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Identification of newly isolated streptomycete which produces actinomycins

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ABSTRACT

A new actinomycete strain designated as *Streptomyces* sp. SZS39 was isolated along with other 61 bacterial strains from a soil in Japan using ISP2 agar medium. As a result of antimicrobial screening, the acetone extract of the strain SZS39 exhibited promising antimicrobial activities against *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. Isolation and identification of the antimicrobial principles from the strain SZS39 was accomplished. As a result of analysis using NMR and MS spectra, the main antimicrobial compound was identified as actinomycin X_2. To identify the strain SZS39, a partial 16S rRNA gene sequence from the strain SZS39 was determined and found to have high identity (99%) with known actinomycin producer *Streptomyces padanus* MITKK-103.

KEYWORDS

Streptomyces;
Actinomycin;
HPLC;
NMR spectra;
16S rDNA.

INTRODUCTION

Actinomycins constitute a family of chromopeptide antibiotics containing an aminophenoxazinone chromophore (actinocin) attached to two pentapeptide lactone rings. The various naturally occurring actinomycins differ by amino acid substitutions in specific positions of their peptide chains, while the actinocin moiety remains conserved[1]. Because of the planar structure of actinocin, actinomycins have the ability to intercalate into GC-rich DNA, which results in inhibition of transcription in both prokaryotes and eukaryotes[2,3]. Among actinomycins, actinomycin D is one of the older chemotherapy drugs, and has been used in treatment of a variety of cancers including gestational trophoblastic neoplasia[4], Wilms' tumor[5] and rhabdomyosarcoma[6]. Due to the significance of actinomycin, the search for the producing bacteria has been accomplished so far. There are many reports regarding streptomycetes which produce actinomycin related compounds[7,8]. *Micromonospora floridensis* NRRL 8020 was reported as only actinomycin producing bacterial strain which was not in *Streptomyces* genus[9]. Still the search for producing strain was an important issue to find more efficient producer.

On these circumstances, we performed screening for actinomycin producing actinomycetes. As a result, we found newly isolated strain SZS39 as an actinomycin producer. Here we describe the detail of identifica-

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tion of *Streptomyces* sp. SZS39 using 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

General methods

All NMR spectra were obtained with JEOL ECA-600 using DMSO-d_6 as solvent at 27.0 °C. The resonances of residual DMSO-d_6 at δ_H 2.49 was used as internal reference for ^{1}H NMR spectrum, respectively. ESI-TOF MS spectra were recorded by JEOL JMS-T100LP mass spectrometer.

Testing bacterial strain for antimicrobial assay

The bacteria strains including *Bacillus subtilis* (NBRC 13719), *Escherichia coli* (NBRC 1002203), *Pseudomonas aeruginosa* (NBRC 12689), *Saccharomyces cerevisiae* (NBRC 2376), and *Schizosaccharomyces pombe* (NBRC 0340) were obtained from Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Japan.

Isolation of bacteria and antimicrobial screening

The soil samples were collected from the ground in Sizuoka University, Shizuoka Prefecture, Japan. The soil samples were spread onto ISP2 agar medium[10]. After 4-5 days of incubation at 30 ºC, colonies developed were isolated and stored in a refrigerator at -80 °C. Total of 62 strains were collected and cultured for antimicrobial screening. ISP2 agar culture (25 mL) of each strain was extracted with equal volume of acetone. Each acetone extract was evaporated and dissolved in DMSO to adjust the concentration to 10 mg/mL. The antimicrobial activity was evaluated by the inhibitory zone that was caused by 10 μg inoculation of acetone extract sample on the testing microorganism's culture using ISP2 agar medium.

Polymerase chain reaction (PCR) amplification, sequencing, and phylogenetic analysis of 16S rRNA genes

The extraction of total DNA from the cells of SZS39 was performed according to the previous paper[11]. The 16S rRNA-encoding sequence was amplified from the total DNA by PCR method using two sets of universal primer pairs: 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 926R (5'-CCGTCAATTCCTTTGAGTTT-3'), 686F (5'-TAGCGGTGAAATGCGTAGA-3') and 1510R (5'-GGCTACCTTGTTACGA-3'). The reaction mixture for PCR was prepared by adding 1.0 μL of total DNA of SZS39 (100 ng), 1.0 μL of Pfu DNA polymerase (Bioneer, Korea), 5.0 μL of X10 reaction buffer (Bioneer, Korea), 4 μL of 2.5mM dNTPmix solution (Bioneer, Korea) and 31 μL of distilled water into the PCR reaction tube. PCR amplification was carried out with a thermal cycler using the following program: initial denaturation for 10 min at 94°C, followed by 34 cycles consisting of denaturation for 40 s at 94°C, annealing for 60 s at 55°C, and DNA synthesis for 1 min at 72°C. A final extension of 5 min at 72°C was added at the end of the 34 cycles. The PCR product was purified with QIAGEN PCR PURE kit following the manufacturer's instruction. The reactions for sequencing were performed using Beckman DTCS-Quick Start Kit following manufacture's instruction. The 4 primers are used for the reacton: 339F (5'-CTCCTACGGGTGAGTAACAC-3'), 536R(5'-GTATTACCGCGG CTGCTG-3'), 686F (5'-TAGCGGTGAAATGCGTAGA-3'), 1099F (5'-GCAACGAGCGCAACCC-3'). The sequencing was performed with capillary DNA sequencer CEQ8000XL (beckman coulter).

