Original Article

Taxonomic identification of a novel strain of *Streptomyces cavourensis* subsp. *washingtonensis*, ACMA006, exhibiting antitumor and antibacteria activity

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ABSTRACT: Taxonomically diverse and genetically specialized, marine microorganisms have great potential in generating bioactive substances. A previous study isolated a novel actinomycete strain designated ACMA006 and revealed that the fermentation broth of ACMA006 (FBA6) significantly inhibited the growth of a series of tumor cell lines. The present study examined various characteristics of the ACMA006 strain, including its morphological, physiological, and biochemical nature, and the 16S rDNA gene sequence of ACMA006 and biological activity of FBA6. The ACMA006 strain grew at an optimal temperature of 28°C on nearly all media tested, except for Czapek's agar, producing an exuberant substrate and aerial hyphae. Phylogenetic analysis showed that the 16S rDNA gene sequence of ACMA006 was closely related to that of *Streptomyces cavourensis* subsp. *washingtonensis*, with a sequence similarity of nearly 100%. However, ACMA006 differed somewhat from *Streptomyces cavourensis* subsp. *washingtonensis* in terms of its morphological, physiological, and biochemical characteristics. According to a bioactivity assay, FBA6 strongly inhibited the growth of hepatocellular carcinoma cell line HepG2, while it was weakly cytotoxic to human normal hepatocytes LO2 according to an MTT assay. In addition, the growth of bacterial strains *Bacillus subtilis* and *Staphylococcus aureus* but not *Escherichia coli*, *B. aerogenes*, *Pseudomonas fluorescence*, and *B. proteus* was significantly suppressed by FBA6 as indicated by the filter paper disc method. Results of this study indicated that the strain ACMA006 represents a new strain of the *Streptomyces cavourensis* subsp. *washingtonensis* and that the active metabolites of this strain are candidates for utilization as anticancer or antibacterial agents.

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1. Introduction

Compounds from marine sources exhibit a variety of bioactivity against tumors, inflammation, allergies, viral infections, and the like (*1*). Searching among metabolites of marine microorganisms for new drugs has proven to be an important approach to current drug development. Many agents derived from these metabolites have been used clinically, *e.g.*, vidarabine is active against herpes simplex and varicella zoster viruses (*2*) and cytarabine is a chemotherapy agent used mainly in the treatment of hematological malignancies (*3*).

Actinomycetes are widely distributed in terrestrial and aquatic ecosystems and especially in soil, where they play a crucial role in the recycling of refractory biomaterials (*4*). Actinomycetes, and particularly species in the genus *Streptomyces*, are widely known to be prolific producers of bioactive compounds, including antitumor and antibacterial agents (*5*). Approximately 45% of the bioactive secondary metabolites produced by microorganisms are reportedly found in the metabolites of actinomycetes (*6*). Therefore, Actinomycetes have great potential in the areas of drug research and development.

A previous study isolated ACMA006, a marine actinomycete, in the laboratory (*7*). The fermentation broth of ACMA006 (FBA6) was found to be significantly cytotoxic to several cancer cell lines such as human hepatoma cell line SMMC 7721, colorectal cancer cell line Lovo, cervical cancer cell line Hela, and mouse myeloma cell line SP2/0 (*7*). However, the taxonomy of ACMA006 was not readily apparent and the wide-ranging biological activity of FBA6, and especially its cytotoxicity to normal cells, needed to

be clarified. The current study examined the cultural, morphological, physiological, and biochemical characteristics and chemotaxonomy of ACMA006. The 16S rDNA gene sequence of ACMA006 was also analyzed and compared with that of *Streptomyces cavourensis* subsp. *washingtonensis*. In addition, this study ascertained the bioactivity of FBA6, including inhibition of growth of both hepatoma cell line HepG2 and normal hepatocytes LO2, and certain bacterial strains.

2. Materials and Methods

2.1. *Materials*

The actinomycete strain ACMA006 was isolated from the marine mud collected in Lianyungang harbor, Jiangsu, China, in a previous study (*7*). The method of isolating ACMA006 has been patented under Chinese patent No. ZL200710025156.5. The strain was preserved in liquid paraffin in the lab and China General Microbiological Culture Collection Center (CGMCC) (No. 2027).

