A NEW MACROMOLECULAR ANTITUMOR ANTIBIOTIC, C-1027

I. DISCOVERY, TAXONOMY OF PRODUCING ORGANISM, FERMENTATION AND BIOLOGICAL ACTIVITY

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(Received for publication March 24, 1988)

Strain C-1027, an actinomycete isolated from a soil sample collected in China, was found to produce the new antibiotic, C-1027. From taxonomical studies on its morphological, cultural and physiological characteristics, this antibiotic-producing strain was identified as *Streptomyces globisporus* C-1027. Antibiotic C-1027 has antimicrobial activity against most Gram-positive bacteria but not against *Mycobacterium* sp. or Gram-negative bacteria. This antibiotic shows remarkable activity in spermatogonial assay and potent cytotoxicity against KB carcinoma cells *in vitro*, and exhibits inhibition on transplantable tumors in mice.

Spermatogonial assay, developed by ZHEN and XUE[1], is a new prescreen method for detection of antitumor antibiotics. In the course of our screening for antitumor antibiotics, we have found many producers of antitumor antibiotics by use of this spermatogonial assay and additionally by use of microbiological tests[2]. Strain C-1027, producer of a new antibiotic, was also found by using this method from approximate 2,000 fermentation broths of actinomycete strains. The fermentation broth of strain C-1027 was observed to inhibit the growth of Gram-positive bacteria and to have cytotoxicity against KB carcinoma cells. Strain C-1027 produced the antitumor protein, termed antibiotic C-1027, in the culture filtrate. It also produced a related protein which antagonistically suppressed the inhibitory activity of antibiotic C-1027 against *Micrococcus luteus*.

The present paper describes the taxonomy of the producing organism, fermentation and biological properties. The isolation and physico-chemical characterization of antibiotic C-1027 are described in the following paper[3].

Taxonomy of the Producing Strain

Strain C-1027 was isolated from a soil sample collected in Qian-jiang county, Hu-bei province, China.

The taxonomic characteristics were performed in accordance with the methods proposed by the International Streptomyces Project (ISP)[4] and WAKSMAN[5]. The color names based on RIDGWAY's description were used[6]. All observations were made after incubation at 27°C or 37°C for 3 weeks.

Microscopic observation showed moderate aerial mycelia with straight to flexious spore chains, belonging to type Rectus-Flexibilis (Plate 1). The mature spore chains generally had 10 to 30 or more than 30 spores per chain. The spores were cylindrical in shape and $0.5\sim0.6\times0.7\sim0.8$ μm in size with smooth surface as observed by a scanning electron microscope (Plate 2). No sporangia,

Plate 1. Photomicrograph of strain C-1027 after 18 days of incubation on ISP medium No. 5 (×300).

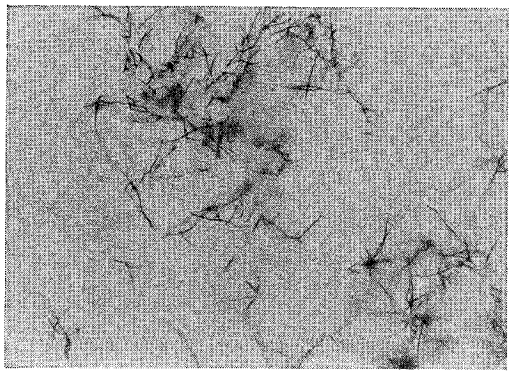

Plate 2. Scanning electron micrograph of strain C-1027 after 18 days of incubation on ISP medium No. 4.

Bar indicates 5 μm.

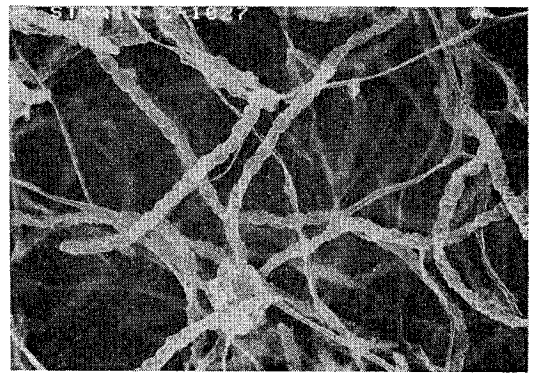

Table 1. Cultural characteristics of Strain C-1027.

