Studies on Antibiotics Produced by Psychrophilic Microorganisms

Part I. Production of Antibiotics by a Psychrophile, *Streptomyces* sp. No. 81[†]

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A screening for antibacterial antibiotics was carried out with psychrophilic microorganisms. The most active microorganism, a soil actinomycete, was selected and characterized to be a facultative psychrophile, *Streptomyces* sp. No. 81. This strain was found to produce antibiotic(s) in the culture fluid only at low temperature cultivation below 20°C but not at moderate temperature. Mycelial growth at low temperature seemed to be indispensable for the antibiotic production. The antibiotic produced by *Streptomyces* sp. No. 81 was isolated and characterized. It appeared that the antibiotic had the selective toxicity against several Gram-positive bacteria. From the comparative studies with several known antibiotics, the antibiotic appears to be a new compound derived from the new metabolic routes involving temperature-sensitive mechanisms.

Although it has generally been known that microorganisms do not grow and multiply well at low temperature, it has recently become a serious problem that foods and biological materials were spoiled by cold-tolerant microorganisms, so-called psychrophiles, during the storage at low temperature. Psychrophiles are widely found in nature, not only in polar regions¹⁾ but also in temperate zones²⁾ and in the oceans.³⁾

Studies on the relationship between cultural temperature and microbial activities have been made by many investigators.⁴⁻⁸⁾ But few

attempts have been performed on the fermentative utilization of these microorganisms.⁹⁾ It may be assumed that the low temperature cultivation of psychrophiles leads to the production of the metabolites different from those produced at moderate temperature.

Up to date, antibiotics have been discovered exclusively by the microbial cultivation at moderate temperature but never at low temperature. During the investigation on the low temperature fermentation,^{10,11)} the authors

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made attempts to isolate antibiotic-producing psychrophiles from various samples, and obtained some strains of bacteria and actinomycetes.

In this paper, the authors will describe the isolation and characterization of a psychrophilic actinomycete, strain No. 81, which produces antibiotic(s) only at low temperature but not at moderate temperature. The production and some characteristics of the antibiotic shall also be presented.

MATERIALS AND METHODS

1) Media

a) *Media for isolation of psychrophiles.* Krainsky's agar medium which is composed of glucose 10 g, L-asparagine 0.5 g, K_2HPO_4 0.5 g and agar 20 g in 1000 ml of tap water, pH 7.0, was used for the isolation of psychrophilic actinomycetes. Nutrient agar medium which is composed of peptone 10 g, meat extract 10 g, NaCl 5 g and agar 20 g in 1000 ml of tap water, pH 7.0, was used for isolation of psychrophilic bacteria. ZoBell's agar medium which is composed of peptone 5 g, yeast extract 1 g, $FeSO_4 \cdot 7H_2O$ 0.1 g and agar 20 g in 1000 ml of sea water, pH 7.2, was used for isolation of marine psychrophiles.

b) *Media for antibiotic formation.* The medium which is composed of soluble starch 15 g, soybean 20 g, KNO_3 2 g, yeast extract 2 g, NaCl 3 g, K_2HPO_4 2 g, KH_2PO_4 0.7 g and $MgSO_4 \cdot 7H_2O$ 0.1 g in 1000 ml of tap water, pH 6.5, was used for the screening of the antibiotics with actinomycetes. The medium which is composed of glucose 10 g, peptone 10 g, meat extract 5 g and NaCl 5 g in 1000 ml of tap water, pH 7.0, was used for the screening of the antibiotics with terrestrial bacteria. The medium which is composed of glucose 10 g, peptone 10 g and meat extract 5 g in 1000 ml of sea water, pH 7.0, was used for the screening of the antibiotics with marine bacteria.

c) *Medium for antibiotic assay.* For the assay of antibiotics, the medium which is composed of peptone 10 g, meat extract 5 g, NaCl 5 g and agar 20 g in 1000 ml of tap water, was used. The pH was adjusted to 7.0 in usual cases, and to 6.0 in the case of assay of the antibiotic produced by *Streptomyces* sp. No. 81.