Fermentation broth of ACMA006 (FBA6) was prepared as described before (*8*). Passaged from Gause's agar, the strain was inoculated in Gause's fermentation medium and cultivated for 4 h at 28°C. Then, the strain was inoculated in 2216E fermentation medium and cultivated for 6 days at 28°C. The broth was harvested, disrupted using ultrasound, and centrifuged at 10,000 × g for 10 min at 4°C. The supernatant was removed, filtered with a 0.22-μm filter, and stored at –20°C for further study.

2.2. *Cell and/or bacterium culture*

Human hepatoma cells HepG2 and normal hepatocytes LO2 were obtained from American Type of Cell Culture (Manassas, Virginia, USA). HepG2 and LO2 cells were maintained in RPMI-1640 media supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin-streptomycin (100 IU/mL-100 μg/mL), 2 mM glutamine, and 10 mM HEPES buffer at 37°C in a humid atmosphere (5% CO_2-95% air) and were harvested by brief incubation in 0.02% (w/v) EDTA in PBS (ICN, Aurora, USA) (*25*).

Bacterial strains employed in the current study including *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, *Proteus vulgaris*, and *Pseudomonas fluorescens* were obtained from American Type of Cell Culture (Manassas, Virginia, USA). All strains except *Pseudomonas fluorescens* were cultured in nutrient broth (Becton Dickinson. Cockeysville, MD, USA) at 37°C. *Pseudomonas fluorescens* was cultured in Pseudomonas CFC agar (Becton Dickinson. Cockeysville, MD, USA) and incubated at 28°C.

2.3. *Cell growth assay*

Cells (5×10^3 per well) seeded in 96-well plates for 24 h were exposed to adriamycin (positive control) or different doses of FBA6 for 96 h at 37°C in a humidified 5% CO_2 atmosphere. The medium was then removed and the cells were washed with PBS. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20 μL MTT (5 mg/mL, Sigma-Aldrich, St Louis, MO, USA) for 4 h. Light absorbance of the solution was measured at 492 nm on a microplate reader (PerkinElmer, Waltham, MA, USA) (*9*). Experiments were performed in triplicate with three samples.

2.4. *Bacteria growth assay*

Antibacterial activity of FBA6 was screened for using the filter paper disc method (*10*). A previously liquefied medium appropriate for the test was inoculated with the requisite quantity of the suspension of microorganisms. The suspension was added to the medium at a temperature between 40-50°C and the inoculated medium was immediately poured into dried Petri dishes to fill a depth of 3 to 4 mm. Paper discs (Whatmann No.2) were cut into smaller discs (6-mm diameter), sterilized at 180°C for 30 min in a hot air oven, and then impregnated with FBA6. The dried discs were placed on the surface of the medium. For strains *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, and *Proteus vulgaris*, the incubation temperature was 37°C, while for *Pseudomonas fluorescens* the incubation temperature was 28°C. The incubation time for all of the bacterial strains was 16-18 h. The diameter of the circular inhibition zones was subsequently measured.

2.5. *Cultural and physical characteristics*

Cultural properties of the strain ACMA006 were evaluated as described by Shirling *et al*. and Locci (*11,12*). ACMA006 was inoculated onto Gause's synthetic agar, glucose asparagine agar, Czapek's agar, inorganic salts/starch agar, glucose-yeast extract agar, Emerson agar, nutrient agar, and glycerol-extractum carnis-peptone agar and incubated at 28°C for 7-10 days to investigate growth. The colors of substrate mycelium, aerial mycelium, spore mycelium, and soluble pigment produced by ACMA006 were also recorded after 14 days of incubation on different agars.

Physical characteristics of ACMA006, including the carbon source utilization pattern and optimal growth temperature requirements, were determined as follows. Utilization of carbon sources was tested by growth on carbon utilization medium (ISP 9) supplemented with 1% carbon sources at 28°C for 2 weeks (*13*). Temperature ranges for growth were determined on

2216E medium (Sigma, USA) using a temperature gradient incubator (Toyo Kagaku Sangyo, Tokyo, Japan).

2.6. *Morphological characteristics*

Substrate mycelium and aerial mycelium morphology of ACMA006 were viewed under a light microscope (Olympus BH-2; Olympus, Tokyo, Japan) after 7 days of incubation on Gause's synthetic agar at 28°C. Morphology of the spore chain and the spore surface ornamentation was examined using scanning electron microscopy (JSM-6390LV; JEOL Ltd., Japan) after 14 days of incubation on Gause's synthetic agar (*14*). Samples for scanning electron microscopy were prepared by fixing the agar block in osmium tetroxide vapor for 12 h, dehydration by critical-point drying, and then sputter coating with palladium under a vacuum.

