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Isolation and Identification of Newly Isolated Antagonistic *Streptomyces* sp. Strain AP19-2 Producing Chromomycins

Xue-Chang Wu*, Wei-Feng Chen, Chao-Dong Qian, Ou Li, Ping Li, and Yan-Ping Wen

Institute of Microbiology, College of Life Sciences, Zhejiang University, Hangzhou, Zhejiang 310058, P. R. China

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A new antagonistic strain of *actinomycete*, designated AP19-2, was isolated from the feces of giant pandas inhabiting the Foping National Nature Reserve in China. Cultural characteristic studies strongly suggested that this strain is a member of the genus *Streptomyces*. The nucleotide sequence of the 16S rRNA gene of strain AP19-2 evidenced profound similarity (97-99%) with other *Streptomyces* strains. Two pure active molecules were isolated from a fermentation broth of *Streptomyces* sp. strain AP19-2 via extraction, concentration, silica gel G column chromatography, and HPLC. The chemical structures of the two related compounds (referred to as chromomycin A_2 and chromomycin A_3) were established on the basis of their Infrared spectra (IR), High Resolution Electrospray Ionization Mass Spectrometry (HR-ESI-MS), and Nuclear Magnetic Resonance (NMR) data, and by comparison with published data.

Keywords: antagonistic, *Streptomyces* sp. AP19-2, chromomycin

Microbial secondary metabolites are important sources of natural compounds with potential therapeutic applications. As one type of versatile microorganisms, the streptomycetes are potent producers of secondary metabolites. Thus far, approximately 10,000 antibiotics have been identified, and almost half of them are generated by streptomycetes (Demain, 1999; Lazzarini *et al.*, 2000). A variety of bioactivities are associated with secondary metabolites generated by streptomycetes, including antibacterial, antifungal, antiviral, antitumor, and enzyme inhibitory activities.

Soil is the most common habitat for streptomycetes. After the initial few decades of intensive screening, which involved probably millions of microorganisms, it has become increasingly difficult to discover new microbes and bioactive metabolites in normal soil. However, a number of new actinomycete strains that generate active compounds have been recently isolated from novel sources, including saline, ocean, and endogenetic microbes of plants (Mukku *et al.*, 2000; Weber *et al.*, 2004; Chen *et al.*, 2006). These facts clearly indicate that valuable microorganisms can still be isolated, given the appropriate sample.

Foping National Nature Reserve is located in South China, and has been maintained in its intact primitive form for the past 300 years. *Bashania fargesii* and *Fargesta spathacea* grow readily there, and thus create an important habitat for giant pandas (*Ailuropoda melanoleuca*) (Yue *et al.*, 1999; Li and Zhao, 2005). Fresh panda fecal samples were recently collected in a giant panda census conducted in this area, and several antagonistic strains were isolated during a study of the diversity of the predominant microbes within the panda excreta. One of them (designated AP19-2) manifested prominent antibacterial and antitumor activities, and inhibited all the tested methicillin-resistant *Staphylococcus aureus* (MRSA) to a significant degree.

This report describes the isolation of actinomycete strain AP19-2 from the fecal samples of giant pandas. The identification of this strain and its extracellular activity have been assessed. The isolation, purification, and structural elucidation of two antibacterial and antitumor compounds purified from the culture broth are described herein.

Materials and Methods

Screening of antagonistic strains

Twenty-one fresh panda fecal samples were collected from the Foping National Nature Reserve. One gram of sample was diluted (10^{-2} to 10^{-5}) with sterile water and plated on Gause's synthetic agar and YG (1.0% yeast extracts, 1.0% glucose, pH 7.2) agar containing 0.01% potassium dichromate in order to minimize fungal contamination. The plates were then incubated for 14 days at 28°C. The cultures were maintained on Gause's synthetic agar. A 3 ml inoculum of each isolated strain precultured in nutrient broth for 24 h at 28°C was transferred to 30 ml YS medium [2.6% yeast extracts, 4.0% starch, 0.5% dextrine, 0.05% K_2HPO_4, 0.25% $MgSO_4 \cdot 7H_2O$, 0.4% $(NH_4)_2SO_4$, and 0.3% $CaCO_3$, pH 7.0]. Fermentation was allowed to occur by 4 days of incubation at 28°C on a rotary shaker at 180 rpm. The mature medium was then centrifuged at 3,000×g and the cell-free supernatant fluid was assessed for antimicrobial activities. Strain AP19-2, which manifested significant inhibitory activity against Gram-positive bacteria, was selected for further evaluation.

