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Kitasatospora sp. MJM383 Strain Producing Two Antitumor Agents, Streptonigrin and Oxopropaline G

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Abstract MJM383, a rare actinomycete sp. strain originated from Chinese soils, was isolated through an antimicrobial screening system. The analysis of 16S rDNA sequences and biochemical characterization determined the strain to belong to genus *Kitasatospora*. Both NMR and ESI mass data of its purified bioactive compounds revealed *Kitasatospora* sp. MJM383 to produce two antitumor agents, streptonigrin and oxopropaline G, which have been known to be produced from *Streptomyces* species. This is the first report to demonstrate the presence of antitumor agents produced by genus *Kitasatospora*.

Key words: *Kitasatospora* sp. MJM383, streptonigrin, oxopropaline, rare actinomycete, 16S rDNA sequence

Actinomycetes, filamentous Gram-positive bacteria, produce structurally diverse bioactive compounds having antineoplastic, immunosuppressive, antifungal, and antibacterial activities. Discovery of anticancer chemotherapeutics such as doxorubicin, mitomycin, geldanamycin, and actinomycin from *Streptomyces* has made much contributions to human health. Even though *Streptomyces* has been regarded as the richest microbial source for bioactive substances, repetitive functional screenings of general *Stereptomyces* spp. isolates led to an abrupt decrease in the chances of finding novel compounds. Therefore, target-directed screening and isolation of rare actinomycetes have been suggested as alternatives and successfully explored [7, 8, 10, 12]. In order to screen new antibacterial and antitumor compounds, we employed a target-directed screening to rare actinomycetes with combination of inhibitors. In the course of these endeavors, MJM383 strain producing two potent antitumor agents, streptonigrin and oxopropaline, was finally isolated. In this paper, the taxonomic characteristics of this isolate are described.

Soil samples were collected from terrestrial regions in Korea and China. Aliquots of serially diluted soil suspensions were plated onto humic-acid vitamin agar (HAV agar) containing 50 μg/ml each of novobiocin and cyclohexamide after heat treatment [8–10]. Following incubation on HAV agar at 28°C for 14 days, 978 isolates were obtained, and each pure isolate was maintained and stored on Benett's slant agar at 4°C. On-going antibacterial and antifungal screening program established a collection of strains producing potent biological activities. Among them, MJM383 was eventually selected for further studies, because of its broad spectra of antimicrobial activities to various indicator microorganisms. The strain MJM383 was obtained from a humus soil sample of a farmland of Guangzhou city in Guangdong Province of China. Its morphological and physiological characteristics were investigated by previously established methods [15, 22, 24]. Isolation of genomic DNA was carried out as described previously [19]. The 16S rDNA gene sequence was amplified with two primers, fD1 and rP2 [26]. An amplified fragment of the 16S rDNA gene was ligated into pGEM T-easy vector (Promega Co., Madison, WI, U.S.A.), and subjected to nucleotide sequence determination with sequencing primers that were designed for 16S rDNAs by Chun and Goodfellow [5]. The sequences of 16S rDNA gene were analyzed inthe GenBank database, and aligned reciprocally using the CLUSTAL W software [25]. The sequence homology values were calculated from the alignment, and evolutionary trees for the data sets were inferred from the neighbor-joining method using the program of MEGA [21].

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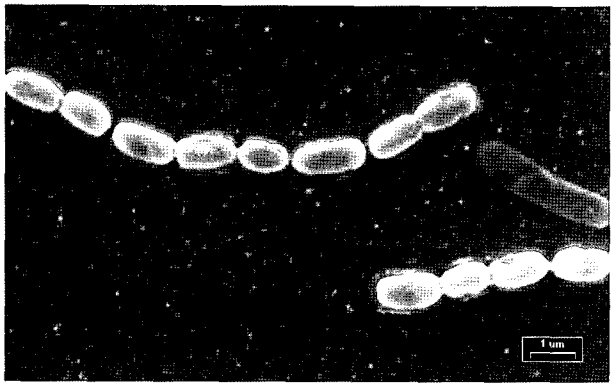

Fig. 1. Scanning electron micrographs (SEM) of the smooth surfaced spores of MJM383.
The microorganism was grown on ISP4 medium at 28°C for 14 days. Bar, μm.

Detailed biochemical and physiological characterization of the isolate were carried out as described previously [27, 28]. Morphological characteristics were examined by light microscopy (Olympus, BX40, Nashua, NH, U.S.A.) and scanning electron microscopy (JEOL, JSM-5410LV, Tokyo, Japan). Isomers of diaminopimelic acid (DAP) in whole-cell hydrolysates were determined by the TLC method [3, 24].

