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Identification of a Newly Isolated Streptomycete, *Streptomyces* sp. SZK1, which Produces Polyene Macrolide Strevertenes

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***Streptomyces* sp. SZK1 was isolated from a soil in Japan using ISP2 agar medium. As a result of antimicrobial screening, the acetone extract of the strain SZK1 exhibited promising antimicrobial activities against *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe*. Isolation and identification of the antimicrobial principles from the strain SZK1 was accomplished. As a result of analysis using NMR and MS spectra, the main antimicrobial compound was identified as strevertene A. To identify the strain SZK1, a partial 16S rRNA gene sequence from the strain SZK1 was determined and found to have high identity (99%) with *Streptomyces racemochromogenes*.**

Key words: *Streptomyces*, Strevertene, HPLC, NMR Spectra, 16S rRNA gene.

The macrolides are a group of organic compounds which possess macrolide ring as carbon skeleton. Most macrolides are isolated as structurally diverse antibiotics such as erythromycin[1, 2] and produced by microorganisms via the polyketide biosynthesis.[3] A soil dwelling bacterium, streptomycete, is a potential producer of macrolide antibiotics. These macrolides exhibit a variety of biological activities including antitumor, antifungal, antiparasitic and immunosuppressant activities. Among the macrolides, strevertenes (Fig. 1) are antifungal pentaene macrolides isolated from *Streptoverticillium* sp. LL-30F848[4, 5]. We recently performed the screening for antifungal compound using newly isolated bacterial strains from field soil. As a result, we found newly isolated strain SZK1 as strevertenes producer. Here we describe isolation and identification of strevertenes from the extract and strain-identification of *Streptomyces* sp. SZK1 using 16S rRNA gene sequence analysis.

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MATERIALS AND METHODS

General Methods

NMR spectra were obtained with JEOL ECA-600 using DMSO-d_6 as solvent at 27.0°C. The resonance of residual DMSO at δH 2.49 was used as internal reference for ^{1}H NMR spectrum. 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), *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 Shizuoka University, Shizuoka Prefecture, Japan. The soil samples were spread onto ISP2 agar medium[6]. After 4-5 days of incubation at 30ºC, colonies developed were isolated and stored in a refrigerator at 80 °C. Total of 63 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 mg 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 SZK1 was performed according to the previous paper[7]. The 16S rRNA-encoding sequence was amplified from the total DNA by the PCR method with a set of universal primer pairs: 9F (52 -GAGTTTGATCCTGGCTCAG-32) and 1510R (52 -GGCTACCTTGTTACGA-32). PCR reaction was performed using EmeraldAmp PCR Master Mix (TakaraBio, Japan) following manufacture's instruction. PCR amplification was carried out using 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 included at the end of the 34 cycles. The PCR product was purified with a AxyPrep DNA Gel Extraction Kit (Axygen Bioscience, USA) following the manufacture's instruction. The reactions for sequencing were performed using a BigDye Terminator Cycle Sequencing Kit following the manufacture's instruction. Four primers were used for the reaction: 339F (52 -CTCCTACG GGTGAGTAACAC-32), 536R (52 -GTATTA CCGCGG CTGCTG-32), 686F (52 -TAGCGGTGAAATGCGTAGA-32), and 1099F (52 -GCAACGAGCGCAACCC-32). The sequencing was performed with the capillary DNA sequencer, ABI 3130 Genetic Analyzer (Applied Biosystems, USA).

Isolation of strevertenes

ISP2 agar medium (500 mL) was used for culture of *Streptomyces* sp. SZK1. 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 (Mitsubishi chemical, CHP20P) eluted with 10%MeOH, 60%MeOH, and MeOH. MeOH fraction was subjected to reversed-phase HPLC using ODS column (Nacalai Tesque, Cosmosil MSII 4.6 ´ 250 mm). Sequential two step elution was performed for HPLC separation; step A: isocratic elution for 5 min with the solvent system consisted of MeCN/H_2O/TFA (40:60:0.1) with 1 mL/min flow rate; step B: gradient elution for 20 min from MeCN/H_2O/TFA (40:60:0.1) to MeCN/H_2O/TFA (90:10:0.1) with 1 ml/min flow rate.

