CRYOMYCIN, A NEW PEPTIDE ANTIBIOTIC PRODUCED ONLY AT LOW TEMPERATURE

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A new antibiotic, cryomycin, was isolated from the culture filtrate of a facultatively psychrophilic streptomycete. As a result of taxonomic studies, it was considered a new subspecies for which the name *Streptomyces griseus* subsp. *psychrophilus*, YOSHIDA, TANI and OGATA, is proposed. The type strain is AKU 2881. This organism grows at 0~37°C and produces cryomycin at 0~18°C. Cryomycin is a peptide antibiotic containing a rather large amount of glycine in its molecule. It darkens at 214~217°C with decomposition. This antibiotic is highly active against Gram-positive bacteria *in vitro*. The LD_{50} in mice by intravenous injection is 150 mg/kg.

In our screening studies for new antibiotics produced by psychrophilic microorganisms, a weakly acidic peptide antibiotic was found in the cultured broth of a *Streptomyces*, which was isolated at low temperature from a soil sample.

This active principle was isolated and designated as cryomycin. The cryomycin-producing strain grew at 0~37°C, and produced the antibiotic at 0°~18°C but never above 20°C. A preliminary description of this organism and its antibiotic formation was made in previous paper.[1] This paper is concerned with additional taxonomic characterization of the organism producing cryomycin and with the production, isolation and properties of the antibiotic.

Taxonomic Studies

The microorganism producing cryomycin was obtained at 15°C. The original culture, No. 81 (laboratory No.: AKU 2881)[1], was isolated from a soil sample collected at Mt. Ushio in Kyoto Prefecture, Japan. The characteristics of the strain were determined in detail according to accepted methods of identification for *Streptomyces*.

Strain No. 81 grew well on both synthetic and natural agar media and developed colonies characteristic of the genus *Streptomyces*. On most agar media, the formation of good to abundant aerial mycelium was observed. Strain No. 81 was cultivated at 15°C or 27°C for 3 weeks on various media used conventionally for characterizing *Streptomyces*. No marked differences were seen between cultures grown at 15°C and those at 27°C. The data are summarized in Table 1, and supplement those reported previously.[1]

Cultures on synthetic media were generally characterized by colorless to dull yellow brown vegetative growth with pale yellowish brown aerial mycelia and no soluble pigment. Cultures on natural media were colorless to pale yellowish orange

Table 1. Cultural characteristics of *Streptomyces* strain No. 81

Medium	Growth	Aerial mycelium	Soluble pigment	Remarks
Sucrose-nitrate agar	good, flat, pale yellow	good, powdery, pale yellow～yellowish gray	none	
Glucose-asparagine agar	good, raised, pale yellow ～yellowish gray	good, powdery, pale yellowish brown～pale brown	none	
Glycerol-asparagine agar	good, raised, pale yellow ～dull yellow	abundant, powdery, water drops on the surface, brownish white ～pale yellowish brown	none	
Inorganic salt-starch agar	good, raised, dull yellow	good, powdery, pale yellow	none	
Tyrosine agar	abundant, pale yellow～ dull yellow	abundunt, powdery, brownish white	none	
Nutrient agar	moderate, pale yellowish brown	good, yellowish white～pale yellowish orange	none	
Cellulose agar	thin, pale yellowish white	moderate, pale yellow	none	
Oat meal agar	good, raised, yellowish gray～dark yellowish brown	pale yellowish brown～grayish yellow brown	none	
Glucose-nutrient agar	abundant, elevated with dark brown reverse	good, brownish white～pale yellow orange	none	
Yeast-malt agar	good, elevated, yellowish brown	good, powdery, pale yellow～ pale yellowish brown, water drops on the surface	none	
Egg	good, spreading, pale yellowish brown	good, yellowish gray	none	medium not liquefied
LÖFFLER's coagulated serum	good, wrinkled, colorless ～pale yellowish brown	good, pale yellow orange～ brownish white	none	medium liquefied
Skimmed milk	ring formation on surface partially flocks on bottom	scant	none	peptonized, pH not changed

and produced no soluble pigment.