The strain MJM383 produced aerial mycelia consisting of 20 or more straight chains of smooth-surfaced rod-shaped spores (Fig. 1). The vegetative mycelia grown on Benett's agar were easily fragmented, and submerged spores were formed in the same broth media. It grew well on various organic media and produced abundant aerial mycelia. The spore mass was whitish-grey, and the reverse side of colonies was yellowish on most agar media. Most carbohydrates

Table 1. Morphological, physiological, and biochemical characteristics of *Kitasatospora* sp. MJM383. (a) Morphological characteristics of *Kitasatospora* sp. MJM383. All the characteristics were observed after 2 weeks of incubation at 28°C. (b) Physiological and biochemical characteristics.

(a)

Medium	Sporulation	Color on the reverse side
Yeast extract malt extract agar (ISP No. 2)	Abundant	Deep brown
Oatmeal agar (ISP No. 3)	Abundant	Grey
Inorganic salt-starch agar (ISP No. 4)	Abundant	Brown
Glycerol-asparagine agar (ISP No. 5)	Poor	Whitish
Peptone/yeast extract iron agar (ISP No. 6)	Moderate	Yellowish
Tyrosine agar (ISP No. 7)	Moderate	Whitish
Tryptic soy agar	None	Yellowish green
Potato dextrose agar	Moderate	White with deep brown
Nutrient agar	Poor	Yellowish
Bennett's agar	Abundunt	Grey
Starch casein	Moderate	Whitish

(B)

Characteristics	MJM383 strain
Aerial mycelia	Rectiflexibiles
Soluble pigment	Yellow to brown
Melanin formation	+
Nitrate reduction	−
Starch hydrolysis	+
Antibiotics produced	Streptonigrin and Oxopropaline G
Utilization of Carbohydrate	
L-Arabinose	+
D-Fructose	+
D-Glucose	+
D-Galactose	+
Inositol	+
D-Mannitol	+
Raffinose	+
Sucrose	+
D-Xylose	−
LL-DAP in whole cells	+ (submerged spores)
Galactose in whole cells	−

(+); positive, (−); negative.

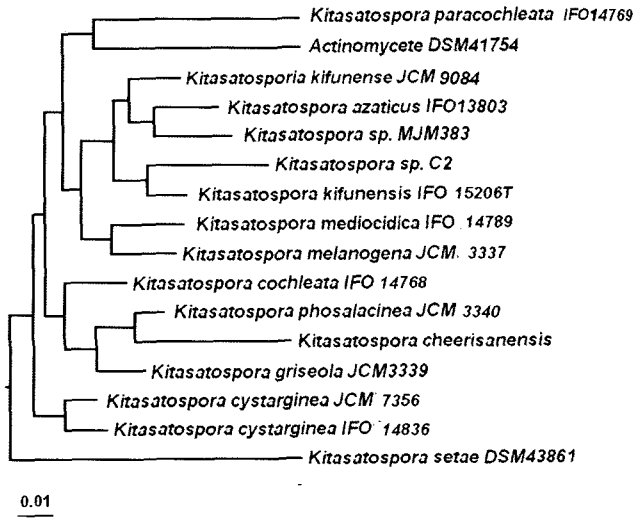

Fig. 2. Neighbour-joining tree, showing the position of MJM383 strain among its phylogenetic neighbors, through the analysis of 16S rDNA sequences.
The bar indicates 1% estimated sequence divergence.

were utilized by the strain MJM383, except D-xylose (Table 1). Analysis of its whole-cell hydrolysates revealed that LL-DAP was contained as a major constituent. Generally, the biochemical characteristics that distinguish *Kitasatospora* from *Streptomyces* are higher ratio of *meso*-diaminopimelic acid (DAP) to LL-DAP and the presence of galactose in the whole-cell hydrolysates [18, 31]. However, MJM383 is an atypical strain which contains LL-DAP and galactose [6]. The amplified and sequenced 16S rDNA gene fragment of MJM383 was 1,482 nucleotides in length. Corresponding sequence was analyzed using the BLAST N program in the NCBI (National Center for Biotechnology Information) site. The analysis showed that the strain MJM383 exhibited a high level of similarity to strains belonging to genus *Kitasatospora*. The phylogenetic position of the strain MJM383 was investigated with all of validly described species of the genus *Kitasatospora*, and the organism formed a monophyletic clade with the type strain *Kitasatospora azaticus* (Fig. 2). Nucleotide sequence similarity between MJM383 and *Kitasatospora azaticus* was 98.0%; however, there is no match between MJM383 and the related *Kitasatospora* species in phenotypic characterizations such as D-mannitol, D-fructose, and sucrose utilization pattern [6, 17].

