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中国南海来源海洋链霉菌 SCSIO 11863 中 Enterocins 的分离鉴定

Kumar Saurav,张庆波,李苏梅,张文军,田新朋,李慧贤,张海波*

中国科学院南海海洋研究所 中国科学院热带海洋生物资源与生态重点实验室
海洋微生物研究中心 广东省海洋药物重点实验室,广州 510301

摘　要:从南海底泥样品中分离到一株具有较强抗菌活性的放线菌株 SCSIO 11863。表型和进化系统分析数据表明该菌属于链霉菌属并命名为 *Streptomyces* sp. SCSIO 11863 (KC904267)。16S rDNA 序列分析表明它与白浅灰链霉菌 *Streptomyces albogriseolus* strain ABRIINW EA1145 (GQ925802)有 99% 的相似性。对该菌发酵液乙酸乙酯萃取物进行活性追踪分离得到了两个结构类似化合物,分别为 Enterocin (**1**) 和 **5**-deoxyenterocin (**2**)。

关键词:南海沉积物;海洋放线菌;*Streptomyces* sp. SCSIO 11863;enterocins

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Isolation and Characterisation of Enterocins from Marine *Streptomyces* sp. SCSIO 11863 Isolated from South China Sea

Kumar Saurav, ZHANG Qing-bo, LI Su-mei, ZHANG Wen-jun, TIAN Xin-peng, LI Hui-xian, ZHANG Hai-bo*

CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, RNAM Center for Marine Microbiology, Guangdong Key Laboratory of Marine Materia Medica, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou 510301, China

Abstract: An actinomycetes strain SCSIO 11863 with strong antibacterial activity was isolated from marine sediment of the South China Sea. Based on the phenotypic and phylogenetic analysis, the strain was identified as a member of *Streptomyces* and designated as *Streptomyces* sp. SCSIO 11863 (KC904267). 16S rDNA sequence showed 99% similarity with *Streptomyces albogriseolus* strain ABRIINW EA1145 (GQ925802). Bioactivity guided extraction with ethyl acetate resulted in the isolation of two analogue compounds. Structures were elucidated with detailed analyses of NMR and MS spectra and identified as Enterocin (**1**) and **5**-deoxyenterocin (**2**).

Key words: marine sediments of South China Sea; marine actinomycetes; *Streptomyces* sp. SCSIO 11863; enterocins

Introduction

Pharmacological screening and usage of natural products for the treatment of human diseases has had a long history from traditional medicine to modern drugs[1]. The majority of clinically used drugs have various drawbacks in terms of toxicity, efficacy, cost and their frequent use has led to the emergence of resistant strains, hence there is a great demand for compounds belonging to a wide range of structural classes, selectively acting on new targets with fewer side effects. Microorganisms from marine environments have gained considerable attention in recent years because of its diversity and biological activities, mainly due to its ability to produce novel chemical compounds of high commercial value. At present there are sixteen compounds isolated from marine actinobacteria are in clinical trial against various diseases. The distribution and abundance of actinomycetes generally depends on various ecological habitats which include beach sand[2] and seawater[3]. Different sea areas contain different kinds of actinomycetes which may produce different novel antibiotic metabolites. South China Sea encompasses a tremendous diversity of natural ecosystems, ranging from coral reefs, rocky and sandy shores, mangroves, estuaries and mudflats to open waters and abyssal habitats, and is home to hundreds of thousands of species of organisms[4]. Various novel actinobacterial genera and spe-

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* Corresponding author Tel: 86-20-89023038; E-mail: zhanghb@scsio.ac.cn

cies have been previously reported from South China Sea [5-7] which focused our attention towards isolating and exploring new genera for its bioactive compounds. This continuous effort of exploring microbial flora from marine environment led to the isolation of *Streptomyces* sp. SCSIO 11863. Bioactivity guided extraction with ethyl acetate led to the isolation of two enterocins. Herein we report the isolation, taxonomical characterisation, structural elucidation of isolated compounds.

Materials and Methods

General

Materials for column chromatography (CC) were silica gel (100-200 mesh; 300-400 mesh; Jiangyou Silica gel development, Inc., Yantai, P. R. China), Sephadex LH-20 (40-70 μm; Amersham Pharmacia Biotech AB, Uppsala, Sweden), and YMC* GEL ODS-A (12 nm S-50 μm; YMC Company Ltd., Kyoto, Japan). Medium pressure liquid chromatography (MPLC) was performed on automatic flash chromatography (EZ Purifier III, Leisure Science Co., Ltd., Shanghai, China). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV-500 NMR spectrometer (BrukerBiospin GmbH, Rheinstetten, Germany) with tetramethylsilane (TMS, δ 0.0 p. p. m.) as the internal standard.

