Marine *Streptomyces chumphonensis* KK1-2[T] produces piericidin A1 as the major secondary metabolite

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ABSTRACT: Strain KK1-2[T] is the type strain of marine *Streptomyces chomphonensis*. Since the discovery of this *Streptomyces* species, the secondary metabolite of the type strain has not been characterized. Based on genome sequence analysis, the biosynthetic gene cluster of piericidin A1 was detected in the genome of strain KK1-2[T]. The isolation of secondary metabolites and spectroscopy data confirm the chemical structure of piericidin A1 produced by the type strain. This study is the first report of the genome analysis and the secondary metabolite produced by the type strain of *S. chumphonensis*.

KEYWORDS: piericidin A1, marine actinobacteria, microbial metabolites, genome analysis

INTRODUCTION

Microorganisms are a primary resource driving drug discovery [1]. They produce a large number of bioactive secondary metabolites which used in pharmaceuticals and agriculture today [2].

The Actinobacteria, Gram-positive bacteria with high G+C contents in DNA, have been well known for the antibiotic producers. Among known microbial metabolites, approximately 45% were produced by actinobacteria. Furthermore, 70% of actinobacterial bioactive secondary metabolites were produced from the members of the genus *Streptomyces* [3]. In general, actinobacteria are widely distributed in terrestrial habitats [4]. Over the past decade, the unexplored and under-explored habitats, such as the marine ecosystem, are believed to be rich sources of actinobacteria [5]. Since the discovery of the obligate genus *Salinispora*, marine actinobacteria are considered as promising sources for the discovery of novel bioactive compounds [6, 7].

The type strain is the strain used when the species was described for the first time. In 2014, we proposed a new marine *Streptomyces* species, *Streptomyces chumphonensis*, isolated from marine sediment collected from Chumphon Province, Thailand [8]. However, the secondary metabolites and the genome analysis of the type strain were not reported. This study aims to isolate the secondary metabolites produced by the type strain of *S. chumphonensis* KK1-2[T] and determine the biological activities of the pure compound. The genome sequence of the type strain was analyzed and discussed in this study.

MATERIALS AND METHODS

Fermentation

The inoculum was prepared using yeast extract-dextrose broth (yeast extract 1.0 g; dextrose 1.0 g; distilled water 100 ml; pH 7.0). One full loop of strain KK1-2[T] was cultured in an Erlenmeyer flask containing 100 ml of yeast extract-dextrose broth and incubated on the incubator shaker at 180 rpm 30 °C for 4 days. Then, 1.25 ml of inoculum was transferred into each of 500 ml Erlenmeyer flasks containing 125 ml the production medium (soluble starch 20.0 g; glycerol 5.0 g, defatted wheat germ 10.0 g; meat extract 3.0 g; yeast extract 3.0 g; $CaCO_3$ 3.0 g; 1 l of 50% (v/v) artificial seawater; pH 7.5). The production medium (6 l) was cultured

under shaking condition at 180 rpm 30 °C for 8 days. Mycelia were separated from the culture broth by centrifugation at 3000 rpm for 5 min. The mycelia cake was extracted with ethanol (95% v/v) for 5 h; and the cell debris were separated by using paper filtration. The filtrate was evaporated to dryness and dissolved in 300 ml of distilled water. This aqueous solution was partitioned with ethyl acetate three times. Finally, the ethyl acetate layer was evaporated to dryness to obtain 1.15 g of crude ethyl acetate extract or yellow gum.

Isolation and structure elucidation of the major secondary metabolite

The major secondary metabolite in the crude mycelial extract was determined and isolated using HPLC (Dionex-Ultimate 3000 series) and open column chromatography (RP18-ODS) and preparative HPLC equipped with the Pegasil ODS sp.100 column (20 × 250 mm), respectively. NMR spectra, including ^{1}H and ^{13}C NMR as well as ^{2}D NMRs (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC), were obtained from Bruker Advance 400 MHz NMR spectrometer. HRESIMS data were obtained from Bruker MicroTOF spectrometer.

DNA extraction and genome analysis

The genomic DNA was extracted from the cells using the PureLink™ genome DNA kit (Thermo Fisher Scientific). Draft genome sequencing was performed on Illumina HiSeq X ten sequencer using 2 × 150 bp paired-end read and assembled using Unicycler, the genome assembly program [9]. Annotation of the assembled genome was carried out using the genome analysis service at PATRIC [10]. The analysis of secondary metabolite biosynthesis gene clusters in the genomes was carried out using antiSMASH 5.0 [11]. A phylogenomic tree was constructed using the TYGS web server (https://tygs.dsmz.de/) [12].

