NEW ANTIBIOTICS, ENAMINOMYCINS A, B AND C
I. PRODUCING ORGANISM, FERMENTATION AND ISOLATION

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(Received for publication May 17, 1978)

New antibiotics, enaminomycins A, B and C, were found in the culture broth of streptomycete strain No. 13120, which was identified as *Streptomyces baarnensis* and designated as *S. baarnensis* No. 13120. Fermentation of enaminomycins were performed by conventional submerged culture in a 30-liter jar fermentor. Isolation of the antibiotics was performed by centrifugation of the culture broth and adsorption of the antibiotics from the supernatant on a column of activated carbon, followed by elution with aqueous acetone. Enaminomycins A, B and C were separated from each other on a column of Sephadex LH-20.

Enaminomycins A, B and C were obtained from the culture broth of strain No. 13120 isolated from a soil sample collected at Sapporo City, Hokkaido, Japan. Taxonomic studies of the producing organism revealed its identity with *Streptomyces baarnensis*, but physico-chemical as well as biological characterization of the antibiotics proved their difference from known antibiotics.

The present paper describes the taxonomy of the antibiotic-producing organism and the fermentation and isolation of the antibiotics. Physico-chemical and biological properties of enaminomycins will be presented in the following paper.

Taxonomic Studies

Taxonomic studies of strain No. 13120 were performed by the methods described by SHIRLING and GOTTLIEB.[1] Observation of the culture was made after cultivation for 2 weeks at 28°C unless otherwise stated. The taxonomic keys of the BERGEY's Manual of Determinative Bacteriology (8th ed.), of WAKSMAN in the Actinomycetes, Vol. 2, and of others were used to compare strain No. 13120 with recognized genera and species of the actinomycetes. Color names were assigned according to "Guide to Color Standard" (a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan).

When observed under the microscope, strain No. 13120 formed sporophores of *Rectus-Flexibilis* with monopodial branching of aerial hyphae as shown in Plate 1. Spore chain usually consisted of $10 \sim 50$ spores, cylindrical in shape and $0.2 \sim 0.4 \times 0.8 \sim 1.5\ \mu$ in size. The surface of the spore was smooth as shown in Plate 2.

The cultural characteristics of strain No. 13120 on various media are shown in Table 1. In general, the vegetative mycelium develops abundantly, and the mass color of the aerial mycelium is white on almost all of the media tested. The reverse of the culture is pale yellowish brown to yellowish brown, and soluble pigment is absent. Physiological properties of strain No. 13120 are summarized in Table 2. Strain No. 13120 grows at $10 \sim 40$°C with an optimum temperature of 28°C. Utilization of carbon sources by strain No. 13120 is shown in Table 3. Among known species of *Streptomyces* many charac-

Plate 1. Photograph of aerial hyphae of strain No. 13120 on glucose-asparagine agar, 14 days (×150).

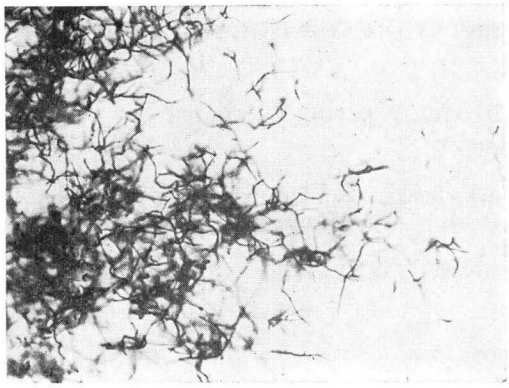


Plate 2. Electron micrograph of spores of strain No. 13120 on soil extract agar, 10 days (×10,000).

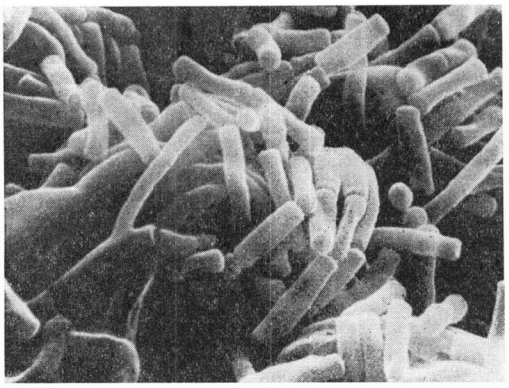


Table 1. Cultural characteristics of strain No. 13120 and *Streptomyces baarnensis* ATCC 23885.