2.7. *Biochemical properties assay*

Biochemical properties of ACMA006 were determined in terms of the following aspects: Gram staining, acid-inhibition staining, decomposition of starch, reduction of nitrate, gelatin hydrolysis, growth on cellulose, coagulation of milk, peptonization of milk, production of hydrogen sulfide, and production of melanoid pigment. Biochemical characteristics were tested using standard techniques from the *Manual for identification of Streptomyces* (*15*).

2.8. *Chemotaxonomic characterization*

Isomers of diaminopimelic acid (DAP) in whole-cell hydrolysates were determined using thin-layer chromatography (TLC) according to the methods of Becker *et al*. and Hasegawa *et al*. (*16,17*). Mycolic acids and whole-cell sugars were analyzed using high-performance liquid chromatography (HPLC) according to the method of Butler *et al*. (*18*).

2.9. *16S rDNA gene-based phylogenetic analysis*

Total DNA was extracted according to the method of Orsini *et al*. (*19*). The 16S rDNA gene was amplified by PCR using the following primers: Primer A, 5′-ATCCTGGCTCAGGACGAACG-3′; Primer B, 5′-GAGGTGATCCCGCCGCACCT-3′. Programmable temperature cycling was performed with the following cycle profile: 95°C for 5 min and then 30 cycles, with each cycle consisting of denaturation for 1 min at 95°C, annealing for 1 min at 58°C, and extension for 1.5 min at 72°C. After 30 cycles, the reaction tubes were stored for 10 min at 72°C and then at 4°C. Samples were electrophoresed in gels containing 1% agarose (FMC; Rockland, ME, USA) and sequenced by Invitrogen (Shanghai, China). The 16S rDNA gene sequences of ACMA006 were aligned using Clustal X1.8. Sequences of the other members of the genus *Streptomyces* used for alignment and calculation of similarity levels were obtained from the GenBank and EMBL database using the BLAST program. Phylogenetic analyses were performed with MEGA3.1. Evolutionary trees were constructed according to the neighbor-joining method and Kimura's two-parameter model (*20,21*).

2.10. *Statistical analysis*

Data were analyzed using a Student's two-tailed *t*-test and are presented as the mean ± S.D. The limit of statistical significance was $p < 0.05$. Statistical analysis was done with SPSS/Win11.0 software (SPSS, Inc., Chicago, Illinois, USA).

3. Results

3.1. *Cultural and physical characteristics*

Table 1 shows the cultural characteristics of ACMA006. It grew vigorously on glucose-asparagine agar, inorganic salts-starch agar, glucose-yeast extract agar, Emerson agar, nutrient agar, and glycerol-extractum carnis-peptone agar. It also grew well on Gause's synthetic agar but poorly on Czapek's agar. Generally, the colors of the substrate mycelium and aerial mycelium were yellowish, yellow, or brown on all of the tested media. The spore mycelium was white, pale yellow, or yellow in color. Yellow or brown soluble

Table 1. Cultural characteristics of the strain ACMA006 on various media

Culture media	Growth status	Substrate mycelium*	Aerial mycelium*	Spore mycelium*	Soluble pigment*
Gause's synthetic agar	Well	Y to B	W to pale Y	W to pale Y	Y to B
Glucose-asparagine agar	Vigorous	W to pale Y	W to pale Y	Y	Y
Czapek's agar	Poor	W to lemon Y	W to pale Y	W to pale Y	Light Y to Y
Inorganic salts-starch agar	Vigorous	W to pale Y	W to pale Y	Lemon Y	Light Y
Glucose-yeast extract agar	Vigorous	Ivory W to lemon Y to Y	Ivory W to lemon Y to Y	W	W to pale Y
Emerson agar	Vigorous	Light Y to Y	W to pale Y	W to pale Y	W to pale Y
Nutrient agar	Vigorous	Yellow to golden yellow	W to pale Y	W to pale Y	Y
Glycerol-extractum carnis-peptone agar	Vigorous	Y to D	Y to D	W to pale Y	Y

* Y: yellow; B: brown; W: white; D: dark brown.

pigments were produced on Gause's synthetic agar, glucose-asparagine agar, nutrient agar, and glycerol-extractum carnis-peptone agar. White or yellowish soluble pigments were observed on the other culture media.