Biological activity of the pure compound

The antibacterial activity against *Bacillus cereus* ATCC 11778 and antifungal activity against *Candida albicans* and cytotoxicity against KB (oral human epidermoid carcinoma), MCF-7 (human breast cancer, ATCC HTB-22), and NCI-H187 (small cell lung cancer) cell lines were determined using resazurin microplate assay [13, 14]. The antiGram-negative bacteria against *Pseudomonas aeruginosa* strain K2733 was determined using an optical density microplate assay [15]. The neuraminidase

inhibition was determined using fluorometric determination (MUNANA-based enzyme inhibition assay) [16]. The antimalarial activity against *Plasmodium falciparum* K1 was determined using the microculture radioisotope method [17]. The green fluorescent protein microplate assay was used to determine anti-*Mycobacterium tuberculosis* H37Ra (ATCC 25177), the cytotoxicity test against Vero cell (African green monkey kidney fibroblasts, ATCC CCL-81) and the anti-Herpes simplex virus type-1 [18].

RESULTS

Isolation and chemical elucidation of the major secondary metabolite

In this study, 1.15 g of yellow gum was obtained from the mycelial extract. The fermentation and isolation procedures of the major secondary metabolite of *S. chumphonensis* KK1-2^T was summarized in Fig. 1. The HPLC analysis of the mycelial extract showed the main compound at retention time (RT) 19.81 min (Fig. S1). To purify this target compound, the crude mycelial extract was applied to an octadecyl silane (ODS) column, equilibrated with water, and eluted using stepwise gradients of H_2O:MeOH 100:0, 90:10, 60:40, 40:60, 20:80, and 0:100. Two fractions, containing the target compound eluted with 20:80 and 0:100, were concentrated to yield 220.6 and 703.9 mg, respectively. Both fractions were dissolved with a small amount of MeOH and purified by HPLC (Pegasil ODS SP100; 20 × 250 mm; Senshu Scientific) using isocratic 85% MeOH with a flow rate of 15 ml/min. The amount of the target compound obtained was 170.4 mg (14.82% yield of the crude extract) and coded as KK1-2 P1 (Fig. 1).

The KK1-2 P1 was colorless oil. It showed maximum absorption at λ 235 and 267 nm in the UV spectrum. The HRESIMS showed a pseudo-molecular ion $[M+H]^+$ at m/z 416.2795, suggesting a molecular formula of $C_{25}H_{37}NO_4$.

The ^{1}H NMR spectrum of KK1-2 P1 (in CDCl$_3$) displayed characteristic proton signals of 6 methyls [at δ_H 1.60 (d, $J = 6.3$ Hz), 1.73 (s), 1.79 (s), 0.78 (d, $J = 6.6$ Hz), 1.62 (s), and 2.20 (s)]; 2 methoxys [at δ_H 3.81 (s) and 3.92 (s)]; 2 methylenes [at δ_H 3.35 (d, $J = 6.9$ Hz) and 2.77 (d, $J = 6.9$ Hz)]; 2 methines [at δ_H 2.66 (m) and 3.61 (d, $J = 9.1$ Hz)]; and 5 olefinic methines [at δ_H 5.39 (t, $J = 6.8, 13.6$ Hz), 5.58 (m), 6.07 (d, $J = 15.6$ Hz), 5.2 (d, $J = 9.71$ Hz), and 5.46 (m)]. The ^{13}C NMR and DEPT 135 spectra gave 25 carbon signals cor-
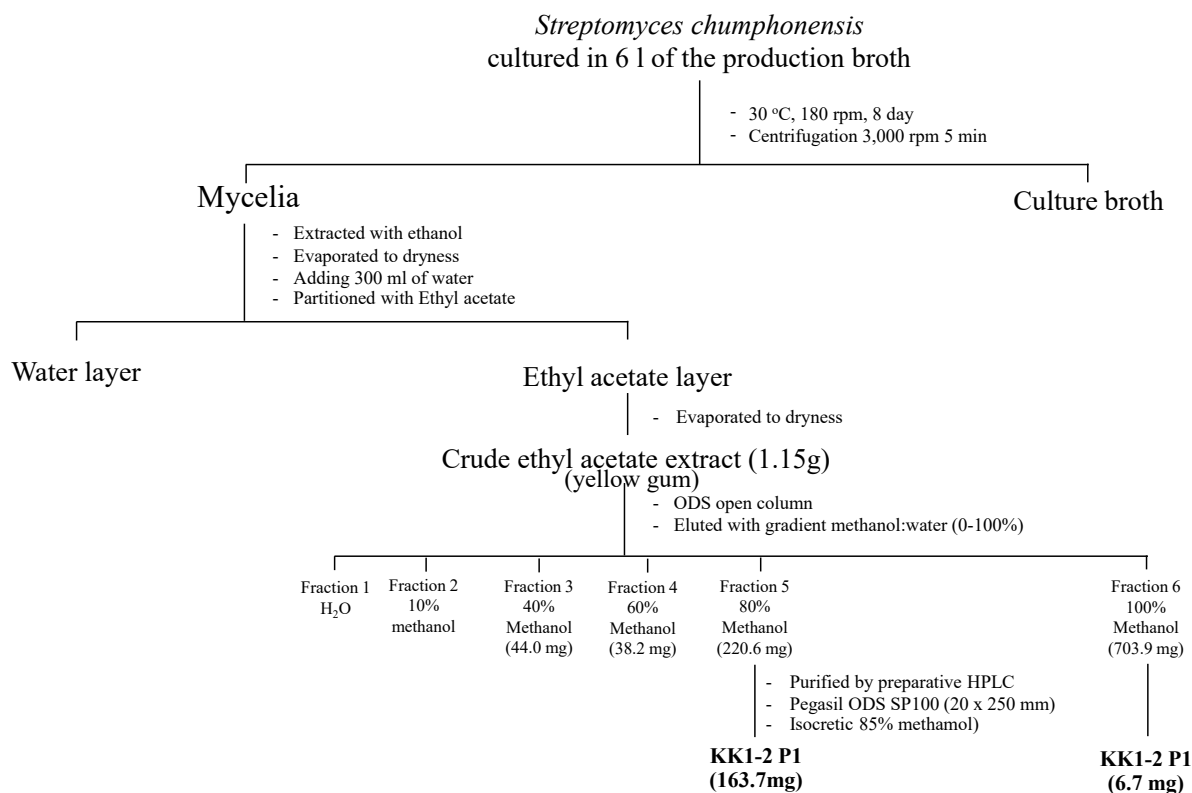
Fig. 1 Scheme of the isolation of the major secondary metabolites from the mycelial extract of *S. chumphonensis* KK1-2[T].
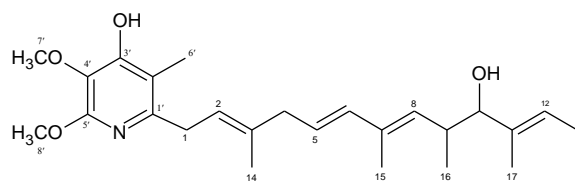