Strain No. 81 grew well at 15°C as well as at 27°C in the pH range of 5～9. The minimum temperature for growth of strain No. 81 was 0°C. Visible colonies occurred at 0°C after about 3 weeks, and at 5°C after 2 weeks. More rapid growth was observed as the incubation temperature increased (Fig. 1). According to the definition of psychrophilic microorganisms by STOKES[2], the strain No. 81 should be designated as a "facultatively psychrophilic" *Streptomyces*.

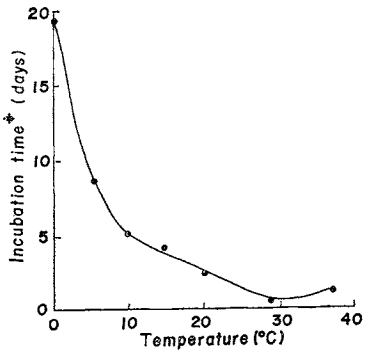
The method of PRIDHAM and GOTTLIEB was employed to dertermine the utilization of carbon sources. As shown in Table 2, most of the carbon sources tested were utilized except for raffinose and *i*-inositol.

Based on the description in the BERGEY's Mannual of Determinative Becteriology, 7 th ed., *Streptomyces griseus*, WAKSMAN and HENRICI, 1948, closely resembled strain No. 81. However, a striking characteristic of strain No. 81 was its ability to grow even at 0°C, whereas *Streptomyces griseus* barely grew at temperatures below 20°C.

In view of the above, strain No. 81 was believed to represent a new subspecies and was named *Streptomyces griseus* subsp. *psychrophilus*, YOSHIDA, TANI and OGATA. The type strain is designated AKU* 2881.

* AKU : Abbreviation of culture collection, Faculty of Agriculture, Kyoto University, Kyoto, Japan.

Fig. 1. Relation between growth rate and incubation temperature.



* Incubation time indicates the period necessary for *Streptomyces griseus* subsp. *psychrophilus*, strain No. 81 to achieve half maximum growth.

Table 2. Utilization of carbohydrates by strain No. 81

Carbohydrate	Response
L-Arabinose	++
D-Glucose	++
D-Galactose	++
D-Lactose	++
D-Fructose	++
Mannose	++
Rhamnose	++
Raffinose	±
Sucrose	+
D-Xylose	++
Salicin	++
Soluble starch	++
Inulin	+
Cellulose	+
Dulcitol	+
i-Inositol	±
D-Mannitol	++
D-Sorbitol	+
No carbon	−

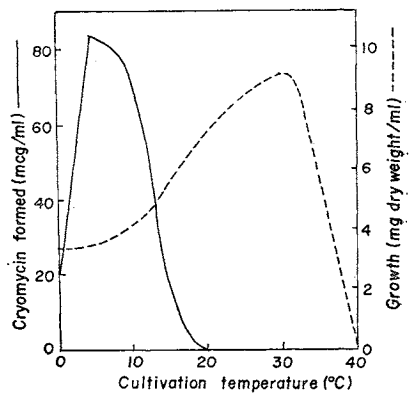
++ : Strongly positive utilization
+ : Positive utilization
± : Weakly positive utilization
− : No utilization

Fermentative Production of Cryomycin

Conditions for producing cryomycin was studied in shake culture using 1% glucose, sucrose, lactose, maltose, starch or glycerol as the carbon source, and 1% peptone, Polypeptone, meat extract, corn steep liquor, soybean powder or ammonium sulfate as the nitrogen source with 0.2% yeast extract, 0.3% NaCl, 0.05% K_2HPO_4 and 0.01% $MgSO_4 \cdot 7H_2O$ as a basal medium. Activity against *Bacillus subtilis* IFO 3037 was highest when glucose and Polypeptone or starch and soybean were used. Using a jar fermenter, the following medium was found to be the most suitable: 2.0% soybean powder, 2.0% glycerol, 0.5% soluble starch, 0.2% yeast extract, 0.2% KNO_3, 0.3% NaCl, 0.2% K_2HPO_4, 0.07% KH_2PO_4 and 0.01% $MgSO_4 \cdot 7H_2O$, pH 7.0 after sterilization.

Growth and antibiotic production of *Streptomyces griseus* subsp. *psychrophilus*, strain No. 81 at various temperatures are shown in Fig. 2. The most suitable temperature for growth was near 30°C, while that for cryomycin production was 5~7°C. The production of cryomycin was noted only at low temperatures below 18°C, and never above 20°C. Antimicrobial activity attained a maximum after cultivation at 5°C for about 10 days.

