lp03 crude extract against (a) *S. aureus*; (b) *B. subtilis*; (c) *E. coli*; (d) *P. aeruginosa*; and (e) *C. albicans*; and (f) The bioautography of the crude extract after being developed on the TLC plate using chloroform:methanol (9:1) showing the active spot at the R_f value of 0.6. Bar 6 mm.

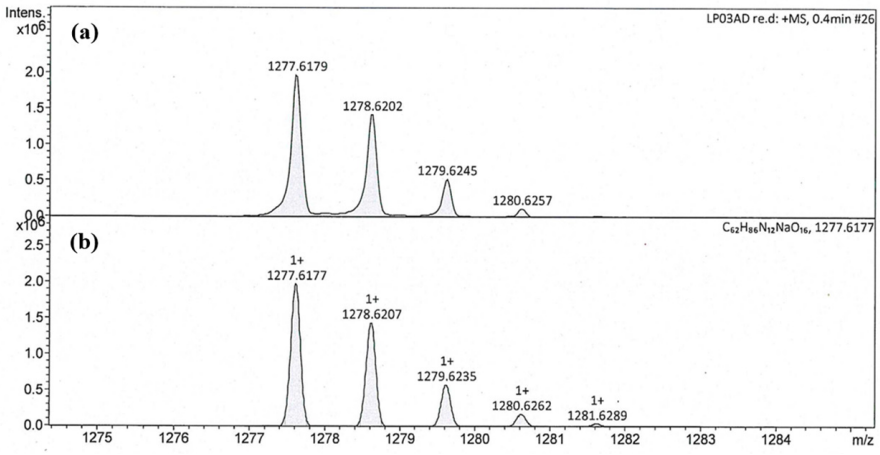

Fig. S3 HRESIMS of the sodium adduct mass ion peak $[M + Na]^+$ of (a) Lp03Ad and (b) the molecular formular of $C_{62}H_{86}N_{12}NaO_{16}$.

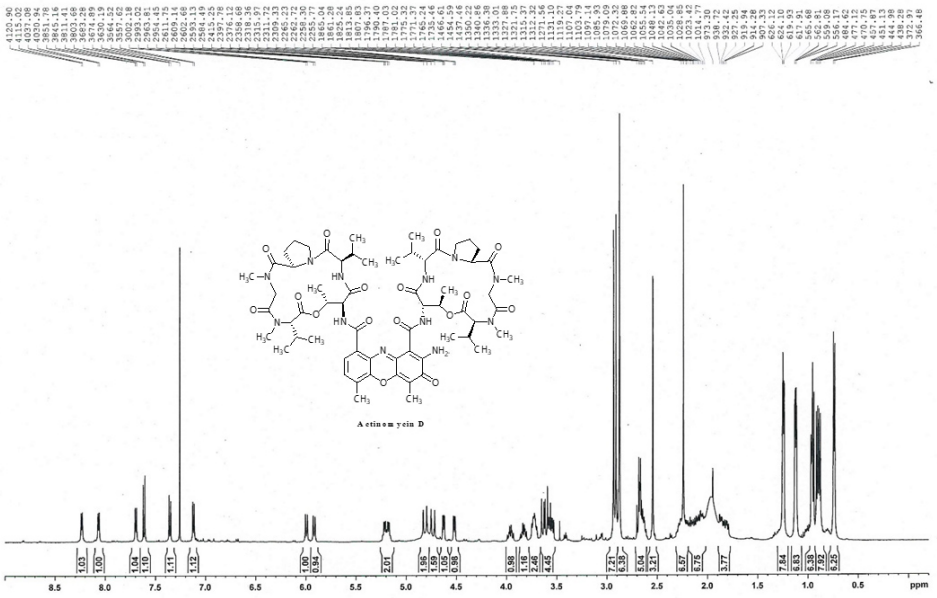

Fig. S4 ^{1}H-NMR spectrum of Lp03Ad (actinomycin D) in $CDCl_3$.

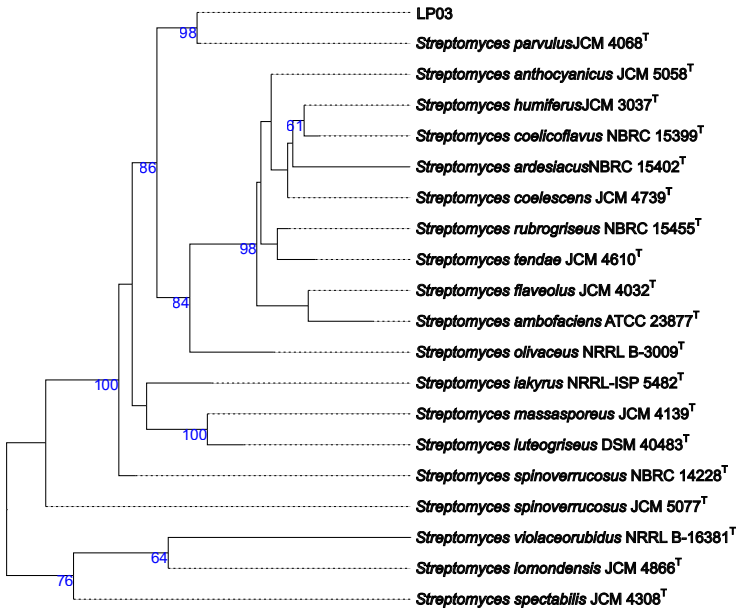

Fig. S5 The phylogenomic tree of strain Lp03 and related *Streptomyces* type strains available on the TYGS database. The numbers of the branches are GBDP pseudo-bootstrap support values from the 100 replications. The tree was rooted at the midpoint.

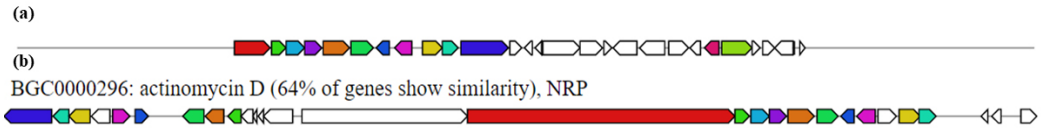

Fig. S6 Biosynthesis gene cluster involved in the actinomycin D biosynthesis of (a) *S. parvulus* strain Lp03 and (b) *S. anulatus* (BGC0000296).