Fig. 2 Chemical structure of piericidin A1.

responding to 6 methyls [at δ_c 10.5 (C-6′), 13.0 (C-13), 16.5 (C-14), 12.9 (C-15), 17.3 (C-16), and 10.4 (C-17)]; 2 methoxys [at δ_C 60.5 (C-7′) and 53.0 (C-8′)]; 2 methylenes [at δ_C 34.3 (C-1) and 43.0 (C-4)]; 1 methine [at δ_C 36.8 (C-9)]; 1 oxymethine [at δ_C 82.8 (C-10)]; 5 olefinic methines [at δ_C 122.2 (C-2), 126.6 (C-5), 135.6 (C-6), 133.0 (C-8), and 123.3 (C-12)]; 3 quaternary olefinics [at δ_C 134.7 (C-3), 135.8 (C-7), and 135.5 (C-11)]; and 5 quaternary aromatics [at δ_C 150.7 (C-1′), 112.1 (C-2′), 154.1 (C-3′), 127.9 (C-4′), and 153.5 (C-5′)] (Table 1).

[1]H and ^{13}C spectral data of KK1-2 P1 and piericidin A1 were identical (Table 1) [19]. Based on NMR and mass spectral data, KK1-2 P1 was, therefore, identified as piericidin A1 (Fig. 2). The NMR spectra of the compound were shown in

Figs. S2 and S3. However, biological activities of KK1-2 P1 showed a negative result in all tests used in this study.

Genome and secondary metabolite biosynthetic gene clusters of strain KK1-2[T]

The draft genome assemblies of strain *S. chomphonensis* KK1-2[T] was submitted to Gen-Bank as accession number JACXYU000000000, and publicly available. The assembled genome sequence of strain KK1-2[T] had 52 contigs, with a total length of 5 823 549 bp and an average G+C content of 73.31% (Fig. S1). The genome had 5406 protein-coding sequences (CDS), 61 transfer RNA (tRNA) genes and 3 ribosomal RNA (rRNA) genes. The result of the subsystems and genes was provided in Fig. S4. Based on the prediction of secondary metabolite biosynthesis gene clusters using anti-SMASH, strain KK1-2[T] showed a total of 28 secondary metabolite biosynthetic gene clusters, as summarized in Table 2. The result of ClusterBlast revealed that one of the T1PKS was 100% similar to the biosynthetic gene clusters of piericidin A1 of *Streptomyces* sp. SCSIO 03032 (BGC0001169) and *Streptomyces piomogenus* (BGC0000124) (Fig. 3).