RESULTS AND DISCUSSION

The new bacterial strain SZK1 was isolated from the ground soil of Shizuoka University in Japan using ISP2 agar medium. The extract of strain SZK1 was subjected to antibacterial assay using 4 bacterial strains (*Bacillus subtilis*, *Escherichia coli*, *Saccharomyces cerevisiae*, and

Strevertene A: R_1=CH_3, R_2=CH_3
Strevertene B: R_1=CH_2CH_3, R_2=CH_3
Strevertene C: R_1=CH_3, R_2=CH_2CH_3
Strevertene D: R_1=CH_2CH_3, R_2=CH_2CH_3
Strevertene E: R_1=CH_3, R_2=$CH(CH_3)_2$
Strevertene F: R_1=CH_2CH_3, R_2=$CH(CH_3)_2$

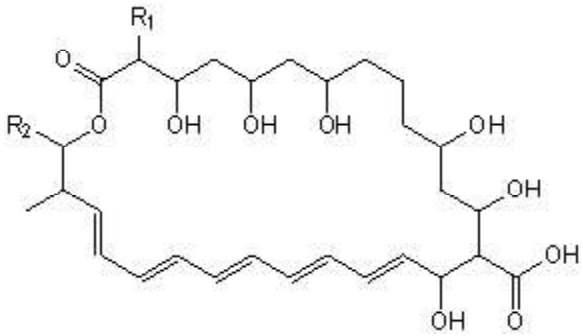

Fig. 1. Chemical structrures of strevertenes

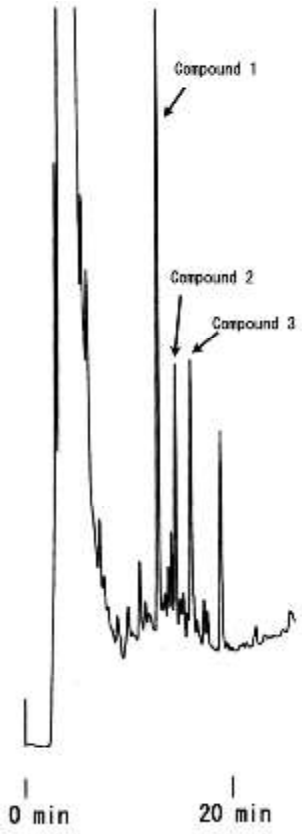

Fig. 2. HPLC chart of extract of *Streptomyces* sp. SZK1

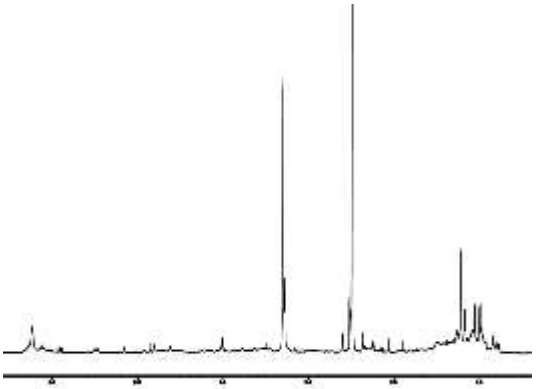

Fig. 3. ^{1}H NMR spectrum of strevertene A (in DMSO-d_6) from *Streptomyces* sp. SZK1

Schizosaccharomyces pombe). As a result, the extract showed intense antibacterial activity against *S. cerevisiae*, and *S. pombe*.

The culture of strain SZK1 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 hydrophobic resin (CHP20P) with H_2O/MeOH solvent system. As a result of antibacterial assay, MeOH fraction showed potent activity and further HPLC analysis was performed on the fraction.