Fig. 2. Growth and cryomycin formation of *Streptomyces griseus* subsp. *psychrophilus*, strain No. 81 at various temperatures.



Isolation and Purification of Cryomycin

The antibiotic was isolated from the culture filtrate; no activity was demostrated in the mycelium.

A procedure representing a modification of that previously described[1] for the isolation of

Fig. 3. Ultraviolet absorption spectrum of cryomycin.

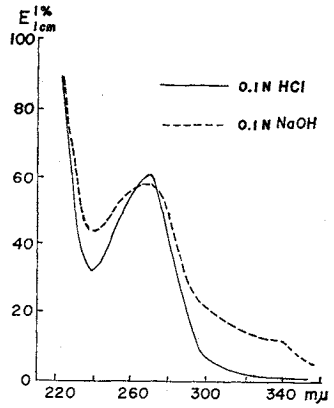
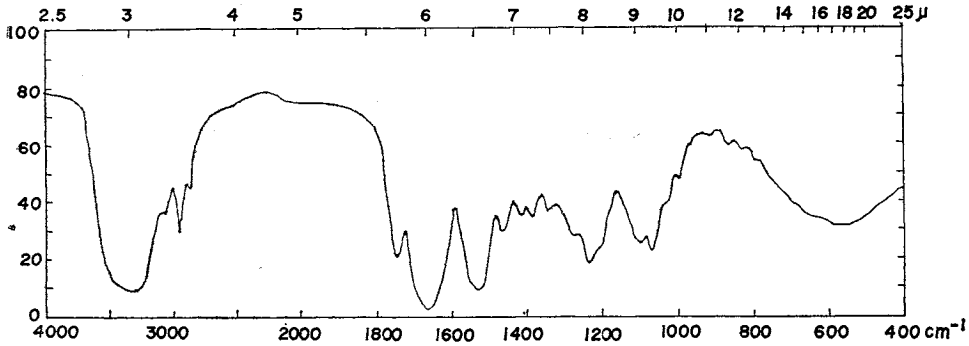


Table 3. Behaviour of cryomycin towards different chemical tests

Chemical test	Result
Ninhydrin	positive (weak)
$FeCl_3$	negative
Alkaline $KMnO_4$	reduction in the cold
Acidic $KMnO_4$	〃
Biuret	positive
Xanthoprotein	positive
MILLON	positive
SAKAGUCHI	positive (weak)
ADAM-KIEWICZ	positive
PAULI	negative
LIEBERMANN	positive
TOLLENS	negative
Dilute I_2 solution	decolorization on cooling
Anthrone	negative
BENEDICT	negative

Fig. 4. Infrared absorption spectrum of cryomycin (KBr).



cryomycin is as follows: The cultured broth filtrate was acidified to pH 2.0 with 10% hydrochloric acid, 1-butanol added, and the mixture stirred for 30 minutes. The organic phase then was separated by centrifugation and concentrated to a small volume *in vacuo*. Dilution of the concentrate with ethyl acetate yielded a precipitate which was dissolved in a small volume of water and applied to a DEAE-cellulose column equilibrated with 0.005 M NaCl. Saline water in increasing concentration was used as an eluant. Cryomycin was eluted with 0.05~0.1 M NaCl. The eluate was passed through Dowex 50W (H^+) ion-exchanger, and the active effluent condensed to a small volume under reduced pressure, further purified on Sephadex G-10 by gel chromatography and precipitated from methanol.

Physical and Chemical Properties

Cryomycin was obtained as a white powder with weakly acidic properties. It darkens at 214~217°C with decomposition. The ultraviolet absorption spectrum shows a maximum at 269 mμ ($E^{1\%}_{1cm}$ *ca.* 60) (Fig. 3). The infrared absorption spectrum has the following frequencies: 3350, 2960, 1740, 1660 and 1530 cm^{-1} (Fig. 4). Elemental analysis gave the following composition (%); C 59.79, H 7.78, N 9.95. Cryomycin is soluble in water, slightly soluble in methanol and ethanol, and insoluble in other common organic solvents.

Fig. 5. Migration of cryomycin on paper chromatograms using different developing solvents.