Glucose, fructose, arabinose, mannitol, glycerol, and sodium succinate were thought to be the main carbon sources used by ACMA006 (Table 2). Xylose and glycerol were mildly or moderately utilized, while sucrose, rhamnose, inositol, and sorbitol were not employed as carbon sources. The temperature range for growth was 10-40°C, whereas the optimal culture temperature was 28°C (data not shown).

3.2. *Morphological characteristics*

Both aerial mycelium and substrate mycelium were amply produced in this strain. The diameter of the mycelium was about 0.8 μm. The substrate hyphae appeared earlier than aerial hyphae and had thin and elongated features (Figure 1A). The septum developed in the substrate hyphae at the incubation time of 36 h at 28°C, and fragmentation was apparent after day 4. The strain produced aerial mycelia that consisted of long straight or flexuous chains of spores with smooth surfaces (Figure 1B). The spores were formed when spore chains fragmented at the sites of septa and had an oval or columnar appearance.

3.3. *Biochemical characteristics*

The biochemical properties of ACMA006 are summarized in Table 3. Among the experiments, strongly or moderately positive results were obtained in the decomposition of starch, reduction of nitrate, Gram staining, coagulation of milk, and peptonization of milk, whereas negative results were obtained in acid-inhibition staining, gelatin hydrolysis, growth on cellulose, production of hydrogen sulfide, and production of melanoid pigment.

3.4. *Chemotaxonomy*

L,L-DAP and glycine were detected in whole-cell hydrolysates of ACMA006 cells (data not shown), suggesting that ACMA006 has a type I cell wall (*22*). In addition, glucose and rhamnose in the C glycoform were found in whole-cell hydrolysates but characterized sugars were not (data not shown). Mycolic acid was not detected.

3.5. *16S rDNA gene analysis*

The complete 16S rDNA gene sequence was determined for the strain ACMA006. The 16S rDNA gene sequence of the ACMA006 strain was 1,507 bp in length. A neighbor-joining tree was generated according to the

Table 2. Utilization of carbon sources in the strain ACMA006

Carbon source	Results*
Glucose	+
Fructose	+
Xylose	Moderate
Sucrose	–
Rhamnose	–
Arabinose	+
Glycerol	Mild
Mannitol	+
Inositol	–
Sorbitol	–
Glycerol	+
Sodium succinate	+

* +, positive; –, negative.

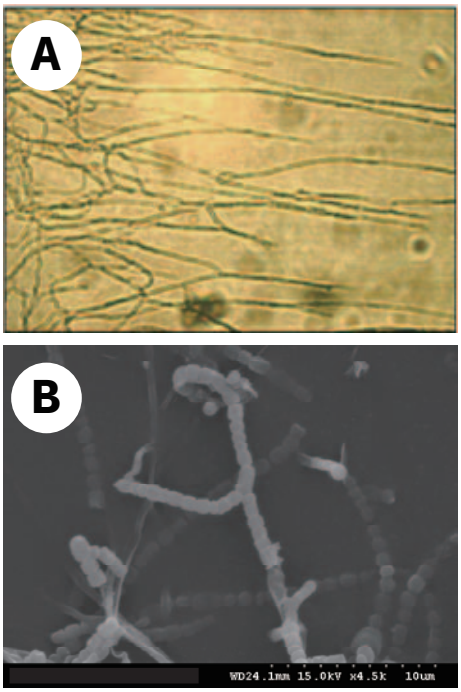

Figure 1. Morphological observation of the strain ACMA006. (**A**) A typical photograph of the strain ACMA006 under a light microscope (400×). (**B**) A typical photograph of spores under a scanning electron microscope (4,500×).

Table 3. Biochemical properties of the strain ACMA006

Tests	Results*
Gram staining	+
Acid inhibition staining	–
Decomposition of starch	++
Reduction of nitrate	++
Gelatin hydrolysis	–
Growth on cellulose	–
Coagulation of milk	+
Peptonization of milk	+
Production of hydrogen sulfide	–
Production of melanoid pigment	–

* ++, strong positive; +, positive; –, negative.