Isolation of actinomycin D and X_2

ISP2 agar medium (500 mL) was used for culture of *Streptomyces* sp. SZS39. After 7 days culture at 30 °C, equal volume of acetone was added to the agar culture for extraction. After concentration, the acetone extract was subjected to open column chromatography (Silica-gel) eluted with $CHCl_3$, $CHCl_3$/MeOH(9:1), $CHCl_3$/MeOH(7:3). The $CHCl_3$/MeOH (9:1) fraction was subjected to reversed-phase HPLC using ODS column (NacalaiTesque, Cosmosil C8, 4.6 × 250 mm) with isocratic elution of 53% MeCN containing 0.05% TFA. The UV detector of HPLC was set at the absorbance of 220 nm.

RESULTS AND DISCUSSION

The new bacterial strain SZS39 was isolated from the ground soil of Shizuoka University in Japan using ISP2 agar medium along with other 61 bacterial strains. With the aim of screening for antibacterial compounds, these strains were cultured with ISP2 agar media for 7 days. Each culture was extracted with acetone, and the acetone extract was concentrated and adjusted to the

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concentration of 10 mg/mL in DMSO. The extract was subjected to antibacterial assay using 5 bacterial strains (*Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*). As a result, high percentage of extracts (80%, 50/62) showed antibacterial activity against at least one testing strain. Among the bacteria, the extract of the strain SZS39 showed intense antibacterial activity against *B. subtilis*, *S. cerevisiae*, and *S. pombe*.

The culture of strain SZS39 using ISP2 agar medium was extracted by acetone and the acetone extract was filtered and concentrated to aqueous residue by the rotary evaporator. The acetone extract was subjected to open column chromatography using silica-gel with $CHCl_3$/MeOH solvent system. As a result of antibacterial assay, $CHCl_3$/MeOH (9:1) fraction showed potent activity and further HPLC analysis was performed on the fraction.

On HPLC analysis, two major peaks at retention time of 16.3 and 20.2 min were observed as shown in Figure 1. The compounds 1 and 2 showed characteristics of yellow powder after lyophylization. High resolution ESI-MS spectrum of compound 2 (retention time 20.2 min in Figure 1) established the molecular formula to be $C_{62}H_{84}N_{12}O_{17}$ based on the HR-ESI-MS ([M + H]$^+$ at *m/z* 1269.6065, calculated for 1269.6155, $C_{62}H_{85}N_{12}O_{17}$). Identification was accomplished by analysis of NMR spectrum data including ^{1}H, COSY, HMBC, HMQC, ROESY spectra. Since the spectral data was identical with literature data, compound 2 was identified as actinomycin X_2. The ESI-MS spectrum of compound 1 (retention time 16.3 min in Figure 1) indicated the ion peak at 1255.1. The compound 1 was deduced to be actinomycin D by judging by the retention time and molecular weight.

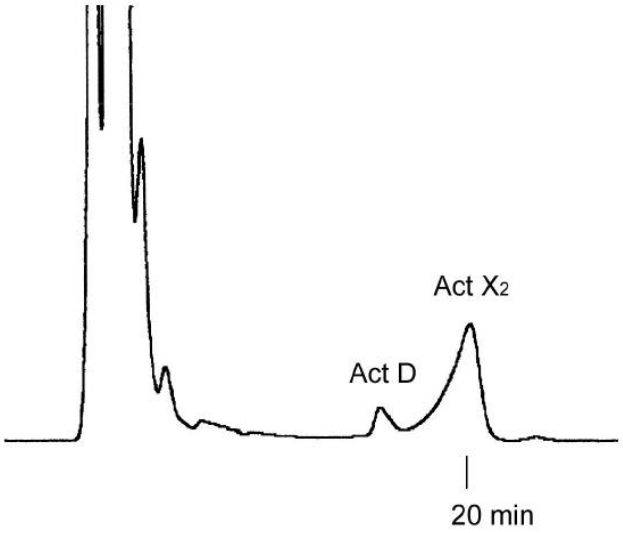

Figure 1 : HPLC chromatogram of extract of strain SZS39 (Act X_2: actinomycin X_2, Act D: actinomycin D)