To trace the bioactive substances produced by MJM383, the isolation of active compounds was conducted [4, 11, 13, 20, 23]. Thus, spores were inoculated into seed culture medium containing 50 ml of Bennett's broth media and then cultured for 48 h at 28°C in a shaking incubator at 220 rpm. Five ml of seed cultures was inoculated into each of 2-l flasks (×25) containing 500 ml of Bennett's broth. The fermentation was carried out for 120 h at 28°C. Each of the following purification procedures of broth cultures was guided by the antibacterial and antifungal assay, employing *Bacillus subtilis* ATCC 6633 and *Candida albicans* ATCC 10131 as respective indicator organisms. The culture broth was obtained by centrifugation and additionally filtered to remove residual cells. The supernatant was extracted with the same volume of ethyl acetate, and the organic phase was evaporated to dryness (1.54 g). The resulting powder was dissolved in 15% aqueous methanol, followed by filtration, and it was then evaporated at 30°C under reduced pressure to obtain a dark-brown powder (0.625 g). The resulting sample was loaded on a RP-18 reverse-phase open column (Kieselgel 60 RP-18, Merck, Darmstadt, Germany), and the column was eluted with water-methanol (50:50, 40:60, 30:70, 20:80, 10:90 and 0:100), acetone, and ethyl acetate in this order. Each fraction was collected, evaporated, and dissolved in methanol. The most active

Table 2. ^{1}H and ^{13}C assignments for 383-A and 383-B. (a) Comparison of ^{1}H- and ^{13}C-NMR data of the compound 383-A with those of streptonigrin [2] (b). Comparison of ^{1}H- and ^{13}C-NMR data of the compound 383-B with those of oxopropaline G [1].

(a)

Atom	Streptonigrin (THF-d8)		383-A ($CDCl_3$)	
	δ_C	δ_H	δ_C	δ_H (J, Hz)
C				
8	181.0	-	179.6	-
5	177.0	-	177.0	-
8'	165.8	-	164.5	-
2	161.2	-	160.0	-
4"	154.5	-	153.0	-
2"	149.6	-	147.2	-
3'	147.9	-	147.0	-
8a	145.4	-	144.2	-
7	141.6	-	139.4	-
5'	138.9	-	139.0	-
3"	138.4	-	137.4	-
6	13.75	-	136.3	-
4'	135.6	-	134.1	-
6'	133.7	-	131.7	-
2'	130.5	-	129.7	-
4a	127.9	-	127.0	-
1"	115.8	-	113.9	-
CH				
4	134.1	8.37	134.2	8.47 (d, 8.4)
3	126.3	9.01	125.2	8.69 (d, 8.4)
6"	125.4	6.72	124.9	6.80 (d, 8.7)
5"	105.2	6.68	105.2	6.68 (d, 8.7)
CH_3				
8"	56.1	3.88	56.0	4.00 (s)
7"	60.7	3.84	61.2	3.96 (s)
9	60.3	3.97	60.7	4.10 (s)
7'	17.5	2.38	17.3	2.50 (s)

Table 2. Continued.

(b)

Atom	Oxopropaline G (CD_3-OD)		383-B (CD_3OD)	
	δ_C	δ_H (*J*, Hz)	δ_C	δ_H (*J*, Hz)
C				
10	203.1	-	203.1	-
8a	143.2	-	143.2	-
9a	136.0	-	136.0	-
1	135.3	-	135.3	-
4	134.1	-	134.1	-
4a	131.1	-	131.1	-
4b	122.1	-	122.1	-
CH				
3	139.7	8.27 (s)	139.7	8.26 (s)
7	129.6	7.59 (dd, 7.1, 7.1)	129.6	7.59 (dd, 7.2, 8.0)
5	124.5	8.25 (d, 8.1)	124.5	8.24 (d, 7.2)
6	121.7	7.34 (dd, 8.1, 7.1)	121.7	7.33 (dd, 7.2, 7.2)
8	113.3	7.72 (d, 7.1)	113.3	7.71 (d, 8.0)
CH_2				
12	58.8	4.08 (t, 6.2)	58.8	4.08 (t, 6.2)
11	41.8	3.54 (t, 6.2)	41.8	3.54 (t, 6.2)
CH_3				
13	17.9	2.92 (s)	17.9	2.92 (s)