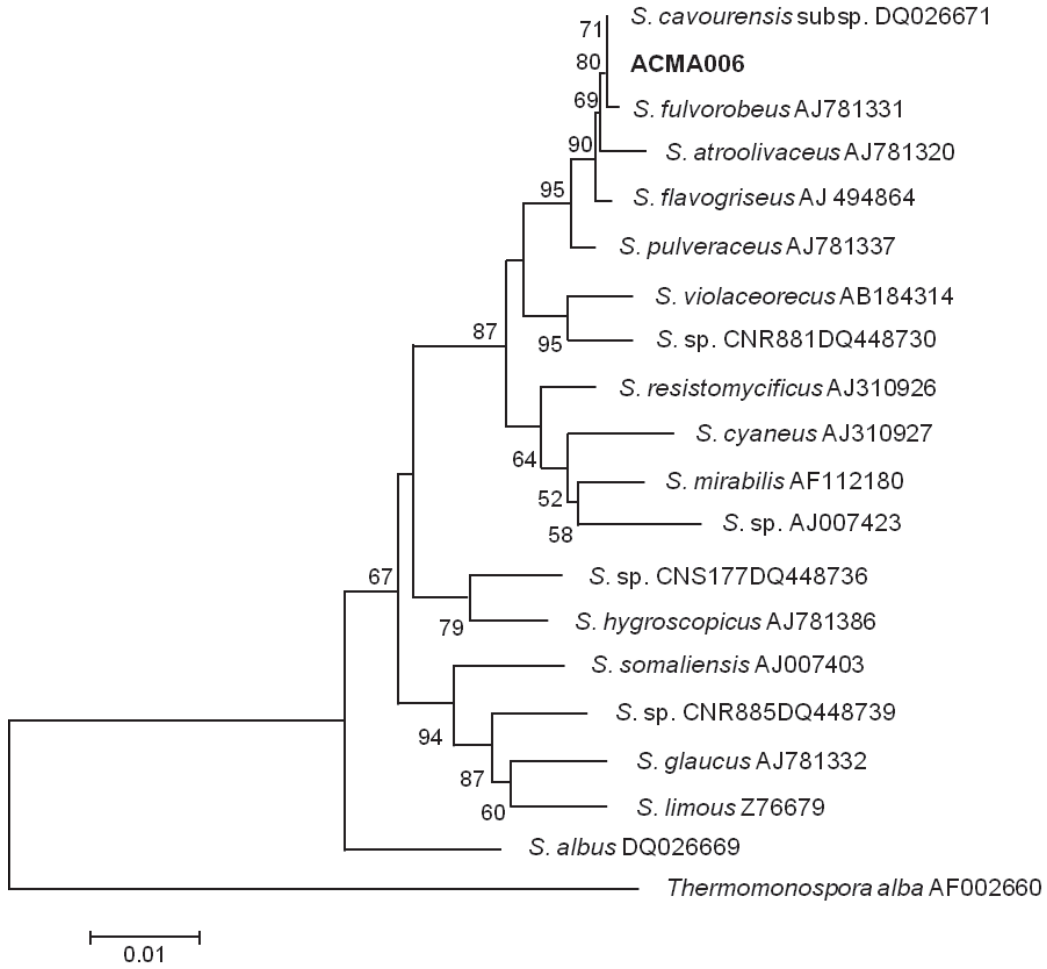

Figure 2. A neighbor-joining dendrogram based on the sequence length of the 16S rDNA gene. The tree was validated by bootstrap analysis (1,000 replications) and values greater than 50% are indicated at the nodes. Bar, 0.01 substitutions per 100 nucleotides.

neighbor-joining method and Kimura's two parameter model (Figure 2). The ACMA006 strain formed a clade with the members of the genus *Streptomyces*. The 16S rDNA gene sequence of the strain was highly similar, with similarity close to 100%, to the 16S rDNA gene sequence (1,490 bp) of the previously described species *Streptomyces cavourensis* subsp. *washingtonensis*.

3.6. *Inhibition of cell growth*

Cell growth inhibition by FBA6 was examined with human hepatoma cells HepG2 and normal hepatocytes LO2. As shown in Figure 3, FBA6 had an anti-proliferative effect on HepG2 cells in a dose-dependent manner for up to 96 h of exposure ($p < 0.05$ *vs*. untreated control). Similar proliferation profiles were observed in LO2 cells exposed to FBA6. However, LO2 cells were less sensitive to FBA6 than HepG2 cells. The survival rate for LO2 cells was higher than that for HepG2 cells at the same concentrations.