Isolation of marine actinomycetes

Actinobacteria were isolated from marine sediment samples collected from South China Sea at the depth of 10m using a large sterile spatula. Carefully measures were taken to remove the upper debris and were transported quickly to the lab in a sterile polythene cover. Pretreatment of sediments were performed before selective isolation. The International *Streptomyces* Project (ISP) No. I-IV, Starch Casein agar, Kuster's agar, modified Nutrient agar, Marine agar, actinomycetes isolation agar, Bennet agar, Glucose asparginate agar and tryptose soya agar medium with 50% sea water was used for the isolation of actinomycetes[8]. Plates prepared with different medium and varying sea salt concentration (3, 5, 7, 9 and 11% [w/v]) were incubated at 28 ℃ and observed for the growth after 4-6 weeks of incubation. The colonies were recognized according to their cultural characteristics and then transferred to slant culture at 4-8 ℃ as well as at 20% (v/v) glycerol stock at -80 ℃.

Preliminary screening for isolated compound

Well grown slant culture of the strain was used for crude extract preparation. A loopful of culture was inoculated to 50 mL AM3 medium (Bacterial peptone 15 g/L, soyabean powder 5 gm/L, Soluble starch 15 gm/L, Glycerol 15 gm/L, $CaCO_3$ 2 gm/L, pH 7.2 and sea salt 3%) contained in 250 mL Erlenmeyer flask and incubated for 7days on rotary shaker (200 rpm) at 28 ℃. After one week of incubation equal amount of butanone was added to the flask and kept in ultrasonication for 30 minutes. Organic phase was separated and concentrated in vacuum to obtain the crude extract. Dried crude extract was used to prepare stock solution using DMSO (16 mg/L). The appropriate concentration of the extract was used for antimicrobial assay (Disc diffusion) and brine shrimp assay as preliminary screening for its bioactivity.

Taxonomy

The morphology of the spore bearing hyphae with entire spore chain along with substrate and aerial mycelium was examined under light microscope as well as scanning electron microscope (Hitachi, S-3400N). Molecular taxonomic charaterisation was performed using 16S rRNA sequencing. The DNA was isolated, PCR conditions, design of the sequencing primers and the methodology for the sequencing were adapted from previous report[9]. The 16S rDNA sequence of the strain was determined on both strands using dideoxy chain termination method. The similarity and homology of the sequence was compared with the existing sequences available in the NCBI data bank using BLAST search. The DNA sequences were aligned and phylogenetic tree was constructed by neighbor joining method[10] using Mega 5.1 (biologist centric software for evolutionary analysis of DNA and protein sequences).

Fermentation and Isolation of active principle compounds

Well grown slant culture of the strain was used for preparation of seed culture. The seed culture was inoculated in 50 mL medium containing the optimized production medium prepared with 3% sea salt (pH 7.2)

and incubated for 2 days in rotary shaker (200 rpm) at 28 ℃. The inocula (10%) were then transferred into 200 ml production medium in 1 liter Erlenmeyer flasks and kept for shake flask growth for a week. A total of 10 L of large scale fermentation was carried out. After fermentation, the broth was centrifuged at 4000 rpm for 10 min at 10 ℃ and the supernatant was separated by using separating funnel. The supernatant was extracted twice with ethyl acetate. The extract was then concentrated in vacuum with rotary evaporator. The crude extracts were stored at -20 ℃ for further use. The crude extract obtained was choromatographed over normal phase silica gel (100-200 mesh) column and eluted with gradient system (Chloroform:Methanol;100:0,99:1,98:2,96:4,92:8,84:16,68:32 and 0:100) to obtain 8 fractions. Each fraction was examined for its compound profile on HPLC-DAD (Varian star work station). Subsequent chromatographical technique including elution on Sephadex LH-20 column and MPLC followed by Preparative HPLC using a reversed phase C_{18} column yielded compound **1** (4.3 mg) and compound **2** (6.5 mg) from fraction **3** and **5** respectively.