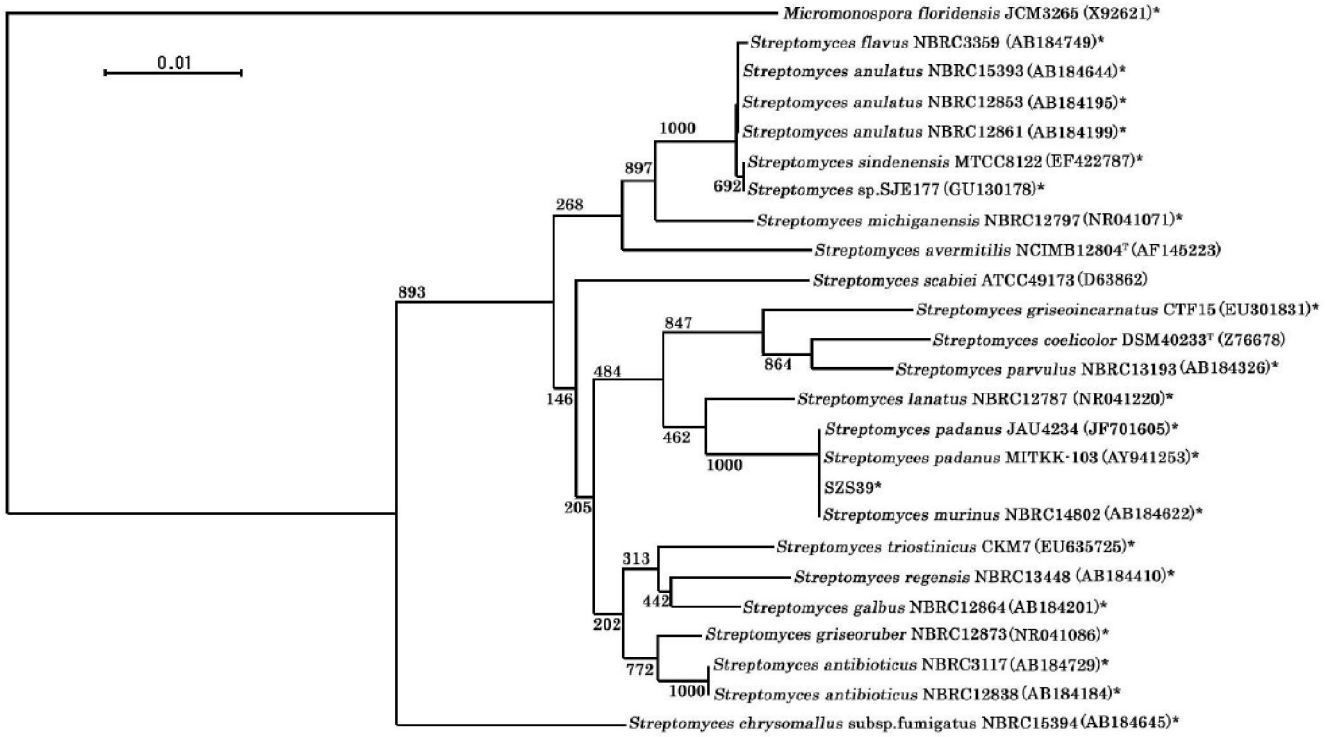

Figure 2 : Phylogenetic position of the strain SZS39 (*: actinomycin producer)

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The sequence of 16S rRNA gene from SZS39 was amplified by PCR method using universal primers and sequenced with automated capillary DNA sequencer. The 16S rDNA sequence of *Streptomyces* sp. SZS39 was deposited in DDBJ database under the accession number AB695291. The nearly complete 16S rRNA gene sequence of SZS39 was compared with 16S rRNA gene sequences of 3 representative *Streptomyces* species (*S. coelicolor*, *S. avermitilis*, and *S. scabiei*) and known actinomycin producers available in databases (Figure 2). So far, 16 S rRNA genes of 20 strains in *Streptomyces* genus and *Micromonospora floridensis*, which produces actinomycins have been deposited in database. As a result, the strain SZS39 was closely related to *Streptomyces padanus* MITKK-103 and JAU4234 with the high identity of 99 %. *S. padanus* MITKK-103 showed high production of actinomycins X_2 and D[7]. Its production pattern of actinomycins was similar to that of the strain SZS39. Recently, *Streptomyces padanus* JAU4234 was also reported to produce actinomycin X_2 along with new polyene macrolide antibiotic[8]. Although there are several clusters of actinomycin producers in this phylogenetic analysis, the actinomycin production seemed to be widely distributed over streptomycetes.

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