Antibiotic Complex SP-351 Produced by a Psychrophile, *Streptomyces* sp. No. 351[†]

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In the course of a screening study for antibiotics using psychrophilic microorganisms a water-insoluble antibiotic complex, SP-351, was found in the culture filtrate of a psychrophilic actinomycete, strain No. 351. This active principle was isolated and characterized as a cyclicpolylactone antibiotic. The SP-351-producing strain was classified as a facultative psychrophile and identified as *Streptomyces phaeochromogenes*.

The main components of the antibiotic complex SP-351 changed with the composition of the culture medium but not with culture temperatures. Component A was exclusively produced in a medium composed of roasted soybean powder and glycerol; components B and C in a medium composed of soybean, glycerol and potassium nitrate; and components A and D in a synthetic medium containing a hydrocarbon, alcohol or ester as the sole carbon source. Maximum production of SP-351 from *n*-paraffin and methyl acetate was 10 and 15 mcg/ml, respectively.

SP-351 showed strong antibacterial activity against Gram-positive bacteria and acid-fast bacteria at 0.1~0.3 mcg/ml concentrations.

In a previous paper,¹⁾ the isolation of a psychrophilic *Streptomyces* sp. No. 81, and its antibiotic production at low temperatures were described. Further investigation has revealed that this organism is a new species of *Streptomyces* which was identified as *Streptomyces griseus* subsp. *psychrophilus* Yoshida, Tani and Ogata,²⁾ and that the antibiotics produced by this organism at low and moderate temperatures, respectively, are new peptide antibiotics, which we named cryomycin²⁾ and M-81.³⁾

During a screening study for antibiotics using psychrophilic microorganisms, another *Streptomyces* strain, No. 351, was found to produce a water-insoluble antibiotic complex to the same degree, at both low and moderate temperatures. This organism is characterized as a psychrophile due to its more abundant

growth at low temperatures than at moderate ones.

The antibiotic complex SP-351 produced by strain No. 351 was separated into the four active components A, B, C and D on a silicic acid thin-layer chromatogram. The main components of this antibiotic are altered by cultivation conditions, especially by the culture medium used.

Strain No. 351 as well as *Streptomyces griseus* subsp. *psychrophilus* assimilated alcohols, esters and *n*-paraffins as the sole carbon source and produced its own antibiotics.⁴⁾ The antibiotic produced from these carbon sources by strain No. 351 contained components A and D.

The properties of strain No. 351 and the isolation and characterization of the antibiotic complex SP-351, as well as its biological activity, are reported in this publication.

MATERIALS AND METHODS

1) Media for production of antibiotic complex

[†] Studies on Antibiotics Produced by Psychrophilic Microorganisms. Part II. Part of this work was presented at the meeting of the Kansai Division of the Agricultural Chemical Society of Japan held in Kyoto, February 19, 1972.

SP-351. The following three media were used to produce the antibiotic.

a) A medium composed of roasted soybean powder (*kinako* in Japanese) 20 g; glycerol 20 g; yeast extract 2 g; KH_2PO_4 2 g; NaCl 2 g and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g in 1000 ml of tap water at pH 6.0, was used to produce mainly SP-351 A.

b) A medium composed of soybean (dried or raw) 20 g; glycerol 20 g; yeast extract 2 g; K_2HPO_4 0.5 g; NaCl 2 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g and KNO_3 2 g in 1000 ml of tap water at pH 7.0, was used to produce mainly SP-351 B and C. Soybean was independently extracted by boiling and freed from the broth before being mixed with other components.

c) To produce SP-351 from hydrocarbons *etc.*, a medium composed of the carbon source of 30 ml; $(\text{NH}_4)_2\text{SO}_4$ 5 g; K_2HPO_4 2 g; KH_2PO_4 1 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5 g and a vitamin solution of 60 ml in a total volume of 1000 ml at pH 7.0, was used. The vitamin solution contained thiamine-HCl 8 mg; riboflavin 8 mg; nicotinic acid 16 mg; folic acid 160 μg ; biotin 64 μg ; Ca-pantothenate 8 mg; *p*-aminobenzoic acid 1.6 mg and pyridoxol 250 μg in a total of 1000 ml. As the sole carbon source; *n*-paraffins, methanol,

ethanol, methyl acetate and ethyl acetate were employed.

