NEW GLUTARIMIDE ANTIBIOTICS, S-632-B_1 AND B_2

I. TAXONOMY OF PRODUCING STRAIN, FERMENTATION AND BIOLOGICAL PROPERTIES

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(Received for publication November 28, 1988)

Strain S-632 was found to produce new glutarimide antibiotics, S-632-B_1 and B_2, which were isolated from the culture fluid. A taxonomic study on strain S-632 was carried out, and the taxonomic characterization demonstrated that it belonged to the species *Streptomyces hygroscopicus*. The strain was given the name *S. hygroscopicus* S-632.

These antibiotics were active against *Saccharomyces* sp., but inactive against filamentous fungi and bacteria, and had cytotoxic activity against KB tissue culture cells.

In the course of our screening program for new antibiotics, we found antifungal antibiotics active against *Saccharomyces cerevisiae* in the culture fluid of a Streptomycete, strain S-632, isolated from a soil sample.

As a result of the isolation and characterization of four antibiotics, designated as S-632-A_1, A_2, B_1 and B_2, S-632-B_1 and B_2 were found to be new members of the glutarimide antibiotics. From the taxonomic studies, strain S-632 was classified as *Streptomyces hygroscopicus*. In this paper, we describe the taxonomy of the producing organism, fermentation and biological activities of the antibiotics. Isolation, physico-chemical properties and structure elucidation are described in the accompanying paper[1].

Taxonomic Studies

Strain S-632 was isolated from a soil collected at Shandong Province, China. Methods and media described by the International Streptomyces Project (ISP)[2] and WAKSMAN[3] were used to determine most of the morphological and physiological characteristics. All observations were made during the first 21 days of incubation at 27°C. Color names were assigned according to the "Guide to Color Standard"[4] and hue numbers were those of the "Color Harmony Manual"[5].

The aerial mycelium of strain S-632 was well branched with sporophores forming open spirals with 10 to 50 or more spores per chain (Fig. 1A). Whirls were not observed. The spores were cylindrical in shape and $0.8 \sim 1.0 \times 1.1 \sim 1.2$ μm in size or had a non-segmented form. They had a smooth or rugose surface showing category for intermediate between a spiny and warty shape (Fig. 1B).

Cultural characteristics of the strain S-632 on the media used for the taxonomic studies are shown in Table 1. The vegetative mycelium was colorless to pale yellowish brown on almost all media. The aerial mycelium was white at first, later becoming light gray to light brownish gray with black moist

Fig. 1. Scanning electron micrographs of strain S-632 with simple drying method from 15 days-culture growth on inorganic salts - starch agar (ISP medium No. 4).

Bars represent 5 μm.

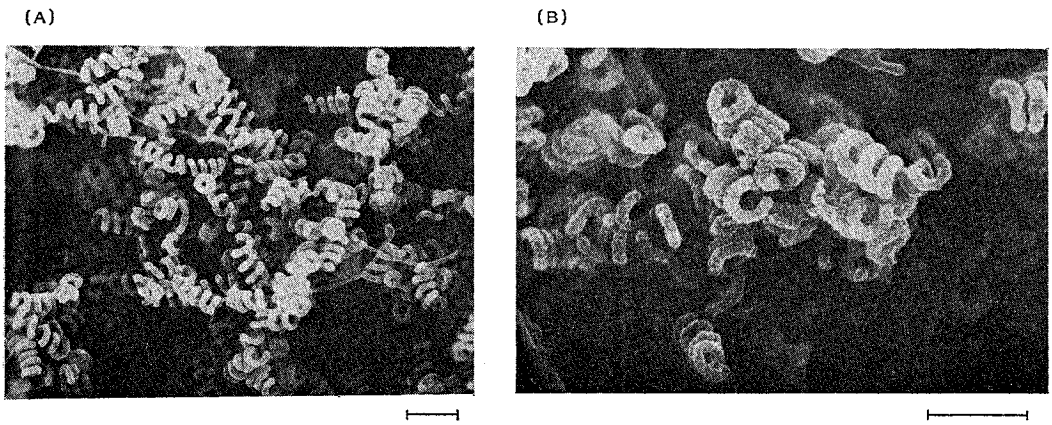


Table 1. Cultural characteristics of strain S-632.