On HPLC analysis, three major peaks at retention time of 11.7, 14.1 and 15.0 min were

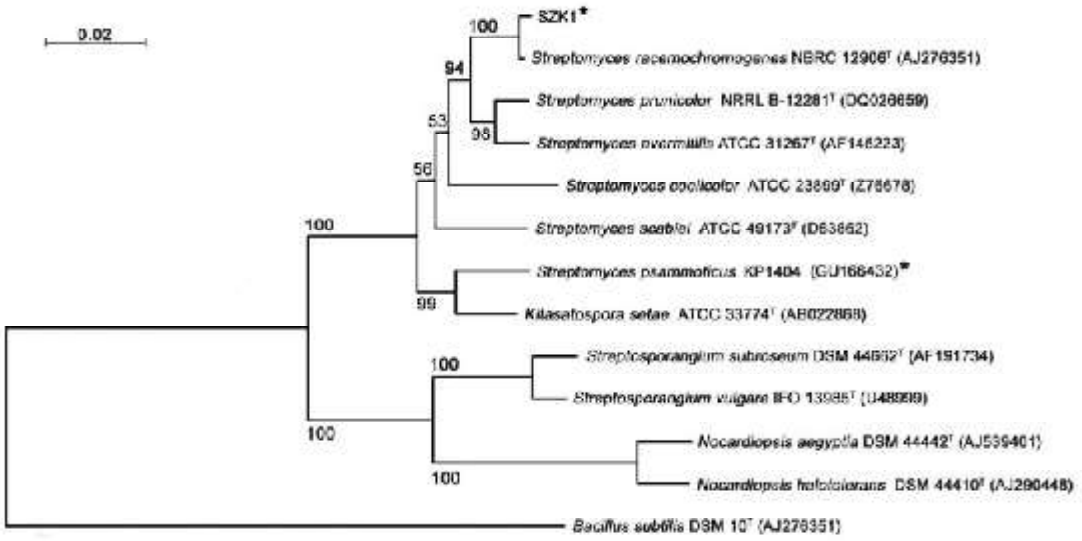

Fig. 4. Phylogenetic position of *Streptomyces* sp. SZK1 (astarisk indicates strevertene producer)

observed as shown in Fig. 2. The compounds 1, 2, and 3 showed characteristics of colorless powder after lyophylization. The ESI-MS spectrum of compound 1 (retention time 11.7 min in Fig. 2) showed the ion peak at 603.3. High resolution ESI-MS spectrum established the molecular formula of compound 1to be $C_{31}H_{48}O_{10}$ based on the HR-ESI-MS. Identification of compound 1 was accomplished by analysis of ^{1}H NMR spectrum data (Fig. 3). Since the ^{1}H NMR spectral data was identical with literature data, compound 1 was identified as strevertene A[4,5]. The ESI-MS spectrum of compound 2 and 3 (retention time 14.1 and 15.0 min in Fig. 2) showed the ion peak at 595.3 and 609.3, respectively. The compound 2 was deduced to be strevertene B or C by judging by the retention time and molecular weight. The compound 3 was deduced to be strevertene D or E in the same manner.

The sequence of 16S rRNA gene from SZK1 was amplified by PCR method using universal primers and sequenced with automated capillary DNA sequencer. The 16S rRNA gene sequence of *Streptomyces* sp. SZK1 was deposited in DDBJ database under the accession number AB770480. The nearly complete 16S rRNA gene sequence of SZK1 was compared with 16S rRNA genes of 12 strains in gram positive bacteria (Fig.4). As a result, the strain SZK1 was found to belong to *Streptomyces* genus. Especially 16S rRNA gene of SZK1 had the high identity (99%) with that of *Streptomyces racemochromogenes*. The identity of 16S rRNA gene sequence of SZK1 to that of strevertene producer *S. psammoticus* KP1404[8] was 95%. Although they produce strevertenes, strain SZK1 and KP1404 were not comparatively close in *Streptomyces* cluster (Fig. 4).

Strevertenes (Fig. 1) were originally isolated as antifungal compounds from *Streptoverticillium* sp. LL-30F848[4, 5]. Recently *S. psammoticus* KP1404 which produced strevertenes was isolated in Korea[8]. Promising disease control effect against tomato fusarium wilt was found by strevertenes produced by strain KP1404. In this report, we found newly isolated strain which produced strevertenes, which would also be promising disease control antagonistic agent.

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