2) *Assay systems for the antibiotic complex SP-351.* The pulp disk method or agar dilution streak method using *Micrococcus lysodeikticus* IFO 3333 as the test organism was used to estimate the biological activity of SP-351. The purity of the antibiotic was assayed with thin-layer chromatography using chloroform-ethyl acetate (1: 2) as the developer.

RESULTS AND DISCUSSION

More than half of the isolated psychrophiles possessing antibacterial activity at low temperatures showed the activity also at moderate temperatures. Of these, one actinomycete, strain No. 351, which showed antibacterial activity to the same degree at both low and moderate temperatures, was used in the following experiments.

TABLE I. CULTURAL PROPERTIES OF STRAIN NO. 351

Medium	Growth	Aerial mycelium	Vegetative mycelium	Soluble pigment	Remarks
Gelatin stab	good, surface	pinkish white		dark brown purple	slow and complete liquefaction
Glucose-asparagine agar	good, spreading	greenish gray to pinkish gray, flocculent	brown	light purplish gray	
Bouillon agar	good	pinkish white	brown	dull red	
Glucose bouillon agar	abundant, elevated	white to greenish gray, flocculent			
Starch agar	good, spreading	light grayish green, lichenous, alveolate	white	violet	
Yeast ext. agar	abundant	dull greenish white, alveolate	dark brown	dark brown purple	
Czapek agar	moderate, spreading	pale green, powdery	white	light brown	
Tyrosine agar	scant	thin, colorless	gray	pale brown	
Milk	ring formation on surface	pinkish gray			alkaline, rapid peptonization
Potato plug	abundant	light purplish gray	pinkish gray	black	

No growth occurred on Carrot plug.

Taxonomical studies on the isolated actinomycete, strain No. 351

Morphological and cultural properties. Vegetative mycelia of this strain developed well on most of the media used (Table I). On various media, it formed abundant aerial mycelia of about $1\ \mu$ wide, which developed into many straight or flexing branches but did not form whorls nor spirals. On an electron-microscopic observation, spore surfaces of this strain were smooth (Fig. 1). Soluble purple pigments were produced in both natural and synthetic media.

Physiological properties. Physiological properties of the strain are summarized in Table II. The organism utilized most of the car-

FIG. 1. Electron Micrograph of Strain No. 351. ($\times 2,500$)

TABLE II. PHYSIOLOGICAL PROPERTIES OF STRAIN NO. 351

Gelatin liquefaction	positive
Action on milk	alkaline, ring formation, rapid peptonization
H ₂ S formation	negative
Indole formation	negative
NH ₃ formation	positive
Nitrate reduction	positive
Cellulolytic activity	positive (weak)
Starch hydrolysis	good
Catalase	present
Urease	present
Tyrosinase	present
Assimilation of citrate	positive
Optimum temperature	25°C
(No growth occurs above 35°C)	

bohydrates tested except sodium acetate and sorbitol. Cellulolytic activity was weak.

Growth temperature. As shown in Fig. 2, the strain grew between 5°C and 32°C. The maximum cell yield was obtained at 12°C, while the most rapid growth was observed near 25°C. No growth occurred above 35°C. These characteristics provide additional evidence that strain No. 351 should be classified as a psychrophilic microorganism.

From these results, we concluded that this strain belongs to the genus *Streptomyces*, based on the description in Bergey's Manual, 7th ed.⁶⁾ Comparative studies among the known species in this genus suggest that the tested strain is closely related to *Streptomyces phaeochromogenes* Waksman and Henrici, 1948. However, all the strains of *S. phaeochromogenes* from AKU* type culture collections could not grow well at low temperatures; their maximum cell yield was observed at moderate temperatures. Thus, we identified strain No. 351 as *Streptomyces phaeochromogenes* AKU 2882.