2) Isolation method of psychrophiles

Suspensions of natural materials such as soil, sewage, stream and river water, and sea water, were streaked on the Krainsky's agar plates for actinomycetes, the nutrient agar plates for terrestrial bacteria and the ZoBell's agar plates for marine psychrophiles, respectively. After the incubation at 5°C or 15°C for 7 to 10 days, the microorganisms grown on the plates were transferred to the agar slants of the same composition. The slants were incubated at 5°C or 15°C for 5 to 7 days and then stored at 5°C.

3) Cultivation

For the investigation of the antibiotic formation, one loopful of organism grown on each agar slant was inoculated into 5 ml of the media mentioned above. The cultivation was carried out on a reciprocal shaker at 5°C for about 2 weeks.

4) Microorganisms

Escherichia coli K-12 IFO 3208, *Proteus vulgaris* IFO 3167, *Bacillus subtilis* IFO 3037 and *Staphylococcus aureus* IFO 3060 were used as test organisms for the assay of antibiotics.

RESULTS AND DISCUSSION

Isolation of antibiotic-producing psychrophiles

About 70 pure cultures of actinomycetes, 1000 pure cultures of terrestrial bacteria and

FIG. 1. Growth and Antibiotic Production of Strain No. 81.

The antibiotic was estimated by agar dilution method with *Bacillus subtilis* IFO 3037 at pH 6.0.

TABLE I. CULTURAL PROPERTIES OF STRAIN No. 81

Medium	Vegetative mycelium	Aerial mycelium	Soluble pigment	Remarks
Czapek's agar	Spreading, white	Good, powdery, yellowish white	None	
Krinsky's agar	Good, grayish white	Abundant, powdery, pinkish white to gray	None	
Ca-malate agar	Good spreading, yellowish white	Abundant, powdery, white	None	
Starch-ammonium agar	Good spreading, brownish white	Abundant, powdery, yellowish white	None	
Bouillon agar	Good spreading, pinkish white	Abundant, powdery, white	None	
Starch agar	Moderate, gray	Scanty, powdery, white	None	Weak hydrolysis
Glucose-Czapek	Poor, spreading, gray	Poor, gray	None	
Glucose-bouillon	Abundant, penetrating, brown	Abundant, white	None	
Litmus milk	Moderate, gray	Moderate, green to white		Alkaline, rapid peptonization, weak coagulation
Cellulose solution	None	None	None	
Gelatin stab	Penetrating into the medium, gray	Moderate, gray to white	None	Liquefaction
Potato plug	Elevated, abundant, yellowish white	Abundant, grayish white		

The strain No. 81 was incubated on each medium at 28°C for 4 days.

600 pure cultures of marine bacteria capable of growing at 5°C, were isolated from natural materials. The antibacterial activity against Gram-positive or -negative bacteria was detected in the cultured broth of one seventh of actinomycetes isolated and one tenth of terrestrial and marine bacteria isolated. Most of them, however, showed stronger antibacterial activity by the cultivation at 28°C. The significant antibacterial activity was obtained by the cultivation of an actinomycete, strain No. 81, at 5°C but not at 28°C (Fig. 1). Then, the strain No. 81 was employed in the following experiments.

Taxonomical studies on the isolated actinomycete, strain No. 81

Morphological and cultural properties. The vegetative mycelia of the strain developed well on the most media used (Table I). On various media, it formed abundant aerial mycelia of about 1 μ in width which developed

FIG. 2. Microscopic Photograph of Strain No. 81.

into many straight or flexuous branches (Fig. 2). Tips of aerial mycelia bore conidial spore chains. The spores were cylindrical or rec-

FIG. 3. Electronmicrograph of Conidia of Strain No. 81. (2,500×4)

tangular and $0.8-1.0\mu \times 1.5\mu$ in size. The spore surface was smooth under electron-microscopy. (Fig. 3).

Physiological properties. Physiological properties of the strain are summarized in Table II. The organism utilized the most of carbohydrates tested.