Results and Discussion

Marine sediments were collected from Weizhou Island, Guangxi province, China and continuous course of our selective isolation and screening procedure resulted in the isolation of SCSIO 11863, which was further deposited in type culture collection of Research Center for Marine Microbes, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China. The isolated strains are aerobic actinomycetes with white branched aerial and substrate mycelium. Spores are arranged in long chain and each chain contains approximately 10-15 spherical spores. Diameter of mature spores is between 0.6-0.8 mm and the length is between 0.9-1.4 mm (Fig. 1). Strain was further identified by molecular taxonomic characterization using 16S rRNA partial gene sequence analysis. The partial sequencing of 16S rRNA gene of the strain on both directions yielded 16S rDNA nucleotide sequence with 1681 base pairs and has been deposited to NCBI under the accession no KC904267. The BLAST search of 16S rDNA sequence of the strain showed highest similarity (99%) with *Streptomyces albogriseolus* strain ABRI-INW EA1145 (GQ925802) and phylogenetic tree was constructed with bootstrap values (Fig. 2). A neighbor-joining tree based on 16S rDNA sequences showed that the isolate occupies a distinct phylogenetic position within the radiation including representatives of the *Streptomycetes* family. The phylogenetic tree based on Maximum-parsimony method also showed that the isolate forms a separate clade. Based on the molecular taxonomy and phylogeny the strain was identified as *Streptomyces* sp. and designated as *Streptomyces* sp. SCSIO 11863. Preliminary screening of crude extract shows it as a potent inhibitory strain against selected human pathogenic bacteria strain and with moderate brine shrimp activity leads us to isolate and elucidate the active principle compounds. Extraction of its culture broth (10 L) yielded 12.5 grams of crude extract. Compounds enterocin (**1**) and 5-deoxy enterocin (**2**) were isolated using chromatographic techniques including column chromatography, Medium pressure liquid chromatography (MPLC) and semi preparative HPLC, and their structures were elucidated by comprehensive spectroscopic analyses (UV, NMR, and MS) and were confirmed by previously reported literatures. Compound 1, differs from 2 only at C-5 with an additional hydroxyl group[11].

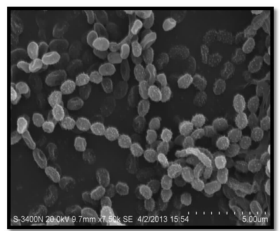

Fig. 1 Scanning electron micrograph of *Streptomyces* sp. SCSIO 11863 grown on Starch Casein Agar at 28 ℃ for 7 days; bar 5 μm

Compound **1**: pale yellowish powder; The molecular mass was determined by ESI-MS at both positive and negative modes and its molecular formular was established as $C_{22}H_{20}O_{10}$ on the basis of ESI-MS data $[M+H]^+$ (*m/z* 445.1), $[M+Na]^+$ signal (*m/z* 467.1). ^1H NMR (CD_3OD, 500 MHz) δ: 4.68 (1H, s, H-3), 4.81 (1H, dd, J = 4.5 Hz, H-5), 4.75

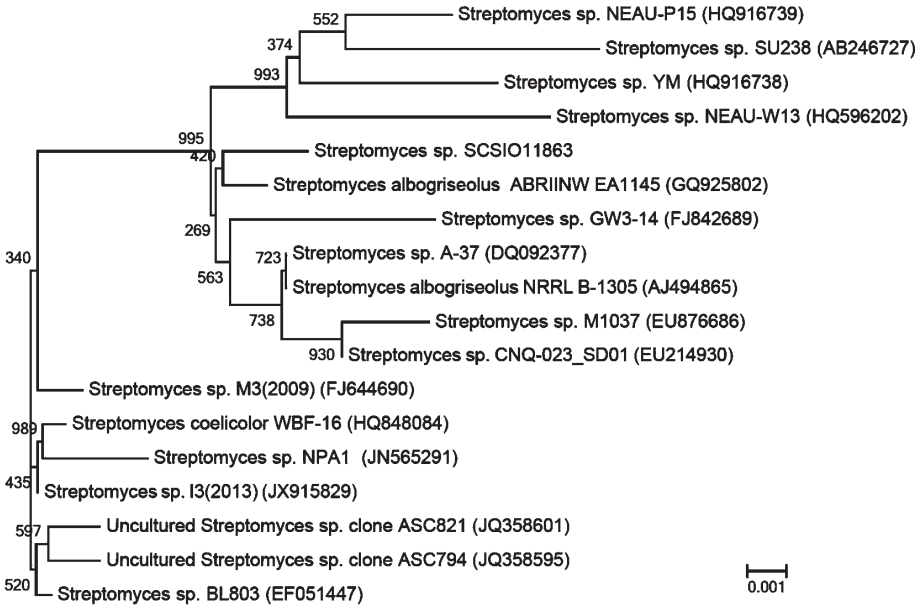

Fig. 2 The phylogram showing the position of the strain *Streptomyces* sp. SCSIO11863 with other *Streptomyces* based on 16S rDNA sequence.

Note: Phylogenetic tree was constructed based on neighbor joining analysis of 1000 replicated data. Number at nodes indicates the percent level of bootstrap support. Score bar represents 1 nucleotide substitution per 100 nucleotides. Bootstrap values of 50 and above are shown.