Effect of temperature on growth and antibiotic production

The temperature range, in which strain No. 351 could grow and produce the antibiotic, was investigated by culturing the organism at various temperatures (Fig. 2). The velocities of growth and antibiotic production accelerated with increased temperature. Almost the same maximum production of antibiotic was observed between 15°C and 28°C. Maximum antibiotic production was reached after cultivation at 15°C for 5 days, and at 20~28°C for 4 days.

Effect of the culture medium on antibiotic production

As shown in Fig. 3, the antibiotic complex SP-351 produced by strain No. 351 separated into the four components on a silicic acid

* AKU: Abbreviation for Culture Collections, Faculty of Agriculture, Kyoto University, Kyoto, Japan.

FIG. 2. Effect of Temperature on Growth and Antibiotic Production of Strain No. 351 in Medium (b).

* The growth is designated as a mycelial dry weight.

** The growth rate is designated as the period necessary for strain No. 351 to achieve a half maximum growth.

FIG. 3. Thin-layer Chromatogram of Antibiotic SP-351.

Solvent: Chloroform-ethyl acetate (1:2).

Detection: 20% H_2SO_4

thin-layer chromatogram. We observed that the main components changed with the culture medium but not with the culturing temperature. The medium (a) composed of roasted

soybean powder and glycerol induced the production of component A, and the medium (b) composed of soybean, glycerol and potassium nitrate induced the production of components B and C.

The product from non-sugar carbon sources was a mixture of two components. One was component A and the other was the substance which closely resembled component D. The most utilizable carbon sources were methyl acetate and ethyl acetate (Table III).

TABLE III. PRODUCTION OF SP-351 FROM VARIOUS NON-SUGAR CARBON SOURCES BY STRAIN NO. 351

Cultivation was carried out at 28°C for 4 days.

Carbon source	SP-351 Produced (mcg/ml)
No carbon (control)	—
Kerosene	—
NP-200 ^{a)}	5
910-363B ^{b)}	trace
Light gas oil ^{c)}	10
Methyl acetate	15
Ethyl acetate	15
Methanol	7
Ethanol	8
Sodium acetate	—

^{a)} *n*-paraffins (C_{14} 68.1%, C_{15} 29.9%)

^{b)} *n*-paraffins (C_{13-17} 75.5%)

^{c)} L.G.O. (*n*-paraffins 40.2%, naphthenes 58.8%)

Isolation of the antibiotic

The antibiotic was produced in the culture broth but not in the mycelia. After removal of the mycelia by centrifugation, the culture filtrate was mixed with a half volume of ethyl acetate. Then the organic phase was separated from aqueous one and condensed *in vacuo* to dryness. Dried matter was extracted with a small volume of petroleum ether, and the petroleum ether-soluble fraction was subjected to silicic acid column chromatography, followed by elution with a mixed solvent of benzene and ethyl acetate (6:4). The active fractions were collected, decolorized with charcoal and condensed *in vacuo* to a small volume. The condensed solution was allowed to stand overnight in a refrigerator to give

TABLE IV. PHYSICOCHEMICAL PROPERTIES OF THE ANTIBIOTIC COMPLEX, SP-351

Properties	SP-351				Nonactin (Corbaz <i>et al.</i>)	Monactin (Beck <i>et al.</i>)
	A	B	C	D		
Melting point	145°~146°C	62°~63°C	66°~67°C	68°C	148°C	63°~64°C
Elemental analysis	C 65.42	C 65.82			C 65.13	C 65.58
	H 8.75	H 9.12			H 8.81	H 8.85
	O 25.83	O 25.05			O 26.20	O 25.56
Molecular weight	736	750	764	778	737	752
	(Mass)	(Mass)	(Mass)	(Mass)		
Formula					C ₄₀ H ₆₄ O ₁₂	C ₄₁ H ₆₆ O ₁₂
Rf value ^{a)}	0.88	0.75	0.46	0.25	0.62	0.48
UV max	end absorption				end abs.	end abs.
Nature	neutral colorless needles, insol. in H ₂ O				insol. in H ₂ O	insol. in H ₂ O
IR	the same spectrum					

^{a)} Developer: CHCl₃-EtOAc (1:2)

crude crystals of antibiotic SP-351. Recrystallization from *n*-hexane gave colorless needles.