	Strain No. 13120	*S. baarnensis*
Sucrose-nitrate agar	G: Good AM: None R: Yellowish gray SP: None	Good White, poor Pale yellowish brown None
Glucose-asparagine agar	G: Good AM: White, poor R: Pale yellowish brown SP: None	Good White, poor Pale yellowish brown None
Glycerol-asparagine agar (ISP 5)	G: Good AM: White, poor R: Pale yellowish brown SP: None	Good White, good Pale yellowish brown None
Inorganic salts-starch agar (ISP 4)	G: Abundant AM: White, good R: Pale yellowish brown SP: None	Abundant White, good Pale yellowish brown None
Tyrosine agar (ISP 7)	G: Abundant AM: White, abundant R: Pale yellowish brown SP: None	Abundant White, abundant Pale yellowish brown None
Nutrient agar (Difco)	G: Moderate AM: None R: Yellowish gray SP: None	Good None Yellowish gray None
Yeast extract-malt extract agar (ISP 2)	G: Abundant AM: White, abundant R: Yellowish brown SP: None	Good White, poor Yellowish brown None
Oatmeal agar (ISP 3)	G: Good AM: White, abundant R: Pale yellowish brown SP: None	Good White, poor Pale yellowish brown None

G: Growth, AM: Aerial mycelium, R: Reverse, SP: Soluble pigment

teristics of *Streptomyces baarnensis*[2] were very similar to those of strain No. 13120. A comparison of the characteristics of strain No. 13120 with those of *S. baarnensis* ATCC 23885, was made by simultaneous cultivation and is given in Tables 1, 2 and 3.

It is likely that strain No. 13120 is closely related to *S. baarnensis*, because their morphological and

Table 2. Physiological characteristics of strain No. 13120 and *Streptomyces baarnensis* ATCC 23885.

	Strain No. 13120	*S. baarnensis*
Tyrosinase reaction	−	−
Gelatin liquefaction	−	−
Starch hydrolysis	−	−
Milk coagulation (26°C)	−	−
(37°C)	−	+
Milk peptonization (26°C)	−	−
(37°C)	−	+
Melanin formation*	−	−
Nitrate reduction	−	−

+: Positive reaction, −: Negative reaction
*: Tryptone-yeast extract broth (ISP 1) and peptone-yeast extract iron agar (ISP 6)

Table 3. Carbon utilization* of strain No. 13120 and *Streptomyces baarnensis* ATCC 23885.

	Strain No. 13120	*S. baarnensis*
D-Glucose	+	+
L-Arabinose	+	+
Sucrose	+	+
D-Xylose	+	+
i-Inositol	−	−
D-Mannitol	+	−
D-Fructose	+	+
Rhamnose	+	+
Raffinose	+	+
Cellulose	−	−
Galactose	+	+
D-Mannose	+	+
D-Cellobiose	+	+
Lactose	−	−
Maltose	+	+
Trehalose	+	−
Melibiose	+	−
Dulcitol	−	−
Inulin	−	−
Dextrin	+	+
Starch	+	+
Salicin	−	−
Na-Acetate	−	−
Na-Succinate	+	−
Glycerol	+	+
Control	−	−

+: Positive utilization, −: Negative utilization
*: ISP medium 9.

cultural characteristics are very similar. The only differences noted were coagulation and peptonization of milk at 37°C, utilization of certain carbon sources, such as trehalose, melibiose, D-mannitol and sodium succinate, and ability to produce enaminomycins. D-Mannitol was reported[2] to be utilized by *S. baarnensis* ATCC 23885, but our studies did not confirm this observation. Furthermore, growth of both strains was observed on raffinose, in contrast to the observation reported in the literature. The differences observed here, however, were not sufficient to consider strain No. 13120 as a new species. Therefore, strain No. 13120 was named *Streptomyces baarnensis* No. 13120.