Identification of AP19-2

The morphological and cultural characteristics of strain AP19-2 were assessed in accordance with the method de-

* To whom correspondence should be addressed.
(Tel) 86-0571-88206627; (Fax) 86-0571-88206627
(E-mail) mblab@163.com

scribed by Shirling and Gottlieb (1966). Colors were assessed on ISCC-NBS Color Charts Standard Sample No. 2106 (Kelly, 1964). Cultural characteristics were observed on yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salt starch agar (ISP4), and glycerol asparagine agar (ISP5) after 14 days of culturing at 28°C. The morphological characteristics were assessed via light microscopy and scanning electron microscopy (SEM) of 7-day cultures grown on ISP4. The isomeric form of diaminopimelic acid (DAP) was determined in accordance with the methods developed by Staneck and Roberts (1974), and the whole-organism sugar pattern was analyzed via the procedure described by Saddler *et al.* (1991).

Genomic DNA was extracted in accordance with the methods described by Edwards *et al.* (1989). The PCR amplification of the 16S rRNA gene was conducted using two primers: 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGG AGGTGATCCAGCCGCA-3', as described by Weisburg *et al.* (1991). The sequence of the 16S rRNA gene (1,478 bp) was obtained using an Applied Biosystems DNA sequencer (model 3730) and deposited in the GenBank (EMBL) database under accession number EF466100. The 16S rDNA sequence was aligned with the related reference sequences retrieved from the DDBJ/EMBL/GenBank databases using the Clustal method (DNASTAR Inc., USA). The phylogenetic tree was constructed via the neighbor-joining algorithm (Saitou and Nei, 1987) based on the 16S rRNA gene sequences of strain AP19-2 and related organisms.

Extraction and purification of active compounds

Streptomyces strain AP19-2 was incubated in a 7.5 L NBS Bioflo 110 Fermentor for 60-72 h and a total of 15 L of culture were obtained. The cells were removed via centrifugation at 3,000×g. The supernatant fluid was extracted twice with an equal volume of ethyl acetate, and the combined organic layers were concentrated to 30 ml with a Rotavapor. The deep brown concentrated solution was applied to a silica gel G column (380×100 mm). The separation was conducted via isocratic elution with chloroform/methanol (92/8, v/v). Each 20 ml tube was collected and analyzed via thin-layer chromatography (TLC) using silica gel plates with a chloroform/methanol solvent system (90/10, v/v).

Compounds with identical retention factors (*Rf*) were combined and assayed for antibacterial activities, and two active fractions were acquired. After the chloroform/methanol was evaporated, a total of 1,326 mg of crude elution was obtained, including 926 mg of fraction 1 and 400 mg of fraction 2, with an *Rf*=0.27 and 0.31 in chloroform/methanol (90/10, v/v), respectively. The crude elution was recuperated in 5-10 ml of 50% methanol, and was further purified via HPLC (Waters: controller 600, pump 600). The column was a Waters C18 reverse-phase column (300×15 mm, 5 μm thick), and the detection wavelength was 280 nm. The column was eluted with a mixture of 45% acetonitrile and 55% acetic ammonium (20 mM, pH 4.0), with a flow rate of 8 ml/min. Each peak was collected and assessed for antibacterial activity. The active fractions were evaporated in order to remove acetonitrile, then freeze-dried with a VIRTIS ADVANTAGE vacuum freeze-drier.

The purity of the active compounds was assessed via by analytical HPLC (a SHIMADZU LC-6A equipped with a diode detector). The column used was a Diamonsil C18 reverse-phase column (250×4.6 mm, 5 μm thick). The elution was conducted with a mixture of acetonitrile and acetic ammonium (20 mM, pH 4.0) at a flow rate of 1 ml/min. Acetonitrile was increased in a gradient from 20% to 80% during elution.

Biological assay of in vitro activities

The *in vitro* antimicrobial activities of strain AP19-2 were assessed via the paper disc method. The Mueller-Hinton (30% meat extracts, 1.95% peptone, 0.15% starch) agar plates were inoculated with 0.1 ml of liquid medium containing the tested strains which had been precultured and diluted to the appropriate concentration (10^5/ml ultimately). A paper disc (6 mm in diameter) impregnated with 5 μl of culture filtrate was placed on the surface of the agar. The diameter of the inhibition zone was determined after 24 h of incubation at 30°C. The tested strains included *Staphylococcus aureus* CMCC(B)26003, *Bacillus subtilis* CMCC(B)63501, *Micrococcus luteus* CMCC(B)28001, *Bacillus pumilus* CMCC(B) 63202, *Escherichia coli* DH5a, *Pseudomonas aeruginosa* B9977, *Saccharomyces cerevisiae* ATCC 4226 and several methicillin resistant *Staphylococcus aureus* strains (B3892, B8208, B6172, 6189, and B4524). During the fermentation and purification of bioactive compounds, *S. aureus* CMCC(B)26003 was selected as the indicator organism.