(1H, d, J = 2.5 Hz, H-6), 1.90 (1H, dd, J = 15.0, 5.0 Hz, H-7), 2.70 (1H, J = 15 Hz, H-7), 4.72 (1H, s, H-9), 6.41 (1H, d, J = 2.0 Hz, H-11), 5.66 (1H, d, J = 2.0 Hz, H-13), 7.05 (2H, d, J = 7.5 Hz, H-17, H-17′), 7.42 (2H, dd, J = 7.5, 8.0 Hz, H-18, H-18′), 7.57 (1H, t, J = 7.5 Hz H-19), 3.80 (3H, s, H-20). ^{13}C NMR (CD_3OD, 125 MHz) δ: 175.6 (s, C-1), 80.0 (s, C-2), 56.6 (d, C-3), 77.4 (s, C-4), 80.7 (d, C-5), 71.1 (d, C-6), 36.6 (t, C-7), 77.6 (s, C-8), 54.6 (d, C-9), 162.3 (s, C-10), 107.2 (d, C-11), 173.5 (s, C-12), 89.0 (d, C-13), 167.1 (s, C-14), 197.8 (s, C-15), 140.8 (s, C-16), 129.5 (d, C-17, 17′), 129.6 (d, C-18, 18′), 134.1 (d, C-19), 57.05 (q, C-20). The ^{1}H and ^{13}C NMR data of compound **1** were identical to those of enterocin thus compound **1** was identified as enterocin[12]. It is also known as vulgamycin.

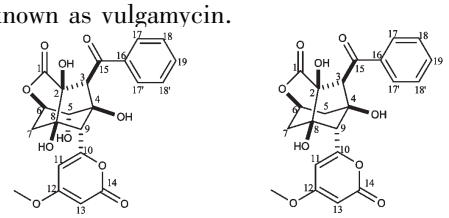

Fig. 2 Chemical structure of compounds 1-2

Compound **2**: pale yellowish powder; The molecular mass was determined by ESI-MS at both positive and negative modes and its molecular formular was established as $C_{22}H_{20}O_9$, ESI-MS data $[M + Na]^+$ (m/z 451.10) and $[2M + Na]^+$ signal (m/z 879.17). ^{1}H NMR (CD_3OD, 500 MHz) δ: 3.48 (1H, s, H-3), 2.96 (1H, d, J = 5.5 Hz, H-5), 2.50 (1H, dd, J = 3.5, 14.5 Hz, H-5), 4.89 (1H, s, H-6), 2.61 (1H, dd, J = 2.5, 4.5 Hz, H-7), 1.74 (1H, d, J = 12.5 H-7), 4.30 (1H, d, J = 1.5 Hz, H-9), 6.34 (1H, d, J = 2.0 Hz, H-11), 5.65 (1H, d, J = 2.0 Hz, H-13), 8.07 (2H, d, J = 7.5, Hz, H-17, H-17′), 7.48 (2H, t, J = 7.5, 8 Hz H-18, 18′), 7.60 (1H, dd, J = 7, 7.5 Hz H-19), 3.90 (3H, s, H-20). ^{13}C NMR (CD_3OD, 125 MHz) δ: 175.1 (s, C-1), 81.5 (s, C-2), 66.7 (d, C-3), 77.0 (s, C-4), 37.5 (t, C-5), 74.6 (d, C-6), 34.1 (t, C-7), 77.9 (s, C-8), 61.8 (d, C-9), 162.7 (s, C-10), 107.1 (d, C-11), 173.8 (s, C-12), 89.4 (d, C-13), 167.4 (s, C-14), 199.6 (s, C-15), 140.6 (s, C-16), 129.7 (d, C-17), 130.5 (d, C-18), 134.7 (d, C-19), 57.4 (q, C-20). The ^{1}H and ^{13}C NMR data of compound **2** were identical to those of 5-deoxyenterocin thus compound **2** was identified as 5-deoxyenterocin[13,14]. The stereochemistry for

both of compounds were assigned as reported[12,15].

Enterocins are well known polyketide antibiotics originally isolated from terrestrial soil derived *Streptomyces* sp. with strong antibacterial activity[11]. Compound **2** was later isolated again from marine Ascidian and successful comprehensive structural determination was done[16]. Since then number of times enterocins and its derivatives has been reported from various microbial sources. Polyketides are clinically important antibiotics requiring a series of organic synthesis pathways due to its complex chemical structures[17] and strong inhibitory activity against numerous gram positive and gram negative pathogenic microorganisms[18-22]. Due to its antagonistic activity against food borne pathogens it has been used as bio-preservatives in the food, making it as one of the most interesting broad spectrum class of antibiotics with a great application in food as well as medical industry. Present finding represents yet another new marine microbial source capable of producing this important class of polyketide antibiotics.

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