Medium	Growth	Aerial mycelium	Substrate mycelium	Reverse side	Soluble pigment
Sucrose - nitrate agar	Poor	Whitish, grayish	Colorless	Pale yellowish brown	None
Glycerol - nitrate agar	Poor	Whitish, grayish	Colorless	Colorless	None
Glucose - asparagine agar	Poor	Whitish, grayish	Colorless to pale yellowish brown	Yellowish	None
Nutrient agar	Moderate	White to grayish white	Pale yellowish brown	Pale yellow	None
BENNETT's agar	Poor	Whitish	Pale yellowish brown (2ie)	Pale yellowish brown (2ic)	Bright yellow
Yeast extract - malt extract agar (ISP medium No. 2)	Moderate	White to light brownish gray (2fe~2ih) or dark grayish brown (2ml~3po)	Pale yellowish brown (2ie)	Pale yellowish brown (2ic)	None
Oatmeal agar (ISP medium No. 3)	Moderate	Light brownish gray (2fe~2nl) to dark grayish brown (2pn~3po) or brownish black	Pale yellow	Yellowish	None
Inorganic salts - starch agar (ISP medium No. 4)	Good	Light brownish gray (2fe~2ih) to dark grayish brown	Colorless	Yellowish	None
Glycerol - asparagine agar (ISP medium No. 5)	Poor	Grayish white to light brownish gray or dark grayish brown (2pn~3po)	Colorless	Colorless	None
Tyrosine agar (ISP medium No. 7)	Poor	White to grayish white	Pale yellowish brown	Pale yellowish brown	None
Starch agar	Moderate	White to light gray (d)	Colorless to yellowish	Yellowish	None

Colors were assigned according to the "Guide to Color Standard"[4]; hue numbers in parentheses were assigned according to "Color Harmony Manual"[5].

patches or hygroscopic masses of spores spreading over the whole surface. Bright yellow diffusible pigmentation was observed on BENNETT's agar medium but no diffusible pigment was observed on most of the media.

Physiological properties of strain S-632 are shown in Table 2. No melanoid pigment was produced on tyrosine agar and peptone - yeast extract - iron agar. Optimum temperature for growth was tested on yeast extract - malt extract agar. The results showed an optimum temperature ranging from 27°C to 30°C. Starch hydrolysis, milk coagulation and peptonization, hydrogen sulfide production and nitrate reduction gave positive reactions.

Table 2. Physiological characteristics of strain S-632.

Melanoid pigment	
Peptone - yeast extract - iron agar (ISP medium No. 6)	Negative
Tyrosine agar (ISP medium No. 7)	Negative
Tryptone - yeast extract agar (ISP medium No. 1)	Negative
H_2S production	Positive
Gelatin liquefaction	
Glucose - peptone - gelatin (27°C)	Positive (weak)
Gelatin (20°C)	Negative
Skim milk (37°C)	
Coagulation	Positive
Peptonization	Positive
Starch hydrolysis (ISP medium No. 4)	Positive (strong)
Nitrate reduction (ISP medium No. 8)	Positive
Cellulose decomposition	Negative
NaCl tolerance	≧7%
Optimum temperature for growth (ISP medium No. 2)	30°C

The chemical analysis of cell wall diaminopimelic acid isomers carried out by the method of BECKER *et al.*[6] showed the presence of LL-diaminopimelic acid. Accordingly, the cell wall of this strain was classified as Type I. Whole-cell sugars were identified by the procedure of LECHEVALIER[7]. However, no characteristic sugar pattern was noted.

The utilization of carbon sources was examined by the method of PRIDHAM and GOTTLIEB[8] on ISP medium No. 9. Cultures of strain S-632 grew well on L-arabinose, D-xylose, D-fructose, sucrose, L-rhamnose, raffinose, inositol, D-mannitol, D-galactose, soluble starch, dextrin, glycerol and maltose. However, they only grew moderately on D-glucose (positive control) and salicin, and grew poorly on carbon-free basal medium (negative control) and cellulose.

Microscopic studies and cell wall analysis of strain S-632 indicated that the strain should be classified in the genus *Streptomyces*. According to the literature[8,9] on *Streptomyces*, strains for which the aerial mycelium becomes moist and forms dark hygroscopic masses at maturity are classified as *S. hygroscopicus*. As shown in the description of DIETZ and MATHEWS[10,11], the sheath is sometimes wrinkled or ridged ("rugose") producing a silhouette which appears to be either warty or even spiny. Based on the spore-chain morphology observed by electron microscopy, DIETZ[12] classified "hygroscopic" *Streptomyces* strains into two species and proposed that "hygroscopic strains" with rugose spore surface should be designated as *S. hygroscopicus* and those with elliptical spore surface as *S. neohygroscopicus* (*S. platensis*). Based on these criteria, strain S-632 resembles a *S. hygroscopicus*-type strain. Accordingly, the strain was compared with the published descriptions[9,13~17] of various "hygroscopic" *Streptomyces* sp. Besides *S. hygroscopicus*, the following strains are known to belong to this type: *Streptomyces endus*, *Streptomyces melanosporofaciens* and *Streptomyces violaceoniger*. Therefore, strain S-632, *S. endus* IFO 12859 (ISP 5187), *S. melanosporofaciens* IFO 13061 (ISP 5318), *S. violaceoniger* IFO 13459 (ISP 5563) and *S. hygroscopicus* IFO 13472 (ISP 5578) were directly compared. *S. melanosporofaciens* and *S. violaceoniger* could be differentiated from strain S-632 as follows: *S. melanosporofaciens* differed from strain S-632 in the color of substrate mycelium, utilization of sucrose and nitrate reduction, and production of a light reddish brown non-melanoid pigment in

Table 3. Comparison of strain S-632 with *Streptomyces hygroscopicus*.