In vitro antitumor activity was determined via sulforhodamine B assays in accordance with the method described by Skehan *et al.* (1990). The tested tumor strains included P388 mouse leukemia cells, BEL-7402 human liver cancer cells, and A-549 human lung cancer cells. In brief, different cell lines were seeded in 96-well plates at 5×10^4 cells/well. After 18-24 h, exponentially growing cells were exposed for 48 h to increasing concentrations of active compound (0.01-1 μmol/L), after which sulforhodamine B (SRB) was added. The plates were read at 540 nm using a FLUOstar OPTIMA microplate muti-detection reader (BMG Offenburg, Germany).

Spectroscopic measurements

The UV-visible absorption spectra of the pure fractions in methanol were determined with a SHIMADZU UV-2550 spectrometer. The infrared spectra were acquired using a NEXUS 870 PT-IR spectrometer. The ^1H and ^{13}C NMR data of the active compounds, in DMSO-d or in a mixed solvent system ($CDCl_3$-CD_3OH, 2:1), were recorded on a Bruker DMX-500 spectrometer at 27°C, and the concentrations were 10 mg/ml and 120 mg/ml, respectively. The high and low-resolution mass spectra were recorded on a Bruker APEX III and Bruker Esquire-LC spectrometer, respectively, equipped with an electrospray ion source (ESI, positive ion mode).

Results

Isolation and identification of strain AP19-2

During a study of the diversity of the predominant microbes in the excreta of giant pandas, actinomycetes strains were isolated and tested for antimicrobial activities. One of the isolated strains, referred to as AP19-2, was determined to

Table 1. The antibacterial activity of the culture filtrate of strain AP19-2 against five MRSA

Tested strains	Culture filtrate of strain AP19-2	Ampicillin	Peillin G	Ciprofloxacin Hydrochloride	Vancomycin
B3892	21.2	0	8.0	0	18.3
B8208	21.5	0	7.3	7.2	18.7
B6172	21.7	0	6.8	0	18.3
B6189	20.7	0	8.3	0	18.0
B4524	18.3	0	0	0	17.2

Antibacterial activity was expressed as an inhibition zone diameter (in mm). Each paper disc contained 0.5 μl culture filtrate of strain AP19-2, or contained 5 μg Ampicillin, Peillin G, Ciprofloxacin Hydrochloride respectively, or 30 μg Vancomycin.

Table 2. Cultural characteristics of strain AP19-2

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
ISP2	Mood	White	Dark greenish yellow	None
ISP3	Moderate	Light gray	Moderate greenish yellow	Brown
ISP4	Moderate	White	Dark greenish yellow	Olivine
ISP5	Moderate	White	Greenish yellow	Light green yellow
Gause's	Poor	White	Brown	None
Czapek's	Poor	White	Brown	None

have significant inhibitory activity against all tested typical Gram-positive strains and several clinic MRSA (Table 1), and was therefore selected for the strain identification and isolation of its bioactive metabolites.

Strain AP19-2 grew on a range of agar media, evidencing the typical morphology of *Streptomycetes* (Table 2). The substrate mycelium was brown or greenish-yellow. The aerial mycelium tended to be white or gray and differentiated into flexuous chains with smooth surface spores (Fig. 1). The organism generated a brown or olivine diffusible pigment on several media. The AP19-2 strain utilized D-fructose, D-galactose, D-glucose, maltose, mannitose, L-rhamnose, and D-xylose as sole carbon sources, but not L-arabinose, raffinose, or sucrose. Tests for nitrate reduction and cellulose and L-tyrosine degradation were positive. Tests for milk litmus coagulation and sulfurated hydrogen were negative. The AP19-2 strain could grow on YG media at 10 to 40°C or at pH 5.0 to 10.0. The AP19-2 strain tolerated up to 10% NaCl. LL-DAP, glucose, and ribose were detected in the whole-cell hydrolysates, but no characteristic sugars were detected. Thus, strain AP19-2 was determined as cell wall type I and sugar type C. The comparison of the biochemical and morphological characteristics of strain AP19-2 with those of the actinomycetes described in Bergey's manual of systematic bacteriology (Buchanan and Gibbons, 1974), strongly indicated that it belonged to the genus *Streptomyces*.

