ENDURACIDIN, A NEW ANTIBIOTIC. I

STREPTOMYCES FUNGICIDICUS NO. B 5477, AN ENDURACIDIN PRODUCING ORGANISM*

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The mycological properties and antibiotic activity of strain No. B 5477, isolated from a soil sample collected in Nishinomiya, Hyogo Prefecture, were investigated. This strain forms spiral sporophores, spiny spores, and gray aerial mycelium. The culture is non-chromogenic. Strain No. B 5477 was compared with known species and identified as a strain of *Streptomyces fungicidicus* OKAMI *et al.* 1954[1]. During the course of selecting a strain with high potency, a yellow mutant with yellowish aerial mycelium was obtained and its properties were investigated.

Streptomyces fungicidicus No. B 5477 produces a new antibiotic, enduracidin, with a strong antibiotic activity against Gram-positive bacteria. Acid-fast bacteria and phytopathogenic bacteria are also inhibited. Enduracidin shows no cross resistance with the known antibiotics examined. It is more active in a basic medium and therefore is a basic antibiotic. The antibiotic activity of enduracidin was not affected by horse serum in the assay medium. Its diffusibility was less than that of the other antibiotics examined. The antibiotic activity of enduracidin was assayed by the paper disc method using *Sarcina variabilis* IFO 3067 as the test organism. Enduracidin was produced mainly in the mycelium. The fermentation conditions for production of the antibiotic were investigated.

Recently, strains resistant to antibiotics have appeared, and discovery of a new useful antibiotic with activity against these resistant strains is desirable.

In the course of screening, a strain of streptomycetes, *Streptomyces* sp. No. B 5477 was isolated from soil collected in Nishinomiya, Hyogo Prefecture. Strain No. B 5477, produces an antibiotic having strong activity against Gram-positive bacteria. The mycological properties of strain No. B 5477 were investigated and the culture was identified as a strain of *Streptomyces fungicidicus*[1]. The new antibiotic, enduracidin[2], is a basic antibiotic showing no cross resistance with known antibiotics.

In this paper, mycological properties of *Streptomyces fungicidicus* No. B 5477 and its yellow mutant, antibacterial properties of these strains and enduracidin, method of assay and fermentation conditions necessary for production of the antibiotic are reported.

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I. Mycological Properties

1. Morphological Properties

The vegetative mycelium develops well on most media. The aerial mycelium is abundant, powdery, Olive Gray to Drab Gray. The sporophores form spirals (Plate 1) and the spores are spherical or oval to ellipsoidal, 0.9~1.2 $\mu \times$ 1.1~1.6 μ. The surface of the spore is spiny under the electron microscope (Plate 2).

2. Cultural Properties

Growth is colorless or pale yellow and well developed. The aerial mycelium is abundant and gray as described above. The aerial mycelium is white on certain media containing an organic nitrogen source.

The cultural characteristics of the original strain shown in Table 1 are based on observation made after 14 days incubation at 28°C, except as noted otherwise. As shown in Table 1, no soluble pigment or only faint yellow pigment is produced in most media. The culture is non-chromogenic, no brown soluble pigment is produced as on peptone agar or on other media containing an organic nitrogen source. The cultures grow well on synthetic or organic media at temperatures ranging from 20°C to 37°C and pH values ranging from 5 to 9. Strong proteolytic activity, starch hydrolysis and nitrate reduction are physiological characteristics of this culture.

In the course of screening, a high potency mutant with yellow aerial mycelium was obtained by ultraviolet irradiation, gamma irradiation and monospore cultivation.

The color of the vegetative mycelium of the mutant is yellow and the aerial mycelium well developed. The morphological properties are the same as those of the original strain. The cultural characteristics of the mutant are shown in Table 2.

Plate 1. Microscopic photograph.