3.7. *Inhibition of bacteria growth*

The antibacteria activity of FBA6 was determined

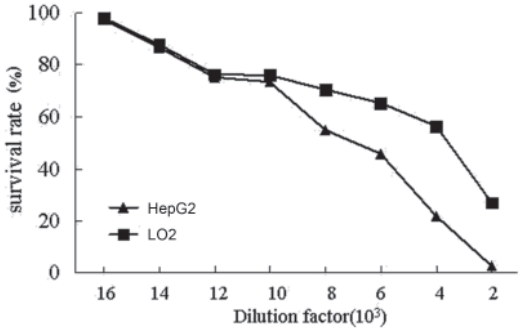

Figure 3. FBA6's inhibition of the growth of hepatoma cells HepG2 and normal hepatocytes LO2. FBA6 was diluted into a series of final concentrations as indicated by the values on the horizontal axis.

with the following bacterial strains: *B. subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, *Proteus vulgaris*, and *Pseudomonas fluorescens*. As shown in Table 4, the growth of strains *B. subtilis* and *Staphylococcus aureus* was inhibited by FBA6, with inhibition (dia) of 16 and 11 mm, respectively. FBA6 was not found to suppress the growth of other bacterial strains.

Table 4. Antibacterial activity of FBA6 on indicated bacterial strains

Bacterial strains	Extent of inhibition (diameter in mm)*
Bacillus subtilis	16
Staphylococcus aureus	11
Escherichia coli	–
Bacillus aerogenes	–
Pseudomonas fluorescence	–
Bacillus proteus	–

* –, negative.

4. Discussion

The present study examined the taxonomic status of the novel strain ACMA006 and evaluated the ability of its fermentation broth to inhibit the growth of human hepatoma HepG2 cells, human normal hepatocytes LO2, and a series of bacterial strains. Complete 16S rDNA gene sequence analysis, cultural, physiological, morphological, and biochemical characteristics, and the results of chemotaxonomic analyses indicated that this strain should be classified as a new strain of the species *Streptomyces cavourensis* subsp. *washingtonensis*. Bioactivity analyses demonstrated that FBA6 was preferentially cytotoxic to hepatoma HepG2 cells. In addition, FBA6 selectively inhibited the growth of the two bacterial strains *B. subtilis* and *Staphylococcus aureus*. These results suggested that metabolites of the strain ACMA006 are candidates for use as antitumor and antibacterial agents in the future.

Members of the genus *Streptomyces* share many phenotypic characteristics and constitute a distinct phyletic line to which over 450 validly described species are assigned (*12*,*23*,*24*). Species in this genus produce extensively branched substrate and aerial mycelia (*12*). Phylogenetic analyses showed that the gene sequence of 16S rDNA of ACMA006 was almost identical to that of *Streptomyces cavourensis* subsp. *washingtonensis*. However, characteristics such as the production of yellow or pale yellow soluble pigments and H_2S, as were found with the strain ACMA006, have rarely been observed with the strain *Streptomyces cavourensis* subsp. *washingtonensis*. Moreover, the mycelium of the strain ACMA006 had septa in the early stage of culture and subsequently fragmented, features that are rarely found in the genus *Streptomyces* except for in the strain *S. limosus* (*15*). Given these findings, the strain was determined to be a new strain of the *Streptomyces cavourensis* subsp. *washingtonensis*, according to the *Manual for identification of Streptomyces* (*15*), *Bergey's Manual of Systematic Bacteriology* (*12*), and *Systematology of Actinomycetes – Principle, Methods, and Practice* (*21*). The metabolites in FBA6 that had bioactivity as indicated by the current study have yet to be specifically identified. Purification and identification of these compounds is now underway.