These results were clearly verified by the results of 16S rDNA sequence analysis. The 1,478 bp 16S rDNA sequence was determined with two putative primers. The alignment of this sequence with the 16S rDNA sequences retrieved from the DDBJ/EMBL/GenBank databases evidenced a high degree of similarity (97-99%) with the *Streptomyces* 16S rRNA genes. The phylogenetic tree was constructed (Fig. 2) on the basis of the 1,458bp 16S rRNA gene sequences of the AP19-2 strain and the closely related *Streptomyces* strains.

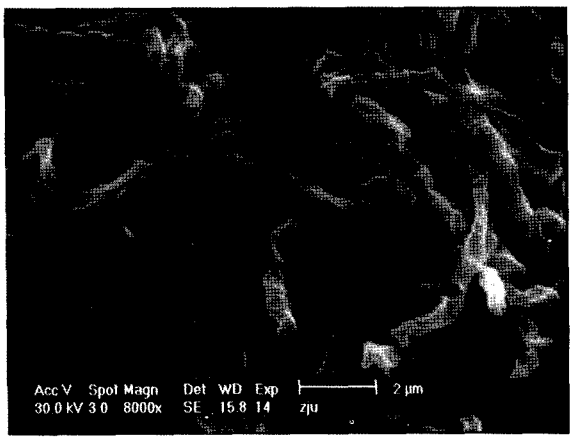

Fig. 1. The electron scanning micrograph of the spore chains of AP19-2 (×8k).

The closest similarity value was obtained between AP19-2 and its neighbors, including *Streptomyces griseorubiginosus* NBRC 13047^T, *Streptomyces alboviridis* NBRC 13013^T, *Streptomyces globosus* NBRC 15874^T, *Streptomyces microflavus* NBRC 13062^T, and *Streptomyces fulvorobeus* NBRC 15897^T. All of these results indicated that strain AP19-2 was a member of genus *Streptomyces*.

Purification and structure elucidation of the active compounds

Mature AP19-2 medium was isolated via extraction, concentration, silica gel G column chromatography, and HPLC, and two active compounds with high purity wereacquired, and designated as AP19-2a and AP19-2b. The retention time of AP19-2a was 20.25 min on a reverse-phase column

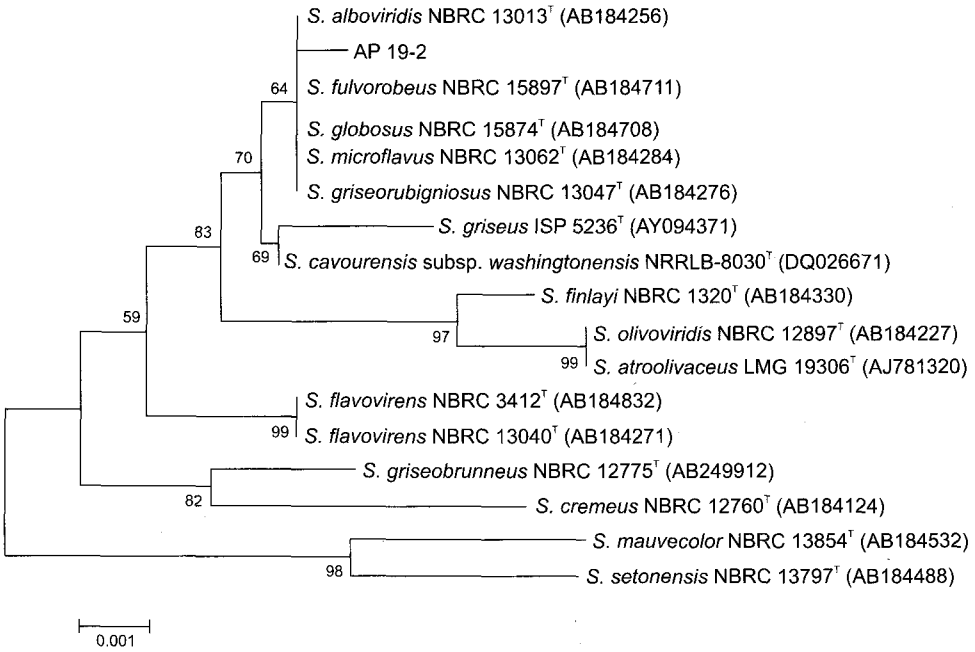

Fig. 2. Phylogenetic tree based on 1,458 bp 16S rRNA gene sequences from strain AP19-2 and related organisms. The tree was constructed using the neighbor-joining algorithm. Bootstrap values (>50%) based on 1,000 replications are shown at the nodes of the tree. Bar, 0.001 substitutions per nucleotide position.