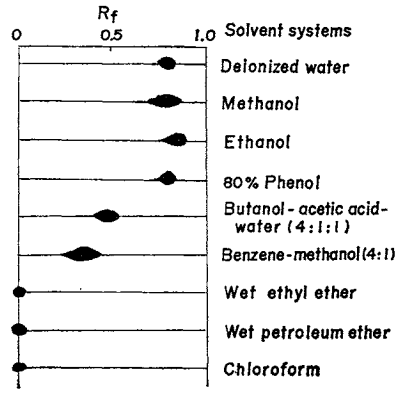


Fig. 6. Paper electrophoresis pattern of cryomycin.

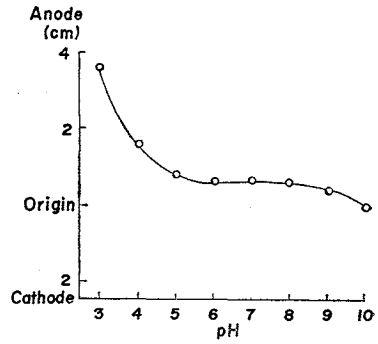


Table 4. Antimicrobial spectrum of cryomycin (1)

Microorganism	M.I.C. (mcg/ml)
Bacillus cereus IFO 3001	0.3
〃 *megaterium* NIH B12	0.2
〃 *subtilis* IFO 3037	0.3
〃 *brevis* IFO 3331	0.6
〃 *pumilus* IFO 3030	0.1
〃 *circulans* IFO 3329	0.6
Micrococcus lysodeikticus IFO 3333	0.2
〃 *flavus* IFO 3242	0.3
〃 *roseus* IFO 3764	0.3
Staphylococcus aureus IFO 3332	1
〃 〃 IFO 3061	3
Sarcina aurantiaca IFO 3064	1
〃 *lutea* IFO 1099	0.5
〃 *marginata* IFO 3066	0.2
〃 *variabilis* IFO 3067	0.5
Flavobacterium flavescens IFO 3085	1
Arthrobacter simplex IFO 3530	10
Brevibacterium divaricatum NRRL 2311	1
Achromobacter aceris IFO 3166	1
Escherichia coli K-12 IFO 3208	—*
Pseudomonas aeruginosa IFO 3080	—
〃 *fluorescens* IFO 3461	—
Aerobacter aerogenes IFO 3320	—
Serratia plymuthica IFO 3055	50
Proteus vulgaris IFO 3045	>200
Alcaligenes faecalis IFO 3160	—
Bacterium cadaveris IFO 3731	>200
Corynebacterium sepedonicum IFO 12188	50
Streptococcus faecalis IFO 3181	20
Pediococcus hennebergi IFO 3884	15
Leuconostoc mesenteroides IFO 3426	5
Lactobacillus plantarum IFO 3070	15
Propionibacterium arabinosum IAM 1714	20

* Growth of test organism was not inhibited at the concentration of 300 mcg/ml.

Table 5. Antimicrobial spectrum of cryomycin (2)

Microorganism	M.I.C. (mcg/ml)
Mycobacterium avium IFO 3154	20
Streptomyces gardneri IFO 3385	10
Nocardia asteroides IFO 3424	15
Endomyces hordei IFO 0104	5
Rhodotorula glutinis IFO 0389	75
〃 *texensis* IFO 0932	150
Schwanniomyces occidentalis IFO 0371	>100
Kloeckera apiculata IFO 0154	>100
Saccharomyces rouxii IAM 4369	>200
Nematospora coryli IFO 0658	>200
Sporobolomyces salmonicolor IFO 0374	>200
Trigonopsis variabilis IFO 0671	>200
Trichosporon cutaneum IFO 0116	>200
Pichia polymorpha IFO 0195	—*
Hansenula anomala AKU 4300	—
Monascus purpureus IAM 8010	> 50
〃 *anka* IAM 8001	> 50
Rhizopus chinensis IFO 4768	>200
Aspergillus niger M-62	>200
Mucor racemosus IFO 4581	—
Penicillium chrysogenum IFO 4626	—
Neurospora crassa IFO 6068	—
Pullularia pullulans IFO 4464	—
Fusarium lini IFO 5880	—
Helminthosporium oryzae	(10)**

* Growth of test organism was not inhibited at the concentration of 300 mcg/ml.
** Hyphae were curled, but the growth not inhibited.