fractions from the RP-18 chromatography were combined, and further separated by a reverse-phase Prep-HPLC column (SymmetryPrep C18, 7.8×300 mm, Waters) with a mobile phase of water/acetonitrile (40:60) at a flow rate of 2.0 ml/min. After the preparative HPLC, we finally obtained 6.6 mg of purified compound of brown powder (named 383-A). The molecular weight of the compound 383-A was determined to be 506.2 by its ESI mass spectrum, having *m/z* 507.2 $(M+H)^+$ and 529.2 $(M+Na)^+$. Its ^1H spectral data (Table 2a) with ^1H-^1H COSY suggested the presence of two 1, 2, 3, 4-tetra substituted benzene rings, three methoxy groups (δ_H 4.10, 3H, s; δ_H 4.00, 3H, s; δ_H 3.96, 3H, s), and one aromatic methyl (δ_H 2.50, 3H, s), which were attached to an aromatic ring. The ^{13}C spectral data showed two carbonyl carbons (δ_C 179.6, and δ_C 177.0) attributable to a quinone, a carboxyl carbon (δ_C 164.5), and several oxygenated or nitrogenated aromatic carbons between δ_C 160 and δ_C 140. These spectral data showed characteristic signals of streptonigrin [2]. By comparison of mass, and ^1H- and ^{13}C-NMR spectral data with those of published values, the compound 383-A was identified to be streptonigrin. The other active compound was purified as a pale-yellow powder, yielding 383-B (2.4 mg). Its molecular weight was determined as 283.1 by ESI mass analysis with *m/z* 284.1 for $[M+H]^+$ and 306.1 for $[M+Na]^+$. Its ^1H spectral data (Table 2b) with ^1H-^1H COSY suggested the presence of 1,2-disubstituted benzene rings (δ_H 8.24, 1H, d, J=7.2; δ_H 7.71, 1H, d, J=8.0; δ_H 7.59, 1H, dd, J=7.2, 8.0; δ_H 7.33, 1H, dd, J=7.2, 7.2), an aromatic singlet proton (δ_H 8.26, 1H, s), one aromatic methyl (δ_H 2.92, 3H, s), and two conjugated methylenes (δ_H 4.08, 2H, J=6.2; δ_H 3.54, 2H, J=6.2). The compound 383-B was identified as oxopropaline G by comparison of MS, and ^1H- and ^{13}C-NMR spectral data with those of published values [1].

Antibacterial and antifungal activities were determined and examined as described previously (data not shown) [14, 16]. Streptonigrin, isolated from *Streptomyces flocculus*, is known to be an antibiotic for leukemia [14], and is the member of a group of antitumor agents that possess the aminoquinone moiety, such as lavendamycin, mitomycin C, porfiromycin, actinomycin, rifamycin, and geldanamycin. The oxopropaline complex (A to G) is also known to possess antitumor activity and is produced by *Streptomyces* sp. G-324 [16]. Even though these compounds do not have any novelty, it is worth mentioning that two antitumor agents are produced by two separate strains of *S. flocculus and Streptomyces* sp. G-324, but they were simultaneously produced by single strain MJM383 [Fig. 3].

It has been known that *Kitasatospora* spp., which are known to produce antifungal and antibacterial agents such as bafilomycin, cystargin, kimorexin, and setamycin, are closely related to *Streptomyces* at the genus level [6,

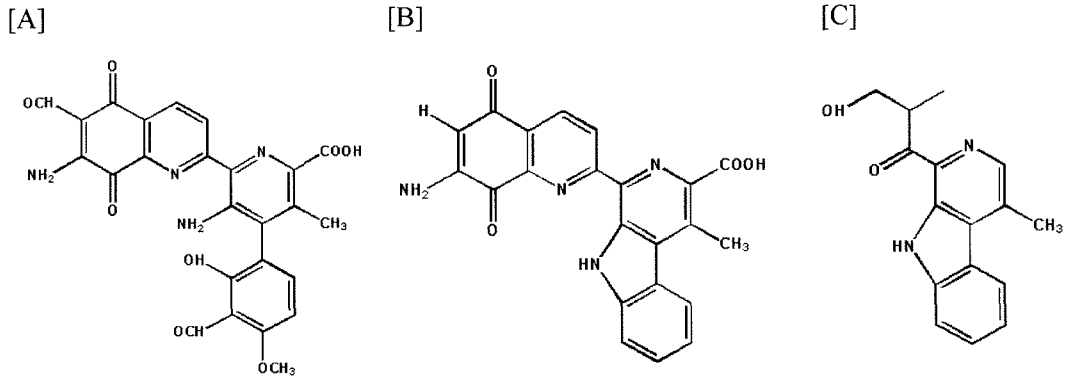

Fig. 3. Structures of streptonigrin (a), lavendamycin (b), and oxopropaline G (c).

15, 29, 30]. To the best of our knowledge, MJM383 is the first strain to show the presence of anticancer agents in genus *Kitasatospora*. Molecular genetic, biochemical, and physiological characteristics of the strain MJM383 revealed many distinctive features to indicate that it belongs to genus *Kitasatospora*. Herein, we established *Kitasatospora* sp. MJM383 as the producer of two antitumor agents, streptonigrin and oxopropaline G.

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