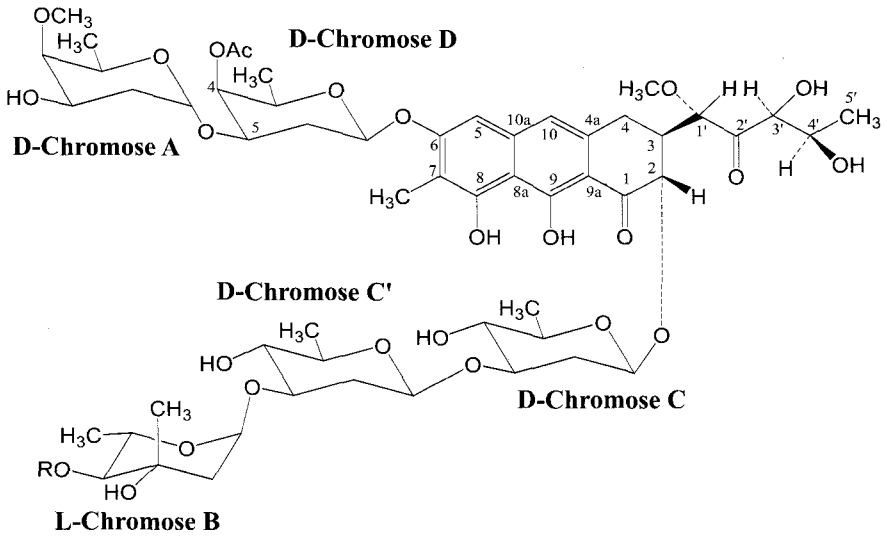

Fig. 3. The structure of AP19-2a (chromomycinA_3, R=Ac) and AP19-2b [chromomycineA_2, R=$COCH(CH_3)_2$].

(Diamonsil C18) with a purity of 99.12%, whereas that of AP19-2b was 34.72 min with a purity of 97.72%. A total of 196 mg of AP19-2a and 85.5 mg of AP19-2b were ultimately obtained from 15 L of medium. The maximum UV adsorption of the two antibiotics was observed at 279 nm in methanol. Their ^1H NMR, ^{13}C NMR, UV, IR, and MS data were studied and compared with the published data, and finally AP19-2a and AP19-2b were identified as chromomycin A_3 and chromomycin A_2 (Fig. 3).

Compound AP19-2a was obtained as a yellow amorphous powder. Its molecular formula was established as $C_{57}H_{82}O_{26}$ by HR-ESI-MS at *m/z* 1205.5001 $[M+Na]^+$ (calcd 1205.4992). Strong IR absorption bands at 3427 and 1727 cm^1 were attributable to hydroxyl and carbonyl, respectively. In accordance with the molecular formula, 57 carbon signals comprising 13 quaternary carbons, 26 tertiary carbons, six secondary carbons, and 12 methyls were observed in the ^{13}C NMR and DEPT spectra. A comparison of the spectral data with the published data (Miyamoto *et al.*, 1967; Yoshimura *et al.*, 1988) showed that compound AP19-2a was chromomycin A_3. The 2D-NMR spectrum (^1H-^1H COSY, HMQC, HMBC, and NOESY) verified this conclusion. The inhibitory rate

of AP19-2a against P388 mouse leukemia cells, BEL-7402 human liver cancer cells and A-549 human lung cancer cells was up to 81.6%, 92.3%, and 100%, respectively, at a concentration of 10^{-8} mol/L, which was consistent with the profound antitumor effects of chromomycin A_3 described in the previously published data (Menendez *et al.*, 2006).

Compound AP19-2b was also a yellow amorphous powder. The positive HR-ESI-MS evidenced a molecular formula of $C_{59}H_{86}O_{26}$ (*m/z* 1233.5309 $[M+Na]^+$, calcd 1233.5305), which was Δm 28 higher than that of AP19-2a. The ^1H and ^{13}C NMR spectra were much closer to those of AP19-2a, which indicated that compound AP19-2b was an analogue of AP19-2a. The difference between AP19-2b and AP19-2a was ascribable to the difference in the 4-*O*-acyl group. The interpretation of the ^1H NMR, ^{13}C NMR, and 2D-NMR data showed that the 4-*O*-acyl group was an isobutyryl group. The comparison of the spectral data with the published data (Miyamoto *et al.*, 1967; Yoshimura *et al.*, 1988) identified compound AP19-2b as chromomycin A_2.