The chemical reactions of cryomycin are given in Table 3. When cryomycin was examined by paper chromatography using nine solvent systems, a single spot active against *Bacillus subtilis* IFO 3037

was observed (Fig. 5). On paper electrophoresis at 15~20 mAmp and 2,000 volts for 40 minutes in each of M/40 acetate (pH 3~5), phosphate (pH 6~8) and glycine (pH 9 and 10) buffers, cryomycin moved towards the anode (Fig. 6).

Although cryomycin showed a very weak ninhydrin reaction, the reaction was strengthened on acid hydrolysis of cryomycin. The nitrogen content, the ninhydrin reaction and the amide band in the infrared absorption spectrum indicate that cryomycin is a peptide antibiotic. Consequently, cryomycin was hydrolyzed in 6 N HCl at 105°C for 24 hours in a sealed tube. The resulting solution was evaporated to dryness *in vacuo* and the mixture of amino acids was analyzed with an automatic amino acid analyzer (Yanagimoto, Model LC-2). The amino acid composition of the hydrolyzate was four moles of glycine, two moles each of threonine, alanine, leucine, tyrosine and lysine for each mole of proline and two unknown ninhydrin-positive substances which closely resemble cysteine and taurine. The optical configuration of these amino acids and their sequence have not yet been determined.

For the detection of fatty acids in the cryomycin molecule, thin-layer chromatography and gas chromatography were carried out on a petroleum ether extract of the hydrolyzate of cryomycin. No fatty acids could be detected.

The activity of cryomycin was reduced by heating, especially in the alkaline pH range.

Biological Properties

The antimicrobial spectrum of cryomycin was determined by an agar dilution streak method. The results are shown in Tables 4 and 5. Cryomycin inhibits Gram-positive bacteria and some yeasts, but not fungi.

The LD_{50} in mice of cryomycin is 150 mg/kg when given intravenously.

Discussion

On the basis of physical and chemical properties, cryomycin can be differentiated from known peptide antibiotics. It is especially unique with reference to the large content of glycine in its molecule.

Cryomycin somewhat resembles saramycetin[3] and antibiotic 362[4] in its ultravio'et absorption maximum near 270 mμ and white powdery nature. However, elemental analysis, melting point and biological activity differentiate cryomycin from saramycetin. Antibiotic 362 also differs from cryomycin in its amphoteric properties and biological activity.

On the basis of the investigations described above, cryomycin is considered to be a new antibiotic.

We have recently reported that the cryomycin-producing organism resembled *Streptomyces globisporus*[5], but the results represented in this paper suggest that *Streptomyces griseus* is a better choice for a specific epithet.

References

1) OGATA, K.; N. YOSHIDA, M. OHSUGI & Y. TANI: Studies on antibiotics produced by psychrophilic microorganisms. I. Production of antibiotics by a psychrophile, *Streptomyces* sp. No. 81. Agr. Biol. Chem. 35 : 79~85, 1971

2) STOKES, J. L.: General biology and nomenclature of psychrophilic microorganisms. In "Recent Progress in Microbiology", VIII. ed. by N. E. GIBBONS, Univ. Toronto Press, Toronto, 1962, p. 187~192

3) BERGER, J.; L. H. STERNBACH, M. MULLER, E. R. LASALA, E. GRUNBERG & M. W. GOLDBERG: X-5079C, a new polypeptide antifungal antibiotic active against systemic mycoses. Antimcr. Agents & Chemoth. -1961 : 436~444, 1962; Saramycetin In "Index of Antibiotics from Actinomycetes", ed. by H. UMEZAWA, Univ. Tokyo Press, Tokyo, 1967, p. 586

4) PHILIPPE, J. *et al.* (to S.I.F.A.): *Streptomyces* 362 and antibiotic 362. Brit. Patent 905,104, Sept. 5, 1962; 362 In "Antibiotics from Actinomycetes", ed. by H. UMEZAWA, Univ. Tokyo Press, Tokyo, 1967, p. 801

5) YOSHIDA, N.; Y. TANI & K. OGATA: Antibiotics produced by hydrocarbon-utilizing actinomycetes. J. Antibiotics 25 : 546~547, 1972