	Strain S-632	*S. hygroscopicus* IFO 13472
Spore shape	Cylindrical (non-segmented)	Cylindrical or oval (non-segmented)
Spore surface	Smooth to ridge	Smooth to ridge (warty)[a]
Color of aerial mycelium	Grayish white to light brownish gray to brownish gray, later becoming moist and black	Grayish white to light brownish gray to brownish gray, later becoming moist and black
Vegetative mycelium	Colorless to pale yellowish brown	Colorless to pale yellowish brown
Melanoid formation	−	−
Soluble pigment	None	None
NaCl tolerance	≧7%	≧7%
Starch hydrolysis	+	+
Peptonization of milk	+	+
Coagulation of milk	+	−
Liquefaction of gelatin	+	±
Nitrate reduction	+	+
Utilization of sucrose	+	−
Utilization of inositol	+	−
Utilization of raffinose	+	−
Utilization of salicin	+	+

Symbols: +, positive; −, negative; ±, doubtful.
[a] These results in parentheses were shown in the reference.

tyrosine agar. *S. violaceoniger* was differentiated from strain S-632 by the spore-chain morphology, nitrate reduction, milk coagulation and a light reddish brown non-melanoid pigment in tyrosine agar, although it resembled strain S-632 in utilizing carbon sources. On the other hand, *S. hygroscopicus* has almost the same morphological, cultural and physiological characteristics, but has a different capability in utilizing carbon sources such as sucrose, inositol and raffinose and in coagulating milk. The results of the comparison with *S. hygroscopicus* are shown in Table 3. *S. endus* was also included in the comparison and was not significantly differentiated from *S. hygroscopicus* in accordance with previous observations[12]. Consequently, strain S-632 was regarded as belonging to the species, *S. hygroscopicus*, and was designated as *S. hygroscopicus* S-632.

The strain S-632 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. S-632 with the accession No. FERM BP-1849.

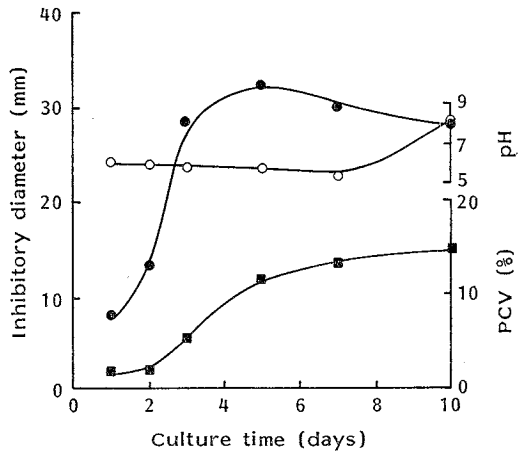
Fermentation

S. hygroscopicus S-632 was cultured and maintained on malt extract or GAUSE No. 1 agar slants. To prepare vegetative inoculum, cultures of *S. hygroscopicus* S-632 were frozen at −20°C as suspensions in deionized water containing 10% glycerol. When needed, a vial was thawed and 2 ml of the frozen seed stock was used to inoculate 100 ml of the seed medium in a 500-ml Erlenmeyer flask, which then was incubated at 27°C for 3 days on a reciprocal shaker (amplitude 7 cm, 140 strokes/minute). The seed medium consisted of glycerol 4.0%, glucose 0.2%, potato starch 0.2%, soybean meal 2.0%, peptone 0.5%, dried yeast 0.5%, NaCl 0.5% and $CaCO_3$ 0.2% (pH 7.0). The resultant vegetative growth was used to inoculate at a rate of 5% in a 500-ml Erlenmeyer flask containing 100 ml of the fermentation medium of the following compositions: Glycerol 3.0%, glucose 0.2%, potato starch 0.2%, soybean meal 2.0%, peptone 0.3%, dried yeast 0.5% and NaCl 0.3% (pH 6.4). The fermenta-

tion was carried out on the same reciprocal shaker as described above at 27°C. Progress of the fermentation was monitored by a paper-disc diffusion method using *S. cerevisiae* IFO 0304 as the test organism. The ratio of respective components produced was also monitored by comparison of inhibition zones on bioautography against *S. cerevisiae* using Silica gel 60 F_{254} TLC plate (Merck No. 5715) developed in ethyl acetate. A typical time course of the fermentation is shown in Fig. 2. Peak antibiotic titers were obtained after 5~7 days of incubation.