Growth temperature. As shown in Table II, the strain grew between 0°C and 37°C . The optimum temperature was near 28°C . It was also observed that no growth occurred above

TABLE II. PHYSIOLOGICAL PROPERTIES OF STRAIN No. 81

Chromogenesis	Negative
Gelatin liquefaction	Positive
Action on milk	Alkaline
	Rapid peptonization
	Weak coagulation
H ₂ S formation	Positive
Indole formation	Negative
NH ₃ formation	Positive
Nitrate reduction	Positive
Methyl red test	Negative
Urease activity	Positive
Catalase activity	Positive
Starch hydrolysis	Positive
Cellulolytic activity	Negative
Temperature range	Facultative psychrophilic, $0^{\circ}\text{C}\sim 37^{\circ}\text{C}$

37°C . According to the description by Stokes¹²⁾ based on optimum growth temperature, it seemed that the organism should be classified in a facultative psychrophile.

From these results, it was concluded that this strain belongs to the genus *Streptomyces*, according to the description in the Bergey's Manual, 7th ed.¹³⁾ The comparative studies

TABLE III. EFFECT OF TEMPERATURE ON THE GROWTH AND ANTIBIOTIC PRODUCTION OF *Streptomyces* sp. No. 81

Temperature	Maximum mycelial amount (dry weight)	Cultivation period (Lag time)	Antibiotic produced
0°C	3.5 mg/ml	26 days (12 days)	40 U/ml
5	4.5	11 " (6 days)	200
10	4.5	6 " (4 days)	100
15	6.2	5 " (3 days)	30
20	8.4	3 " (30 hr)	5
28	8.8	3 " (8 hr)	—
37	2.5	3 " (10 hr)	—
42	No growth	3 "	—

One loopful spores of *Streptomyces* sp. No. 81 were inoculated into test tubes containing 5 ml of sterile starch-soybean medium, and incubated on a shaker at each temperature. Mycelial dry weight and antibacterial activity were estimated periodically. The antibiotic was estimated as described in Fig. 1.

12) J. L. Stokes, "Recent Progress in Microbiology," VIII, ed. by N. E. Gibbons, Univ. Toronto Press, Toronto, 1962, p. 187.

13) R. S. Breed, E. G. D. Murray and N. R. Smith, "Bergey's Manual of Determinative Bacteriology," 7th ed., The Williams and Wilkins Co., 1957.

among the known species in the genus suggested that the tested strain was closely related to *Streptomyces griseus*. However, the former differed from the latter in a few characteristics such as growth temperature, and then was tentatively named as *Streptomyces* sp. No. 81.

Effect of temperature on the growth and antibiotic production

The temperature range, in which the strain No. 81 could grow and produce the antibiotic(s), was investigated by cultivating the organism at various temperatures (Table III). The growth and its rate were increased with increased temperature. The antibiotic was produced by the cultivation below 20°C but not above 20°C. The activity of the antibiotic in the cultured broth reached a maximum value after 11 days cultivation at 5°C and then gradually decreased. It was also recognized that the organism showed a good growth and produced considerable quantity of the antibiotic even at 0°C.

Effect of temperature shift on the antibiotic production

An attempt was made to shift the temperature from 28°C to 5°C during the cultivation

of the organism at the following three points: 1) at the beginning of macroscopical multipli-

FIG. 4. Effect of Temperature Shift from 28°C to 5°C on the Growth and Antibiotic Production of *Streptomyces* sp. No. 81.

Cultivation temperature was shifted from 28°C to 5°C at the times indicated by the arrows (0, 8, 18 and 48 hr). The antibiotic was estimated as described in Fig. 1.

TABLE IV. DEGRADATIVE ACTIVITY OF INTACT MYCELIUM OF *Streptomyces* sp. No. 81 ON ITS OWN ANTIBIOTIC

Incubation temperature	Mycelium	Residual antibacterial activity ^{a)}					
		Incubation time (hr)					
		0	1	3	6	12	24
5°C	None	200 U/ml	200 U/ml	200 U/ml	200 U/ml	200 U/ml	200 U/ml
	Mycelium A ^{b)}	200	200	200	200	200	200
	Mycelium B	200	200	200	200	200	150
28°C	None	200	200	200	200	150	150
	Mycelium A	200	200	200	200	200	200
	Mycelium B	200	200	200	150	150	100

Each reaction mixture consisted of 500 units of the antibiotic, 250 μ moles of potassium phosphate buffer, pH 6.5, and 12.5 mg of intact mycelium in a final volume of 2.5 ml. Incubation was carried out with shaking at 5°C and 28°C, respectively.