Table 1 The selected ^{1}H and ^{13}C spectral data of KK1-2P1 and piericidin A1.

Position	KK1-2 P1 (in CDCl$_3$)		Piericidin A1 (in CDCl$_3$) [19]	
	δ_C (ppm)	δ_H (ppm) multiplicity (J in Hz)	δ_C (ppm)	δ_H (ppm) multiplicity (J in Hz)
1	34.3	3.35 (d, 6.9)	33.4	3.36 (d, 6.9)
2	122.2	5.39 (t, 13.6, 6.8)	122.2	5.43 (t, 13.1, 6.9)
3	134.7	–	134.8	–
4	43.0	2.77 (d, 6.9)	43.1	2.78 (d, 6.9)
5	126.6	5.58 (m)	126.8	5.65 (m)
6	135.6	6.07 (d, 15.6)	135.7	6.08 (d, 15.6)
7	135.8	–	136.0	–
8	133.0	5.20 (d, 9.7)	133.1	5.23 (d, 9.6)
9	36.8	2.66 (m)	36.3	2.68 (m)
10	82.8	3.61 (d, 9.1)	82.8	3.62 (d, 9.1)
11	135.5	–	135.6	–
12	123.3	5.46 (m)	123.5	5.5 (m)
13	13.0	1.60 (d, 6.3)	13.2	1.60 (d, 5.3)
14	16.5	1.73 (s)	16.6	1.75 (s)
15	12.9	1.79 (s)	13.1	1.73 (s)
16	17.3	0.78 (d, 6.6)	17.3	0.79 (d, 6.7)
17	10.4	1.62 (s)	10.5	1.59 (s)
1′	150.7	–	150.8	–
2′	112.1	–	112.0	–
3′	154.1	–	154.0	–
4′	127.9	–	127.8	–
5′	153.5	–	153.5	–
6′	10.5	2.20 (s)	10.5	2.28 (s)
7′	60.5	3.81 (s)	60.6	3.85 (s)
8′	53.0	3.92 (s)	53.0	3.95 (s)

Table 2 The distribution of biosynthetic gene clusters in *S. chumphonensis* KK1-2[T].

Cluster	Type	From	To	Most similar known cluster
1	PKS-like, transAT-PKS	13924	95509	Lagriamide (NRP + polyketide) (9%)
2	NRPS, T1PKS, ectoine	110663	175514	Ectoine (100%)
3	siderophore	250565	266816	Macrotetrolide (polyketide) (33%)
4	NRPS-like	528197	572051	Indigoidine (saccharide) (27%)
5	Lanthipeptide	708929	731553	SapB (Ripp:Lanthipeptide) (75%)
6	T2PKS	6838	79107	Hiroshidine (polyketide) (41%)
7	NRPS, terpene	138149	210149	Mirubactin (NRP) (78%)
8	terpene	264024	286014	Hopene (terpene) (30%)
9	NRPS, T1PKS	522691	566031	Telomycin (NRP) (8%)
10	NRPS-line, betalactone	428781	458923	A-500359/A-500359 B (NRP)(5%)
11	NRPS-like, butyrolactone	252937	296371	Lomofungin (21%)
12	T1PKS	394317	425142	Fulvirucin B2 (polyketide) (11%)
13	Siderophore	68596	83262	Ficellomycin (NRP) (3%)
14	T1PKS	267077	304194	Piericidin A1 (polyketide) (91%)
15	T1PKS	1	47303	Lorneic acid A (polyketide) (23%)
16	NRPS, T1PKS	54784	134351	Incednine (polyketide) (4%)
17	CDPS	26119	46859	Primycin (polyketide) (5%)
18	T2PKS	183082	237461	Prejedomycin/rabelomycin/gaudimycin C/gaudimycin D /UEM6/gaudimycin A (polyketide: Type II+saccharide: hybrid/tailoring) (14%)
19	NRPS	32292	78681	Diisonitrile antibiotic SF2768 (NRP) (66%)
20	T1PKS, PKS-like	79108	119567	Arsono-polyketide (polyketide) (62%)
21	Ladderane, arylpolyene	1	38990	Atratumycin (NRP) (34%)
22	NRPS, lanthipeptide	14797	89168	Argimycin PI/argimycin PII/nigrifactin/argimycin PIV /argimycin PV/argimycin PVI/argimycin PIX (polyketide: modular type I) (8%)
23	T1PKS	38500	91497	pyrrolomycin A/pyrrolomycin B/pyrrolomycin C /pyrrolomycin D (polyketide) (40%)
24	Terpene	1	14644	Geosmin (terpene) (100%)
25	T1PKS	1	2874	Nigericin (polyketide) (55%)
26	LAP, thiopeptide	396291	428740	–
27	Other	108822	149922	–
28	T1PKS	1	2542	–