Medium	Growth	Aerial mycelium	Reverse side	Soluble pigment
Yeast extract - malt extract agar (ISP medium No. 2)	Abundant	Ivory yellow to yellowish gray or pale olive-buff (XXX 21″f, XL 21″f)	Light buff (XV 17″f)	None or light yellow
Oatmeal agar (ISP medium No. 3)	Moderate	Yellowish white, pale olive-buff	Colorless-cream	None
Inorganic salts - starch agar (ISP medium No. 4)	Moderate	Yellowish white, pale olive-buff (XXIX 17″f)	Colorless-cream	None
Glycerol - asparagine agar (ISP medium No. 5)	Abundant	Yellowish gray or pale olive-buff (XL 21″f)	Colorless-cream	None
Tyrosine agar (ISP medium No. 7)	Abundant	Yellowish gray or pale olive-buff	Colorless or pale gray yellow	None
Sucrose - CZAPEK's agar	Thin	Yellowish white to pale olive-buff	Colorless	None
Glucose - CZAPEK's agar	Abundant	Yellowish white to pale olive-buff	Colorless or cream	None
Gauze No. 1 agar	Abundant	Yellowish gray, pale olive-buff (XL 21″f)	Colorless or yellowish gray	None
Glucose - asparagine agar	Moderate	Yellowish gray, pale olive-buff	Colorless	None
BENNET's agar	Moderate	White to yellowish gray	Light yellow	Light yellow
Potato agar	Abundant	Pale olive-buff	Colorless to olive-buff	None

Color names are assigned according to "Color Standards and Color Nomenclature" by RIDGWAY (1912).

scleotia or flagellated spores were observed.

Aerial mass color was white to pale yellow, yellowish-gray or pale olive-buff on various synthetic and organic agar media. Pinkish-buff color occurred in aged cultures on inorganic salts - starch agar (ISP medium No. 4). Accordingly, strain C-1027 is considered to be a strain of the white to yellow

Table 2. Physiological characteristics of strain C-1027.

Melanoid pigment	
Tryptone - yeast extract broth (ISP medium No. 1)	Negative
Peptone - yeast extract iron agar (ISP medium No. 6)	Negative
Tyrosine agar (ISP medium No. 7)	Negative
H_2S production	Positive
Gelatin liquefaction	Positive
Skim milk (37°C)	
Coagulation	Positive
Peptonization	Positive
Nitrate reduction	Positive
Starch hydrolysis	Positive
Cellulose decomposition	Negative
NaCl tolerance	≧7%
Temperature for growth	
28~30°C	Abundant, rapidly
37°C	Moderate, slowly
45°C	No growth

Table 3. Utilization of carbon sources by strain C-1027.

Carbon sources	Growth
L-Arabinose	++
D-Xylose	++
D-Glucose	+
D-Fructose	++
Sucrose	−
Inositol	−
L-Rhamnose	++
Raffinose	−
D-Mannitol	++
D-Galactose	+
Salicin	±
Soluble starch	+
Dextrin	++
Glycerol	+
Maltose	++

++: Good growth, +: moderate growth, ±: doubtful growth, −: no growth.

or gray color series of TRENSNER and BACKUS's grouping[7]. The color of substrate mycelium was colorless to cream, pale yellow gray or pale gray yellow on almost all media. No melanoid pigment was produced on tyrosine agar, peptone - yeast extract iron agar or in Tryptone - yeast extract broth. No soluble pigment was produced on any of the media tested. These cultural and physiological characteristics of strain C-1027 are summarized in Tables 1 and 2, respectively. The utilization of carbon sources of strain C-1027 was examined by growth on PRIDHAM and GOTTLIEB's medium containing 1% of each carbon source. The results obtained are shown in Table 3.