Discussion

According to the sequence alignment and phylogenetic tree based on the 16S rRNA genes, *Streptomyces* AP19-2 was closest to *S. microflavus* NBRC 13062^T, *S. griseorubiginosus* NBRC 13047^T, *S. alboviridis* NBRC 13013^T, *S. globosus* NBRC 15874^T, and *S. fulvorobeus* NBRC 15897^T. However, there are many differences between strain AP19-2 and these strains according to the published data (Buchanan and Gibbons, 1974). Strain AP19-2 generates chromomycins, whereas *S. microflavus* NBRC 13062^T generates streptothricin, and no antibiotics are produced by the latter four streptomyces. Strain AP19-2 forms a white aerial mycelium on oatmeal agar, which can be distinguished from *S. microflavus* NBRC 13062^T (light yellow), *S. griseorubiginosus* NBRC 13047^T (light grayish reddish brown), *S. alboviridis* NBRC 13013^T (white or light yellow), *S. globosus* NBRC 15874^T (dark gray), and *S. fulvorobeus* NBRC 15897^T (gray). Additionally, the spore chain of strain AP19-2 is flexuous, whereas that of *S. fulvorobeus* NBRC 15897^T is spiral; organism AP19-2 endures a 10% concentration of NaCl, whereas *S. griseorubiginosus* NBRC 13047^T and *S. microflavus* NBRC 13062^T endures NaCl concentrations of 5% and 7%, respectively; strain AP19-2 can degrade L-tyrosine and reduce nitrate, whereas *S. alboviridis* NBRC 13013^T cannot; strain AP19-2 can utilize L-rhamnose but not L-arabinose, whereas *S. globosus* NBRC 15874^T can utilize L-arabinose but not L-rhamnose. These results indicate that strain AP19-2 is different from the above-mentioned type strains.

Although *Streptomyces cavourensis* subsp. *washingtonensis* ATCC 27732^T, *Streptomyces griseus* ATCC 13273, and strain AP19-2 are placed on different branches of the phylogenetic tree, the former two strains can also generate chromomycins (Buchanan and Gibbons, 1974; Lombo *et al.*, 2006), in a fashion similar to that of *Streptomyces* AP19-2. Comparison of the cultural and biochemical characteristics of *Streptomyces* AP19-2 with those of strains ATCC 27732^T and ATCC 13273 (purchased from American Type Culture Collection, ATCC) under identical culture conditions, indicates that three strains are quite close (data not shown). However, there are some differences between *Streptomyces* AP19-2 and the other two strains. After 14 days of culture at 28°C, ATCC 27732^T generated a diffuse grayish-yellow pigment on ISP2 and ISP4 media, but *Streptomyces* AP19-2 generated a similar diffusible pigment only on ISP4 medium; *Streptomyces* AP19-2 produced a diffuse light greenish-yellow pigment on ISP5 medium, whereas ATCC 13273 produced no pigment on the same medium. Additionally, *Streptomyces* AP19-2 could utilize L-rhamnose, whereas the other two could not.

In conclusion, our studies show that *Streptomyces* AP19-2 is quite similar to *S. cavourensis* subsp. *washingtonensis* ATCC 27732^T, and *S. griseus* ATCC 13273. However, there are some differences between *Streptomyces* AP19-2 and the other two strains. Whether AP19-2 is a new *Streptomyces* strain or another previously identified *Streptomyces* strain must be determined by other tests in the future.

Previous studies have suggested that the known microorganisms occupy only 0.1% of the microbial resources in soil (Amann *et al.*, 1995). Valuable microorganisms can still be isolated, given the appropriate soil sample. It is well known that many microbes can be isolated from the excreta of animals. Despite the fact that the microorganisms in the metabolites of wild animals ultimately derive from the soil of the forest, the micro-environment of animal feces is idiosyncratic, and the predominant composition of microbial species is somewhat different from that of the nearby soils. Certain strains of rare microbes in soil may be easily obtained from the metabolites of wild animals. This can be demonstrated by the isolation of *Streptomycetes* strain AP19-2 from the feces of giant pandas. Although chromomycin A_2 and A_3 generated by strain AP19-2 are not new compounds, our work indicates that the feces of wild animals may constitute a new source of microorganisms with pharmaceutical value.

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