In this study, the genes involved in piericidin A1 production including, PieR, PieA1, PieA2, PieA3, PieA4, PieA5, PieA6, PieB1, PieC, PieD, PieB2, and PieE, were detected in the genome of *S. chumphonensis* KK1-2[T].
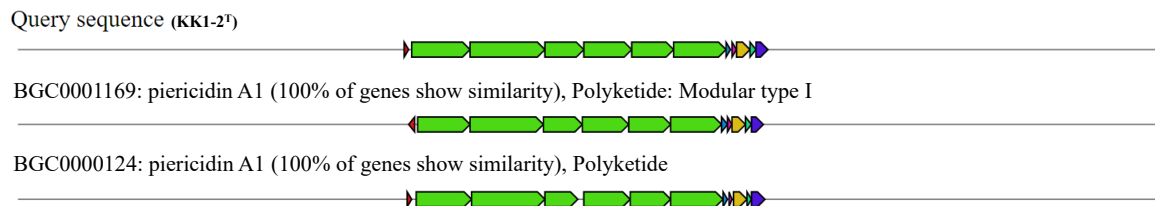
Based on the phylogenomic tree analysis, strain

Query sequence **(KK1-2^T)**

BGC0001169: piericidin A1 (100% of genes show similarity), Polyketide: Modular type I

BGC0000124: piericidin A1 (100% of genes show similarity), Polyketide

Fig. 3 The BLAST result using antiSMASH 5.0 showing the type 1 polyketide synthase (T1PKS) in the genome of strain KK1-2^T, which is 100% similar to the known biosynthetic gene clusters of piericidin A1 of *Streptomyces* sp. SCSIO 03032 (BGC0001169) and *Streptomyces piomogenus* (BGC0000124).

KK1-2^T shared the same cluster with *Streptomyces durbertensis* DSM 104548^T, *Streptomyces qinglanensis* CGMCC 4.6825^T, *Streptomyces radiopugnans* CGMCC 4.3519^T, *Streptomyces barkulensis* RC 1831^T, *Streptomyces taklimakanensis* TRM43335^T, and *Streptomyces megaspores* NRRL B-16372^T (Fig. S5). The genome analysis of these closely related *Streptomyces* species revealed no piericidin biosynthetic gene cluster in the genome. This result indicated that the piericidin A1 biosynthetic gene cluster is not related to the species within this phylogenetic cluster.

DISCUSSION

Piericidin A1, α-pyridone antibiotics, was first isolated from the type strain of *Streptomyces mobaraensis* [20]. This compound is mainly isolated from various *Streptomyces* species from both terrestrial and marine origins [21]. Although piericidin A1 did not show any biological activities against tested bacteria, yeast, virus, and cancer cell lines used in this study, in a previous study by other researchers, this compound exhibited insecticidal activity against green caterpillars, the larva of *Pieris rapae crucivora*, silkworms, and the larva of *Bombyx mori* [20]. Piericidin A1 was first discovered as potent inhibitors of complex I of the electron transport chain in mitochondria and some bacteria. Jeng et al [22] showed that at low concentrations, piericidin A1 reacted at a site near the reduced NADH dehydrogenase. Meanwhile, at high concentrations, the compound inhibited the succinic dehydrogenase system [22]. In 1970, Mitsui et al [23] studied the piericidin antagonistic effects of vitamin K3 on the respiratory chains of insects and mammals in the presence of piericidin A1. Vitamin K3 could overcome the inhibitory effect of piericidin A on the respiratory chain of the mammals, but not the insects. In that study, 0.167 mg/kg of piericidin A1 increased the respiratory rate and lowered the

blood pressure rapidly. In addition, vitamin K3 (10–40 mg/kg) could refresh the response to piericidin A in rats [23]. Recently, Morgan et al [24] found that piericidin A1 can block *Yersinia* Ysc Type III secretion system (T3SS), a bacterial virulence factor found in several Gram-negative pathogens. Lately, the total synthesis of piericidin A1 was proposed by Schnermann et al [25] and Lipshutz & Amorelli [26]. In addition, the proposed biosynthetic pathway of piericidin A1 has been described in the study of Liu et al [19].

In this study, it was noted that the piericidin A1 was mainly obtained from the mycelia of marine *S. chumphonensis* KK1-2^T. In the cell-free broth, the compound was not detected or seen at a very low level (data not shown). The detection of piericidin A1 biosynthetic gene cluster in the genome could support the strain KK1-2^T's ability to produce the piericidin A1. Based on the results obtained from this study, it can be concluded that the type strain of *S. chumphonensis* KK1-2^T is one of the piericidin A1 producers.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2021.024.