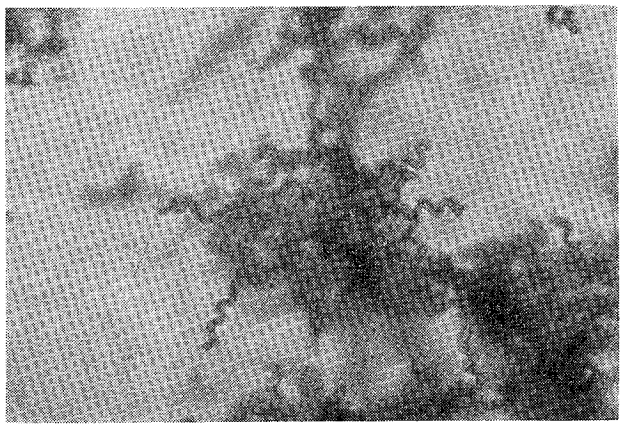
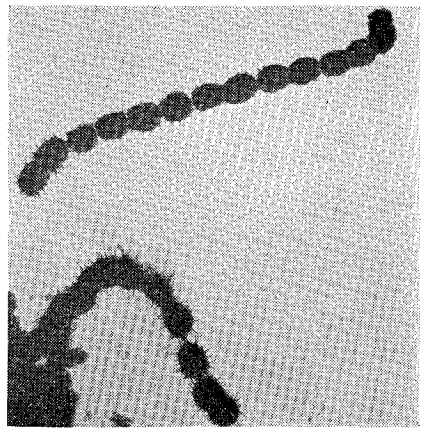


Plate 2. Electron-microscopic photograph.



3. Utilization of Carbon Sources

Carbon utilization by strain No. B 5477 and the yellow mutant were investigated using the method of PRIDHAM and GOTTLIEB[3]. As shown in Table 3, the pattern of carbon utilization for these strains is almost the same.

Table 1. Cultural properties of *Streptomyces fungicidicus* No. B 5477

	Vegetative mycelium	Aerial mycelium	Reverse	Soluble pigment
CZAPEK's agar	Abundant, spreading, colorless, penetrating into the medium	Abundunt, powdery, Quaker Drab (Rdg.* LI, 1''''')	Warm Buff (Rdg. XV, 17' -d)	None
Glucose CZAPEK's agar	Abundant, spreading, colorless	Abundant, powdery, Pale Quaker Drab (Rdg. LI, 1'''''-d)	Warm Buff (Rdg. XV, 17' -d)	None or faint yellow
Glycerin CZAPEK's agar	Abundant, folded, colorless	Abundant, Deep Olive Gray(Rdg. LI, 23'''''), becoming to Light Mouse Gray (Rdg. LI, 15'''''-d) to Light Quaker Drab (Rdg. LI, 1'''''-b)	Warm Buff (Rdg. XV, 17' -d)	None or faint yellow
Glucose asparagine agar	Abundant, spreading, colorless	Abundant, Light Olive Gray (Rdg. LI, 23''''' -d) to Pale Quaker Drab(Rdg. LI, 1'''''-d) or sometimes poor, white	Cream Color (Rdg. XVI, 19' -f) or colorless	None
Nutrient broth	Surface growth good, colorless, producing heavy sediment	Moderate, white		None
Nutrient agar	Abundant, spreading, colorless	Moderate, white to Pale Quaker Drab (Rdg. LI, 1'''''-d)	Warm Buff (Rdg. XV, 17' -d)	None
Glucose nutrient agar	Abundant, folded, spreading, colorless	Abundant, Deep Quaker Drab (Rdg. LI, 1'''''-i)	Yellow Ocher (Rdg. XV, 17')	Faint brown or none
Glycerin nutrient agar	Abundant, spreading, colorless	Abundant, powdery, Quaker Drab (Rdg. LI, 1''''') to Deep Quaker Drab(Rdg. LI, 1'''''-i)	Isabella Color (Rdg. XXX, 19''-i)	None or faint brown
Starch agar	Abundant, spreading, colorless	Abundant, Light Quaker Drab (Rdg. LI, 1'''''-b), becoming to Quaker Drab (Rdg. LI, 1''''')	Light Buff (Rdg. XV, 17' -f)	None
Egg (37°C)	Abundant, spreading, wrinkled	Moderate, white to white with yellowish tinge and sometimes poor, white		None, later becoming pale gray to brown
Potato plug	Abundant, spreading, lichenoid, colorless	Abundant, Quaker Drab (Rdg. LI, 1''''')		None, later becoming dark brown after three weeks
Carrot plug	Abundant, spreading, lichenoid, colorless	Abundant, Quaker Drab (Rdg. LI, 1''''')		None, later becoming Backthorn Brown(Rdg. XV, 17'-i)
Cellulose	Moderate, spreading, colorless	Moderate, powdery, Quaker Drab (Rdg. LI, 1''''')		None