Physicochemical properties of SP-351

Physicochemical properties of antibiotic SP-351 are listed in Table IV. The amounts of components C and D isolated were too

small to use for elemental analysis. All components of SP-351 are neutral, colorless needles, and are insoluble in water. The ultraviolet and infrared absorption spectra of all components were the same (Figs. 4 and 5). Almost the same NMR spectra were obtained for SP-351 A and B (Figs. 6 and 7). From these characteristics, SP-351 was assumed to be a cyclicpolylactone antibiotic. From

FIG. 4. Ultraviolet Absorption Spectrum of Antibiotic SP-351.

FIG. 6. NMR Spectrum of SP-351 A in CCl₄ Solution with TMS as Internal Reference.

FIG. 5. Infrared Spectrum of SP-351 in KBr Tablet.

FIG. 7. NMR Spectrum of SP-351 B in CCl_4 Solution with TMS as Internal Reference.

comparative studies with known substances, SP-351 A was found to be quite similar to nonactin⁶⁾ and lustericin.⁷⁾ However, lustericin has a melting point at 130°C, and its

molecular weight is about 780. As shown in Table IV, SP-351 A and B are, respectively, quite similar to nonactin and monactin.⁸⁾

Biological properties of SP-351

The biological activity of SP-351 was studied with components A and B. No difference between their activities was observed. As shown in Table V, SP-351 had a strong inhibitory activity against Gram-positive bacteria and some yeasts.

TABLE V. ANTIMICROBIAL SPECTRUM OF SP-351 (continued).

Microorganism	M.I.C. (mcg/ml)	Microorganism	M.I.C. (mcg/ml)
<i>Escherichia coli</i> K 12 IFO 3208	— ^{a)}	<i>Mucor mucedo</i> IFO 5776	—
<i>Aerobacter aerogenes</i> IFO 3319	—	<i>Rhizopus oryzae</i> M-21	—
<i>Serratia polymuthicum</i> IFO 3055	0.2	<i>Absidia lichteimi</i> IFO 4009	—
<i>Proteus vulgaris</i> IFO 3045	—	<i>Aspergillus oryzae</i> M-61	—
<i>Alcaligenes faecalis</i> IFO 3160	0.2	<i>Penicillium chrysogenum</i> IFO 4626	—
<i>Flavobacterium arborescens</i> IAM 1100	0.2	<i>Monascus purpureus</i> IAM 8010	—
<i>Bacillus subtilis</i> IFO 3037	0.8	<i>Neurospora crassa</i> IFO 6068	—
<i>Micrococcus lysodeikticus</i> IFO 3333	0.1	<i>Oospora viscosa</i> IFO 4604	—
<i>Staphylococcus aureus</i> IFO 3060	0.8	<i>Fusarium lini</i> IFO 5880	—
<i>Sarcina lutea</i> IFO 1099	0.2	<i>Gibberella fujikuroi</i> IFO 5268	—
<i>Corynebacterium equi</i> IAM 1038	0.8	<i>Ustilago zeae</i> IFO 5346	—
<i>Arthrobacter simplex</i> IFO 3530	0.6	<i>Keratinomyces ajelloi</i> IFO 7865	—
<i>Brevibacterium ammoniagenes</i> IFO 12071	0.2	<i>Sporotrichum schaeenkii</i> IFO 5983	—
<i>Bacterium cadaveris</i> IFO 3731	—	<i>Verticillium arbo-atrum</i> IFO 5922	—
<i>Pseudomonas aeruginosa</i> IFO 3080	—	<i>Isaria kogane</i> IFO 5299	—
<i>Streptococcus faecalis</i> IFO 3181	0.3	<i>Gliocladium deliquescens</i> IFO 6617	—
<i>Pediococcus hennevergi</i> IFO 3884	0.3	<i>Phytophthora infestans</i> IFO 4872	—
<i>Leuconostoc mesenteroides</i> IFO 3426	0.3	<i>Trichophyton sulfureum</i> IFO 5945	—
<i>Lactobacillus plantarum</i> IFO 3070	0.3	<i>Saccharomyces cerevisiae</i> AKU 4100	—
<i>Mycobacterium avium</i> IFO 3154	1.5	<i>Eremascus fertilis</i> IFO 0691	—
<i>Nocardia gardneri</i> IFO 3385	1.5	<i>Endomyces hordei</i> IFO 0104	0.8
<i>Streptomyces albus</i> AKU 2201	4.5	<i>Shizosaccharomyces pombe</i> IFO 0346	—
" <i>rimosus</i> IFO 3226	—	<i>Zygosaccharomyces soja</i> IFO 0495	—
" <i>griseus</i> IFO 3430	—	<i>Pichia polymorpha</i> IFO 0195	—
" <i>griseus</i> IFO 3237	2.2	<i>Hansenula anomala</i> AKU 4300	—
" <i>griseus</i> IFO 3357	3.5	<i>Schwanniomyces occidentalis</i> IFO 0371	—
" <i>fradiae</i> IFO 3439	1.0	<i>Debaryomyces japonicus</i> IFO 0039	—
" <i>fradiae</i> IFO 3123	1.0	<i>Saccharomycodes ludwigii</i> IFO 0339	6.0
" <i>antibioticus</i> IFO 3117	4.5	<i>Hanseniaspora valbyensis</i> IFO 0115	—
		<i>Lipomyces lipoferus</i> IFO 0673	—
		<i>Sporobolomyces salmonicolor</i> IFO 0374	6.0
		<i>Torulopsis candida</i> IFO 0768	—
		<i>Candida albicans</i> IFO 0197	—
		<i>Trigonopsis variabilis</i> IFO 0671	1.6
		<i>Torula rubra</i> AKU 4730	—
		<i>Trichosporon cutaneum</i> IFO 0174	—