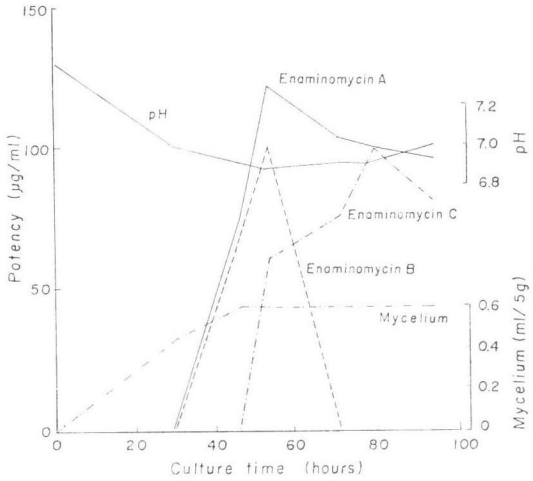
Fermentation

One loopful growth of *S. baarnensis* No. 13120 was inoculated into 500-ml flasks each containing 80 ml of a medium composed of: glucose, 1.5%; soluble starch, 1.5%; fish meal, 2.0%; $CaCO_3$, 0.2% (pH 7.4 before sterilization); inoculated flasks were incubated on a rotary shaker at 28°C for 24~30 hours. Fermentation was carried out in a 30-liter jar fermentor containing 15 liters of the same medium as described above. After inoculation of 300 ml of a seed culture the fermentation was conducted at 28°C for 40~60 hours with agitation (200 rev/min.) and aeration (15 liters/min.).

Mycelial growth was expressed as the packed cell volume (ml) after centrifugation of 5 g of the culture broth at 3,000 rpm for 10 minutes. Antibiotic production during fermentation was monitored by a cylinder-plate method using *Proteus vulgaris* OX19 as the test organism for the determination of enaminomycin A, and by densitometry with Dual-Wave Length TLC Scanner (Model CS-900, Shimazu

Co., Ltd., Japan) on silica gel plate (silica gel F_{254}, E. Merck, Darmstadt, Germany) using $CHCl_3$ - MeOH - AcOH (9 : 1 : 1) as a solvent system for enaminomycins B and C. Each authentic sample was applied on the plate simultaneously with the samples to be tested. A typical time course of fermentation in a 30-liter jar fermentor is shown in Fig. 1. Both enaminomycins A and B reached a maximum potency of about 100 μg/ml after 50 hours; C yielded a peak of about 100 μg/ml after 80 hours.

Fig. 1. Fermentation of enaminomycins A, B and C in 30-liter jar fermentor.



Isolation

The supernatant obtained by centrifugation of the fermentation broth (30 liters) was chilled, adjusted to pH 5.0 with 20% phosphoric acid and adsorbed on a column of 900 ml of activated carbon at 4°C. The column was washed with 5 liters of cold distilled water and the antibiotics were eluted with 50% aqueous acetone, and the eluate collected in 500-ml fractions. The active fractions (No. 2 through No. 8) were pooled and concentrated under reduced pressure to remove acetone. The concentrate (2.4 liters) containing enaminomycins A, B and C (460, 960 and 155 mg, respectively), was adjusted to pH 3.0 with 20% phosphoric acid, and the antibiotics were extracted three times each with 2.4 liters of ethyl acetate.

The extract was concentrated to 100 ml, the concentrate transformed to the top of a column consisting of 2 liters of Sephadex LH-20 equlibrated with ethyl acetate - methanol (95 : 5), and the column was developed with the same solvent. The eluate was collected in 14.5-ml fractions. Every component of enaminomycins was effectively separated from the others on this column chromatogram: 375 mg of A were obtained from fractions No. 141 to No. 193; 750 mg of B from No. 103 to No. 140; and C from No. 194 to No. 223.

Table 4. Rf values of enaminomycins A, B and C on TLC plates in various solvent systems.

Solvent	Support	Rf		
		A	B	C
Chloroform	Silica gel*	0.01	0	0
Ethyl acetate		0~0.3	0~0.3	0~0.3
Acetone		0.72	0.72	0.72
Methanol		0.79	0.79	0.79
Butanol - acetic acid - water (3 : 1 : 1)		0.72	0.59	0.67
Chloroform - methanol - acetic acid (9 : 1 : 1)		0.64	0.52	0.39
Propanol - pyridine - acetic acid - water (15 : 10 : 3 : 12)	Cellulose**	0.94	0.94	0.94
Butanol - acetic acid - water (3 : 1 : 1)		0.84	0.86	0.85
Butanol - methanol - water (4 : 1 : 2)		0.79	0.76	0.74

* Eastman Chromagram sheet 6060 Silica gel
** Eastman Chromagram sheet 6065 Cellulose

Each component was then subjected to a column of Sephadex LH-20 under the conditions described above. Enaminomycins A (300 mg) was obtained as a white amorphous powder; enaminomycin B (110 mg) crystallized in colorless needles from ethyl acetate - acetone; and enaminomycin C (56 mg) formed colorless needles crystallized from methanol. Each purified component showed a single spot on TLC using several solvent systems (Table 4).

References

1) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Internat. J. System. Bacteriol. 16: 313~340, 1966
2) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Internat. J. System. Bacteriol. 18: 279~392, 1968