Chemical analysis of whole cell hydrolysates was carried out by the methods of BECKER *et al.*[8], and LECHEVALIER[9]. Whole cell hydrolysates of strain C-1027 contained LL-diaminopimelic acid, glucose and ribose, and the strain was assigned to cell wall type I.

From the results obtained for the taxonomical studies on morphological, cultural and physiological characteristics, strain C-1027 belonged to genus *Streptomyces*, and thereafter this strain was compared with published descriptions[10~13] of various *Streptomyces* species. *Streptomyces setonii* and *Streptomyces globisporus* were selected using NONOMURA's key[14]. Strain C-1027 was found to be closely related to *S. setonii* (Millard and Barr) Waksman and *S. globisporus* (Krassilnikov) Waksman. Further detailed comparisons were directly made between strain C-1027, *S. setonii* IFO 13085 and *S. globisporus* As 4.52.

S. setonii differed from strain C-1027 as follows; *S. setonii* produces ordinarily yellowish white, occasionally white aerial mycelium on oatmeal agar (ISP medium No. 3) and inorganic salts - starch

agar (ISP medium No. 4), and doesn't or slightly utilizes inositol for growth and coagulate milk.

The aerial mass color of *S. globisporus* on glucose - CZAPEK's agar and sucrose - CZAPEK's agar, showing Marguerite yellow to pale glass green, was differentiated from that of strain C-1027. However, in spite of these minor differences, strain C-1027 and *S. globisporus* were quite similar in other morphological and physiological properties, including their ability to utilize various carbon sources. Therefore, on the basis of these results, it is concluded that strain C-1027 is classified as *Streptomyces globisporus* C-1027. This strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaraki Prefecture, Japan, with the accession No., FERM BP-1299.

Fermentation

Stock cultures of *S. globisporus* C-1027 were maintained in skim milk at −20°C and working cultures were maintained on malt extract agar slants (glucose 0.4%, malt extract 1.0%, yeast extract 0.4%, agar 1.5%, pH 7.0). A loopful spores and mycelia from a 2 week-old slant were inoculated into 100 ml of the seed medium containing glycerol 2%, dextrin 2%, Bacto-soytone (Difco) 1%, yeast extract 0.3%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (pH 7.0) in a 500-ml Erlenmeyer flask. The flask was incubated at 27°C for 2 days on a rotary shaker with 7.6 cm throw at 220 rpm. Four ml (4%) of seed culture thus obtained were transferred to 100 ml of the fermentation medium consisting of glycerol 2%, dextrin 2%, fish meal 1%, peptone 0.5%, $(NH_4)_2SO_4$ 0.2% and $CaCO_3$ 0.2% (pH 7.0) in a 500-ml Erlenmeyer flask. The fermentation was conducted at 27°C for 4~5 days on a rotary shaker.

Antibiotic production was monitored during the fermentation by the agar well diffusion method against *M. luteus* ATCC 9341 as the test organism. C-1027 production in the culture filtrate started at 80~90 hours after inoculation, then rapidly increased and reached a maximum level at 110~120 hours. The antibiotic activity didn't decrease until 144 hours. At the harvest time, pH of the culture supernatant was approximately 8.0.

Biological Properties

The antimicrobial spectrum of antibiotic C-1027 was determined by serial agar dilution method using Mueller-Hinton agar at an inoculum of 10^6 cells/ml. The MIC was measured after 21 hours-incubation at 37°C. As shown in Table 4, this antibiotic was mainly effective against Gram-positive bacteria, but inactive against *Mycobacterium* sp. and Gram-negative bacteria tested.

Antibiotic C-1027 was extremely active in the spermatogonial assay, with a minimal effective concentration of 0.0039 μg/ml.

Table 4. Antimicrobial spectrum of antibiotic C-1027.