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Appendix A. Supplementary data

Table S1 Assembly details.

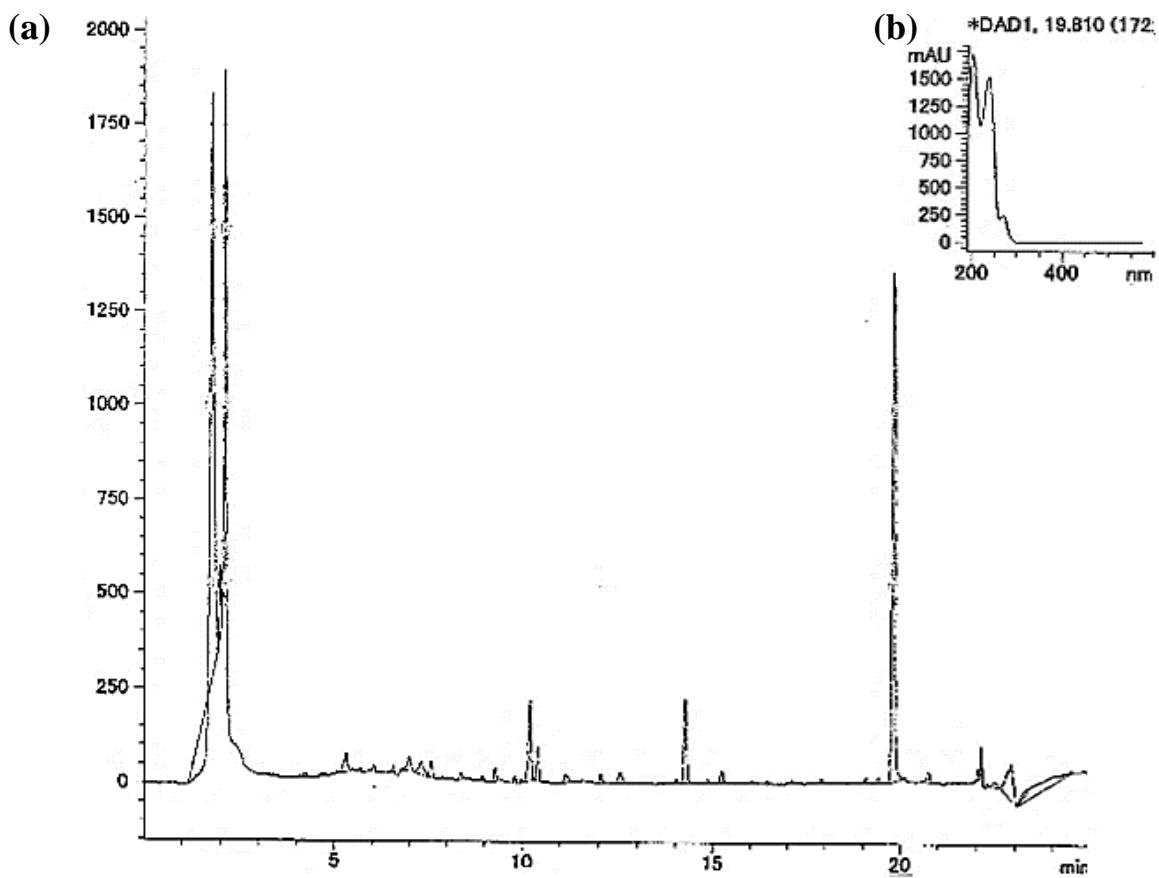| Assembly detail	
Contigs	52
G+C content	73.31
Plasmids	0
Contig L50	5
Genome length	5 823 549
Contig N50	350 958