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References

1. Newman DJ, Cragg GM. Marine natural product and related compounds in clinical and advanced preclinicatrials. J Nat Prod. 2004; 67:1216-1238.
2. Shen W, Kim JS, Kish PE, Zhang J, Mitchell S, Gentry BG, Breitenbach JM, Drach JC, Hilfinger J. Design and synthesis of vidarabine prodrugs as antiviral agents. Bioorg Med Chem Lett. 2009; 19:792-796.
3. Advani AS, Gundacker HM, Sala-Torra O, Radich JP, Lai R, Slovak ML, Lancet JE, Coutre SE, Stuart RK, Mims MP, Stiff PJ, Appelbaum FR. Southwest Oncology Group Study S0530: A phase 2 trial of clofarabine and cytarabine for relapsed or refractory acute lymphocytic leukaemia. Br J Haematol. 2010; 151:430-434.
4. Kirby R. Actinomycetes and lignin degradation. Adv Appl Microbiol. 2006; 58:125-168.
5. Okami Y, Hotta K. Search and discovery of new antibiotics. In: Actinomycetes in Biotechnology (Goodfellow M, Williams ST, Mordarski M, eds.). Academic Press, London, UK, 1988; pp. 33-67.
6. Nett M, Ikeda H, Moore BS. Genomic basis for natural product biosynthetic diversity in the actinomycetes. Nat Prod Rep. 2009; 26:1362-1384.
7. Yang RL, Yuan XW, Zheng J, Wang SJ, Li D. Preliminary study on antitumor activity of culture broths from a marine actinomycete. Journal of Oceanography in Taiwan Strait. 2007; 26:351-355. (in Chinese)
8. Yuan XY, Yang RL, Cao X. Studies on the optimum fermentation conditions of marine actinomycete ACMA006 with antitumor activity. Chinese Journal of Marine Drugs. 2008; 27:5-9. (in Chinese)
9. Inagaki Y, Tang W, Zhang L, Du G, Xu W, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.
10. Qadrie ZL, Jacob B, Anandan R, Rajkapoor B, Rahamathulla M. Anti-bacterial activity of ethanolic extract of *Indoneesiella echioides* (L) nees. evaluated by the filter paper disc method. Pak J Pharm Sci. 2009; 22:123-125.
11. Shirling EB, Gottlieb D. Methods for characterization of Streptomyces species. Int J Syst Bacteriol. 1966; 16:313-340.
12. Locci R. Streptomycetes and related genera. In: Bergey's manual of systematic bacteriology (Williams ST, Sharpe ME, Holt JG, eds.). Williams & Wilkins, Baltimore, MD, USA, 1989; pp. 2451-2508.
13. Pridham TG, Gottlieb D. The utilization of carbon compounds by some actinomycetales as an aid for species determination. J Bacteriol. 1948; 56:107-114.
14. O'Donnell AG, Falconer C, Goodfellow M, Ward AC,

Williams E. Biosystematics and diversity amongst novel carboxydotrophic actinomycetes. Antonie Van Leeuwenhoek. 1993-1994; 64:325-340.
15. Zhang JZ. Manual for identification of Streptomyces. Fudan University Press, Shanghai, China, 1990; pp. 111-155.
16. Becker B, Lechevalier MP, Lechevalier HA. Chemical composition of cell-wall preparation from strains of various form-genera of aerobic actinomycetes. Appl Microbiol. 1965; 13:236-243.
17. Hasegawa T, Takizawa M, Tanida S. A rapid analysis for chemical grouping of aerobic actinomycetes. J Gen Appl Microbiol. 1983; 29:319-322.
18. Butler WR, Floyd MM, Silcox V, Cage G, Desmond E, Duffey PS, Guthertz LS, Gross WM, Jost KC, Ramos LS, Thibert L, Warren N. Standardized Method for HPLC Identification of Mycobacteria. U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES Public Health Service.
19. Orsini M, Romano-Spica V. A microwave-based method for nucleic acid isolation from environmental samples. Lett Appl Microbiol. 2001; 33:17-20.
20. Saitou N, Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic tree. Mol Biol Evol, 1987, 4:406-425.
21. Xu LH, Li WJ, Liu ZH. Systematology of Actinomycetes – Principle, Methods and Practice. Science Press, Beijing, China, 2007; pp. 150-201.
22. Lechevalier MP, Lechevalier HA. The chemotaxonomy of actinomycetes. In: Actinomycete Taxonomy (Dietz X, Thayer Y, eds.). Arlington, Texas, USA, 1980; pp. 227-291.
23. Embley TM, Stackebrandt E. The molecular phylogeny and systematics of actinomycetes. Annu Rev Microbiol. 1994; 48:257-289.
24. Goodfellow M, Manfio GP, Chun J. Towards a practical species concept for cultivable bacteria. In: Species: the Units of Biodiversity (Claridge MF, Dawah HA, Wilson MR, eds.). Chapman & Hall, London, UK, 1997; pp. 25-69.
25. Yan G, Duan RH, Yin K, Zhu S, Liu Q, Gong M, Wang H, Sun C, Pu D, Tang N, Huang AL. Inhibition of survivin expression to induce the apoptosis of hepatocarcinoma cells by adenovirus-mediated siRNA. Biosci Trends. 2008; 2:88-93.

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