Test organism	MIC (μg/ml)	Test organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P	0.78	*Escherichia coli* NIHJ	>100
S. aureus Smith	0.39	*Proteus vulgaris* IID OX19	>100
S. citreus	0.78	*Klebsiella pneumoniae* ATCC 10031	>100
Micrococcus luteus ATCC 10240	0.78	*Salmonella typhimurium*	>100
M. luteus ATCC 9341	0.78	*Serratia marcescens* IFO 12648	>100
Bacillus subtilis ATCC 6633	0.78	*Pseudomonas aeruginosa* IFO 13275	>100
B. cereus IFO 3001	1.56		
Mycobacterium smegmatis ATCC 607	>100		

The cytotoxic activity of antibiotic C-1027 was determined by a proliferating cell inhibition assay against KB carcinoma cells. When KB cells were exposed to antibiotic C-1027 for 3 days in a tissue culture, the potent cytotoxic activity exceeded that of doxorubicin. The ED_{50} value obtained was 0.001 μg/ml[3].

The toxicity of antibiotic C-1027 by a single ip injection in CDF_1 mice was intensely observed. The LD_{50} value was approximately 0.5 mg/kg, as calculated from mortality for 48 to 54 days following injection, and no mice was died at a dose of 0.3 mg/kg. However, the toxicity was reduced in the case of an iv injection. The dose of 2 mg/kg was not toxic and no influence of body weight was observed.

In vivo antitumor activity was evaluated against lymphocytic leukemia P388 in CDF_1 mice in several administration schedules. Though the maximum effect (92% increase of life span) was observed at a dose of 0.33 mg/kg on a day 1, 5 and 9 treatment schedule, there was no 30-day survivors. The therapeutic efficacy seemed to be low from the results of the toxicity. Therefore, we were now investigating the antitumor effect by iv injections.

Acknowledgments

The authors wish to thank Dr. M. HAMADA, Institute of Microbial Chemistry, Prof. M. NAKAYAMA, University of Osaka Prefecture, and Dr. N. ISHIDA in our labolatory, for their valuable advice and encouragement. We also thank Mrs. R. YAMASAKI for the technical assistance to cytotoxicity tests.

References

1) ZHEN, Y.-S. & Y.-C. XUE: Use of murine spermatogonial assay as prescreen for antitumor drugs. *In* Proc. 13th Int. Congr. Chemother., *Eds.*, K. H. SPITZY & K. KARRER, Part 223, No. 7, pp. 38~44, Vienna, 1983
2) HU, J. L.; Y. Y. ZHAO, Y. C. XUE, W. Y. LU, L. F. SU, Z. Y. YAO & Y. Y. LI: Screening for producing microorganisms of antitumor antibiotics by using spermatogonial assay as Guide and microbiological tests as Follow-up method. Proceedings of the 5th National Congress of Antibiotics Research, pp. 126~128, Ji-nan, Oct., 1985
3) OTANI, T.; Y. MINAMI, T. MARUNAKA, R. ZHANG & M.-Y. XIE: A new macromolecular antitumor antibiotic, C-1027. II. Isolation and physico-chemical properties. J. Antibiotics 41: 1580~1585, 1988
4) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
5) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, Identification and Descriptions of Genera and Species. Williams & Wilkins Co., Baltimore, 1961
6) RIDGWAY, R. (*Ed.*): Color Standards and Color Nomenclature. Press of A. Hoen & Co., Baltimore, 1912
7) TRESNER, H. D. & E. J. BACKUS: System of color wheels for Streptomycete taxonomy. Appl. Microbiol. 11: 335~338, 1963
8) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
9) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968
10) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type culture of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. 18: 69~189, 1968
11) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type culture of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 279~392, 1968
12) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type culture of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391~512, 1969
13) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type culture of *Streptomyces*. V. Additional descriptions. Int. J. Syst. Bacteriol. 22: 265~394, 1972
14) NONOMURA, H.: Key for classification and identification of 458 species of the *Streptomyces* included in ISP. J. Ferment. Technol. 52: 78~92, 1974