Fig. S1 (a) HPLC chromatogram using UV 210 nm detector of mycelial extract of *S. chumphoensis* KK1-2[T] and (b) UV-visible spectrum of the target compound at the retention time of 19.81 min.
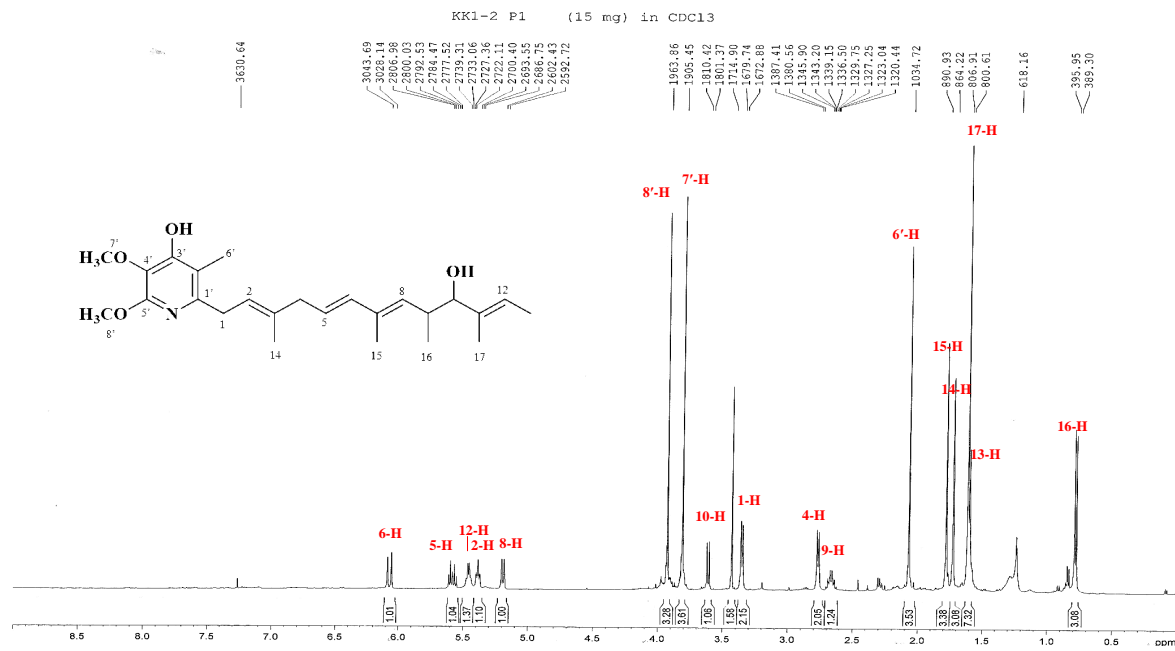
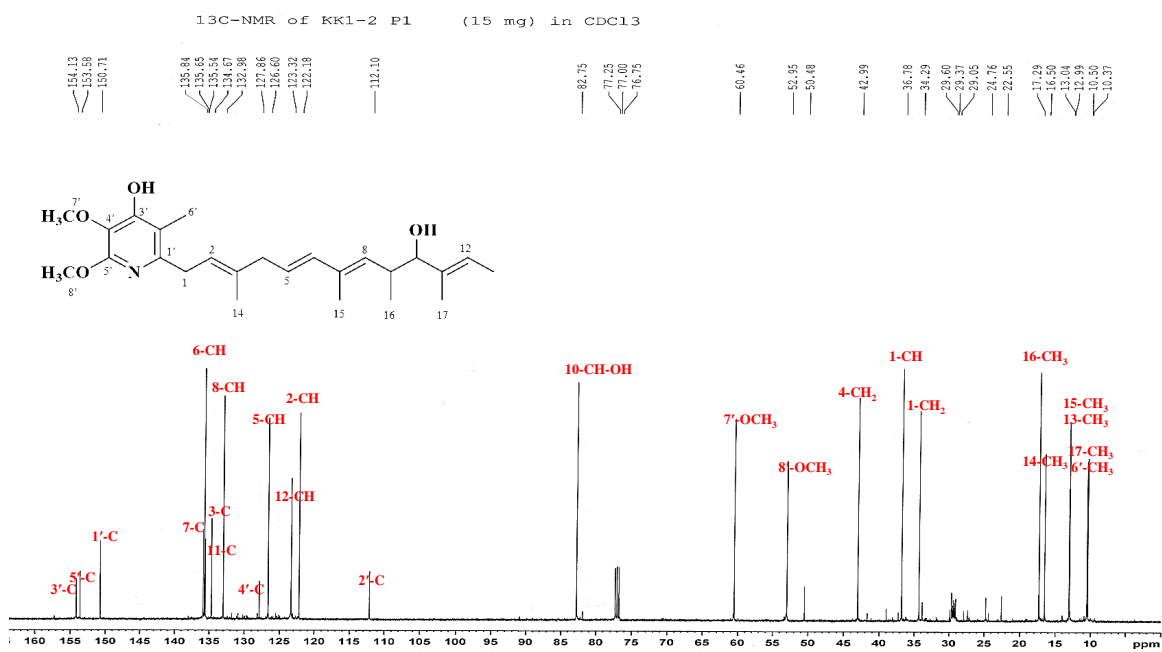**Fig. S2** ¹H NMR spectrum (400 MHz, CDCl₃) of KK1-2 P1 (piericidin A1).