^{a)} No inhibition was observed at 20 mcg/ml concentration.

CONCLUSION

SP-351 possesses properties characteristic to cyclicpolylactone antibiotics such as lustericin, N-329A,⁹⁾ nonactin and monactin. However, SP-351 differs from all of these in its isolation procedure. Lustericin, N-329A, nonactin and its related compounds are extracted from mycelia but not from the culture broth, while SP-351 is extracted from the culture filtrate. Extraction of the mycelial cake of strain No. 351 with methanol or acetone produced no antibiotic.

Furthermore, SP-351 does not show antifungal activity against phytopathogenic fungi as does *Piricularia oryzae* and *Helminthosporium sigmoideum*.¹⁰⁾ Other cyclicpolylactone antibiotics inhibit the growth of *Piricularia oryzae* at a concentration of 0.1~0.2 mcg/ml.

The minimum inhibitory concentration of lustericin or nonactin against *Micrococcus* sp. is 0.5 mcg/ml, and that of SP-351 is 0.1 mcg/ml.

The SP-351-producing organism differs from *Streptomyces* sp. 10400,⁷⁾ the producer of lustericin and from *Stm. tsushimaensis*,⁹⁾ *Stm.* SC 3763¹¹⁾ and *Stm. werraensis*,¹²⁾ the producers of nonactin, in the following respects: 1) Strain No. 351 produces soluble purple pigments while the others produce none or brown soluble pigments. 2) Strain No. 351 is psychrophilic, whereas the others are mesophilic and show poor growth at low temperatures.

Strain No. 351 is notable for its good growth and antibiotic production at low temperatures as well as at moderate ones. And, it has the striking characteristics of assimilating non-sugar carbon sources, *i.e.* alcohols, esters,

n-paraffins *etc.* to produce SP-351.

Based on these results and comparisons, SP-351 is seen as a new cyclicpolylactone antibiotic which closely resembles nonactin and its related compounds.

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