Fig. 2. Time course of the fermentation by *Streptomyces hygroscopicus* S-632.

■ Packed cell volume, ● inhibitory diameter against *Saccharomyces cerevisiae*, ○ pH.



Biological Activity

The antifungal spectra of component S-632-B_1, B_2 and A_2 (9-methylstreptimidone) as well as cycloheximide as reference compound were determined by a serial agar dilution method using SABOURAUD's agar after 48 hours-incubation at 27°C. Due to the poor solubility in water, these antibiotics were initially dissolved in dimethyl sulfoxide and subsequently diluted with the medium. The antifungal activities of these antibiotics were compared, as shown in Table 4. The components S-632-A_2, B_1 and B_2 were active against *Saccharomyces* sp. but inactive against filamentous fungi, Gram-positive and

Table 4. Antifungal spectrum of compounds S-632-B_1, S-632-B_2 and S-632-A_2 (9-methylstreptimidone).

Test organism	MIC (μg/ml)			
	S-632-B_1	S-632-B_2	S-632-A_2	Cycloheximide
Penicillium chrysogenum mut. *fulvescens* IFO 6223	>100	>100	>100	12.5
P. rubrum IFO 6580	>100	>100	>100	50
P. citrinum IFO 4631	>100	>100	>100	100
P. notatum IFO 4640	>100	>100	>100	12.5
Aspergillus nutans IFO 7869	>100	>100	>100	3.13
Trichophyton mentagrophytes IFO 5812	>100	>100	>100	>100
T. rubrum IFO 5467	>100	>100	>100	>100
Helminthosporium sacchari IFO 9283	>100	>100	>100	>100
Arthroderma tuberculatum IFO 8165	>100	>100	>100	>100
Candida albicans IFO 1060	>100	>100	>100	>100
C. guilliermondii IFO 0838	>100	>100	>100	>100
C. lusitaniae IAM 12189	>100	>100	>100	1.56
C. utilis IAM 4220	>100	>100	>100	3.13
C. krusei IFO 0011	100	>100	>100	1.56
Saccharomyces cerevisiae IFO 0304	0.78	3.13	3.13	≦0.05
S. cerevisiae Kyokai No. 7	1.56	12.5	6.25	≦0.05
S. delbrueckii IAM 12236	25	>100	>100	3.13
S. rosei IFO 1143	25	>100	100	3.13
Schizosaccharomyces pombe IFO 0362	>100	>100	>100	12.5
Rhodotorula rubra IFO 0001	>100	>100	>100	6.25
Pichia farinosa IFO 0193	>100	>100	>100	6.25

Gram-negative bacteria. The antifungal spectrum of component S-632-B_1 is similar to that of component S-632-B_2. Meanwhile, cycloheximide (Sigma Chemical Company) showed little activity against filamentous fungi.

The cytotoxic activity *in vitro* of components S-632-A_2, B_1 and B_2 was determined on KB carcinoma cells. KB cells (2×10^4 cells/ml) were cultured in EAGLE's minimal essential medium supplemented with 10% calf serum at 37°C under a 5% CO_2 - 95% air atmosphere. After incubation for 24 hours, the various concentrations of the antibiotics were added to the culture medium. Concentration of the antibiotic required for 50% inhibition of cell growth (IC_{50}, μg/ml) was determined by plotting the logarithms of the concentration versus the growth rate of the treated cells. When the cells were exposed to the antibiotics for 3 days, S-632-B_1, B_2 and A_2 possessed cytotoxic activity with IC_{50} values of 0.03, 3 and 1 μg/ml, respectively. Cycloheximide exhibited an IC_{50} value of 0.08 μg/ml. In addition, the antitumor activity of the antibiotic S-632-B_1 *in vivo* was determined by ip injection in BDF_1 mice. No activity was observed against leukemia P388 at the dose up to 80 mg/kg. The acute toxicity of S-632-B_1 in mice was $LD_{50}>80$ mg/kg (ip).

The effect of S-632-B_1 on the incorporation of [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]leucine at concentrations of 1~100 μg/ml into acid precipitable macromolecules of KB carcinoma cells was determined. When cells were exposed to the antibiotic for 5 hours, inhibition of incorporation of [^{3}H]-leucine into 5% TCA insoluble materials was relatively more marked than that of the other two precursors at lower concentrations of the antibiotic. The results suggest that protein synthesis is the primary target of antibiotic S-632-B_1.

Acknowledgments

The authors are indebted to Professor M. NAKAYAMA of University of Osaka Prefecture, Dr. M. HAMADA of Institute of Microbial Chemistry and Dr. Y. YAMADA in our laboratory, for their valuable comments and suggestions during the course of this study.

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