Fig. S3 ¹³C NMR spectrum (400 MHz, CDCl₃) of KK1-2 P1 (piericidin A1).
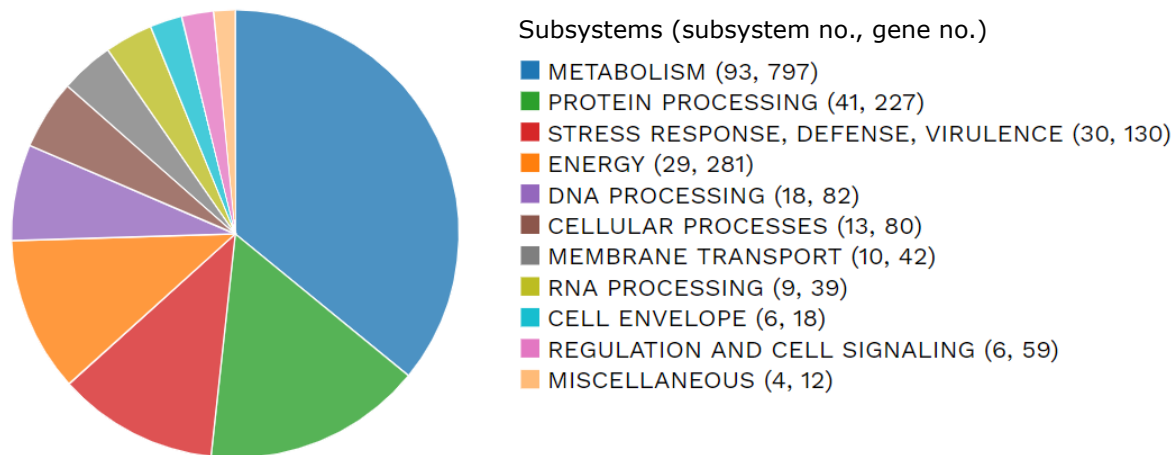
Fig. S4 An overview of the subsystems, a set of proteins that together implement a specific biological process or structural complex, of the genome of strain KK1-2ᵀ.
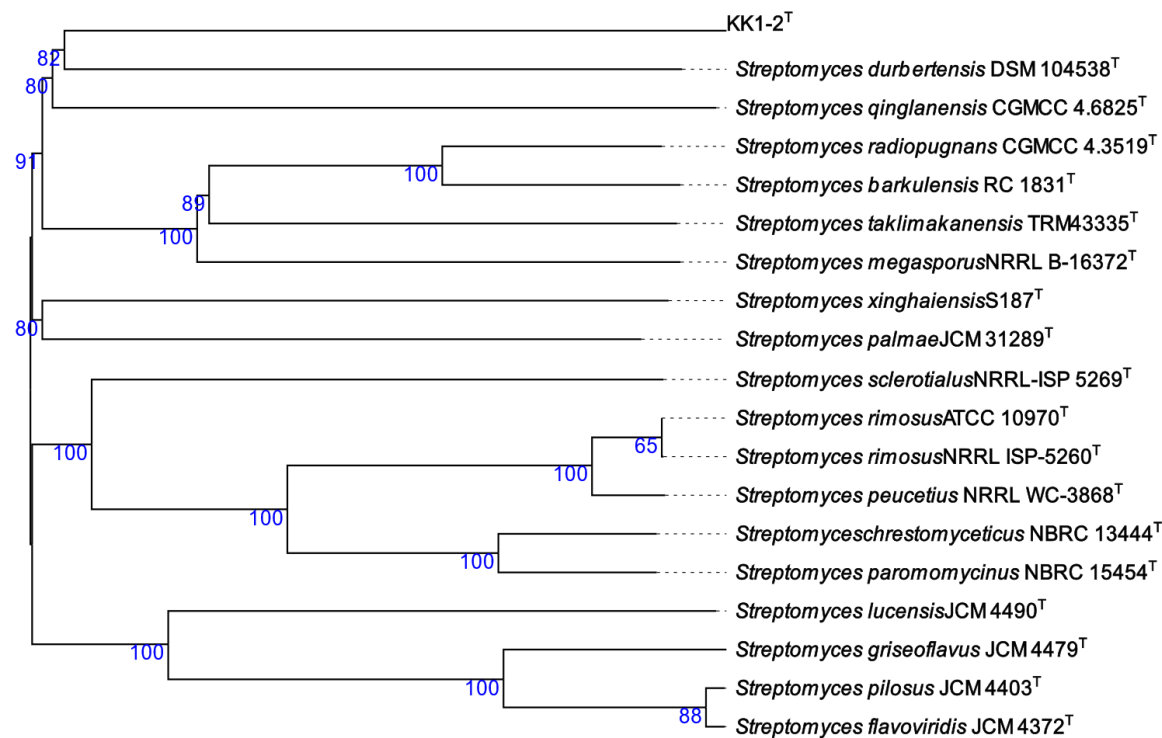

Fig. S5 The phylogenomic tree of *S. chumphonensis* KK1-2ᵀ and related type strains of the genus *Streptomyces* available on the TYGS database. The numbers above branches are GBDP pseudo-bootstrap support values from 100 replications. The tree was rooted at the midpoint.