^{a)} The antibiotic was estimated as described in Fig. 1.

^{b)} The mycelium A and B were obtained by the cultivation of *Streptomyces* sp. No. 81 at 5°C for 12 days, and at 28°C for 4 days, respectively.

cation after 8 hr incubation; 2) in the midst of the exponential growth after 18 hr incubation; and 3) at the end of the exponential growth after 48 hr incubation. Preincubation of the organism at 28°C hastened the growth and shortened the over-all cultivation period, but decreased the yield of the antibiotic. When cultivation was carried out at 28°C for 48 hr and then the temperature was shifted to 5°C, the antibiotic production no longer proceeded (Fig. 4). This may indicate that the multiplication of the organism at 5°C is indispensable for the antibiotic production.

Followingly, an attempt was made to investigate whether the mycelium grown at 28°C possessed the stronger activity of destroying the antibiotic accumulated than that grown at 5°C with intact mycelia. The each mycelium cultivated at 5°C for 12 days and at 28°C for 4 days was aseptically harvested, washed and transferred into the potassium phosphate buffer solution, pH 6.5, which contained 200 units of the antibiotic per ml, and then incubated on a reciprocal shaker at 5° and 28°C, respectively. The antibacterial activity was periodically assayed. As shown in Table IV, the mycelia grown at 28°C as well as at 5°C possessed a weak or no degradative activity to the antibiotic. Moreover, the antibiotic was recognized to be heat-stable because the antibacterial activity in the cultured broth was not reduced by boiling for 3 min.

From these results, it seemed that the biosynthetic mechanisms of the antibiotic might be temperature-sensitive ones.

Isolation of the antibiotic

The antibiotic produced by *Streptomyces* sp. No. 81 was more stable at acidic pH range and showed the stronger antibacterial activity in the assay media in which pH was adjusted to 6.0 than those adjusted to 7.0 or 8.0 (Table V). According to the general consideration that antibiotics which show strong activity at acidic pH range are acidic compounds, the

TABLE V. RELATION BETWEEN pH AND MINIMAL INHIBITORY CONCENTRATION OF THE GROWTH OF TEST ORGANISMS

Organism	MIC (U/ml) ^{a1}		
	pH		
	6	7	8
<i>Bacillus subtilis</i> IFO 3037	1	2	5
<i>Bacillus megaterium</i> NIH B12	0.5	1	2
<i>Staphylococcus aureus</i> IFO 3060	5	10	20
<i>Micrococcus lysodeikticus</i> IFO 3333	1	2	5
<i>Sarcina lutea</i> IAM 1099	2	3	5

^{a1} Agar dilution streak method was employed.

The pH of the medium was adjusted to 6, 7 and 8, respectively. The antibiotic was estimated as described in Fig. 1.

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Broth filtrate
|
| -adjusted pH to 2.0 with HCl
| -extracted with n-butanol
|
Butanol layer
|
| -condensed to a small volume
|
Butanol solution
|
| -extracted with NaHCO3 solution
|
Aqueous solution
|
| -adjusted pH to 2.0 with HCl
| -extracted with n-butanol
|
Butanol layer
|
| -dehydrated with Na2SO4
| -ethyl acetate was added
|
Residue
|
| -dissolved in ethanol
|
Ethanol solution
|
| -ethyl ether was added
|
Residue
|
| -dried up
|
Light-brown powder

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FIG. 5. Isolation and Purification of the Antibiotic Produced by *Streptomyces* sp. No. 81.

antibiotic produced by this organism is thought to be an acidic one. The isolation procedure of the antibiotic was comparable to that used for other acidic antibiotics as outlined in Fig. 5.

Twenty liters of the filtrate of the cultured broth was adjusted to pH 2.0 with hydrochloric acid and extracted three times with 4 liters of

TABLE VI. ANTIMICROBIAL ACTIVITY OF THE ANTIBIOTIC PRODUCED BY *Streptomyces* sp. No. 81

Test organism	M.I.C. (mcg/ml) ^{a)}
<i>Bacillus cereus</i> IFO 3001	1
<i>Bacillus megaterium</i> NIH B12	0.5
<i>Bacillus subtilis</i> IFO 3037	1
<i>Bacillus brevis</i> IFO 3331	2
<i>Bacillus pumilus</i> IFO 3030	0.5
<i>Bacillus circulans</i> IFO 3329	2
<i>Micrococcus lysodeikticus</i> IFO 3333	0.5
<i>Micrococcus flavus</i> IFO 3242	1
<i>Micrococcus roseus</i> IFO 3764	1
<i>Micrococcus glutamicus</i> ATCC 13032	25
<i>Staphylococcus aureus</i> IFO 3332	3
<i>Sarcina aurantiaca</i> IFO 3064	1
<i>Sarcina lutea</i> IFO 1099	2
<i>Sarcina marginata</i> IFO 3066	0.5
<i>Sarcina variabilis</i> IFO 3067	2
<i>Corynebacterium pseudodiphtherium</i> AKU 601	40
<i>Arthrobacter simplex</i> IFO 3530	25
<i>Brevibacterium divaricatum</i> NRRL 2311	2
<i>Achromobacter asceris</i> IFO 3166	0.5
<i>Flavobacterium flavescens</i> IFO 3085	2
<i>Escherichia coli</i> K-12 IFO 3208	— ^{b)}
<i>Aerobacter aerogenes</i> IFO 3320	—
<i>Serratia plymuthicum</i> IFO 3055	—
<i>Proteus vulgaris</i> IFO 3045	—
<i>Alcaligenes faecalis</i> IFO 3160	—
<i>Pseudomonas aeruginosa</i> IFO 3080	—
<i>Pseudomonas fluorescens</i> IFO 3461	—
<i>Bacterium cadaveris</i> IFO 3731	—
<i>Xanthomonas campestris</i> IAM 1671	—
<i>Mucor mucedo</i> IFO 5776	—
<i>Rhizopus oryzae</i> AKU 3101	—
<i>Aspergillus oryzae</i> IFO 4117	—
<i>Aspergillus niger</i> IFO 4380	—
<i>Penicillium chrysogenum</i> IFO 4626	—
<i>Neurospora crassa</i> IFO 6068	—
<i>Saccharomyces cerevisiae</i> AKU 4100	—
<i>Endomyces hordei</i> IFO 0104	—
<i>Candida utilis</i> IFO 0396	—
<i>Mycobacterium avium</i> IFO 3154	—

^{a)} Agar dilution streak method was employed.

^{b)} Growth of test organism was not inhibited at the concentration of 100 mcg/ml.

Antibiotic used was the partially purified light-brown powder which was obtained in Fig. 5.

n-butyl alcohol followed by reextraction with 500 ml of dilute sodium bicarbonate solution. Condensed aqueous solution was again adjusted to pH 2.0 and extracted with 100 ml of *n*-butyl alcohol. Then, several parts of ethylacetate were added to the alcohol solution to give brown crude powder of the antibiotic. The crude powder was dissolved in small amount of ethyl alcohol and then precipitated with ethyl ether. The partially purified light-brown powder showed antibacterial activity more than 1000 units per mg against *Bacillus subtilis* IFO 3037.

Improved isolation and purification procedures shall be shown in later paper.

Biological properties

The biological activity was studied by using the partially purified antibiotic, obtained as shown in Fig. 5 possessing antibacterial activity more than 1000 units per mg against *Bacillus subtilis* IFO 3037. The antibacterial and antifungal spectra obtained by the agar dilution streak method are shown in Table VI. As shown in this table, the antibiotic produced by *Streptomyces* sp. No. 81 showed a strong inhibitory activity against Gram-positive bacteria, but a weak or no activity against Gram-negative bacteria, yeasts and fungi.

Comparative studies of the antibiotic, including the bioautographies and chemical reactions, indicated that the compound produced by *Streptomyces* sp. No. 81 differed from several known antibiotics such as tetracyclines, streptomycins, erythromycins, pyrrolic compounds, etc. Assuming that new metabolic reactions involving temperature-sensitive mechanisms may take place for the production of the antibiotics at low temperature, it is likely that the antibiotic produced by *Streptomyces* sp. No. 81 at low temperature is a new compound.

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