(To be continued)

Table 1 (Continued)

	Vegetative mycelium	Aerial mycelium	Reverse	Soluble pigment	
Calcium malate agar	Thin, spreading, colorless	Moderate, Pallid Quaker Drab (Rdg. LI, 1′′′′′-f) to Pallid Mouse Gray (Rdg. LI, 15′′′′′-f)	Naples Yellow (Rdg. XVI, 19′-d)	None or faint yellow	
Tyrosine agar	Poor, thin, spreading	None	Colorless	None	
Yeast extract agar	Abundant, wrinkled, spreading, colorless	Abundant, powdery, Light Mouse Gray (Rdg. LI, 15′′′′′-b) to Quaker Drab (Rdg. LI, 1′′′′′)	Isabella Color (Rdg. XXX, 19′′-i)	None	
Peptone agar	Abundant, spreading, colorless	Poor, Drab Gray (Rdg. XLVI, 17′′′′-d)	Cream Color (Rdg. XVI, 19′-f)	None	
Starch casein agar	Abundant, spreading, colorless	Abundant, velvety, Deep Mouse Gray (Rdg. LI, 15′′′′-i)	Light Olive Gray (Rdg. LI, 23′′′′′-d)	None	
Maltose tryptone agar	Abundant, spreading, colorless	Abundant, Mouse Gray (Rdg. LI, 15′′′′′) to Quaker Drab (Rdg. LI, 1′′′′′)	Light Buff (Rdg. XV, 17′-f)	None or faint yellow	
BENNETT's agar	Abundant, spreading, colorless	Quaker Drab (Rdg. LI, 1′′′′′) to Deep Quaker Drab (Rdg. LI, 1′′′′′-i) or poor, white	Colorless to Warm Buff (Rdg. XV, 17′-d)	None	
Litmus milk (37°C)	Moderate, colorless to Pinkish Buff (Rdg, XXIX, 17′′-d)	Poor, white to Pearl Gray (Rdg. LII, 35′′′′′-f)			Peptonization without coagulation. Reaction unchanges or becomes faintly acid.
LÖFFLER's medium (37°C)	Abundant, wrinkled colorless	Poor or moderate, velvety, white		None	Liquefaction.
Gelatin (24°C, one month)	Poor, colorless	None		None	Slow liquefaction.
Nutrient gelatin (24°C, one month)	Abundant, pale brown	Moderate, white		None	Rapid liquefaction.

Nitrate reduction in CZAPEK's solution : Positive.
Starch hydrolysis : Enzymatic zone/Growth zone=33 mm/10 mm, 28 mm/10 mm

* Rdg. : R. RIDGWAY, Color Standard and Color Nomenclature.

Table 2. Cultural properties of *Streptomyces fungicidicus* No. B5477 yellow mutant

	Vegetative mycelium	Aerial mycelium	Reverse	Soluble pigment
Czapek's agar	Abundant, spreading, penetrating into the medium, pale yellow	Abundant, powdery, Cream Color (Rdg.* XVI, 19′-f) to Pale Ochraceous-Buff (Rdg. XV, 15′-f)	Cream Color (Rdg. XVI, 19′-f)	None or faint yellow
Glucose Czapek's agar	Abundant, spreading, colorless to faint yellow	Moderate, powdery, white to Naples Yellow (Rdg. XVI, 19′-d)	Cream Color (Rdg. XVI, 19′-f) to Mustard Yellow (Rdg. XVI, 19′-b)	None
Glycerin Czapek's agar	Abundant, spreading, folded, Mustard Yellow (Rdg. XVI, 19′-b)	Abundant, powdery, Cream Color (Rdg. XVI, 19′-f) to Naples Yellow (Rdg. XVI, 19′-d)	Light Ochraceous-Buff (Rdg. XV, 15′-d) to Ochraceous-Buff (Rdg. XV, 15′-b)	Pale yellow
Glucose asparagine agar	Moderate spreading, colorless to Mustard Yellow (Rdg. XVI, 19′-b)	Moderate, Straw Yellow (Rdg. XVI, 21′-d) or sometimes scant, white	Colorless to yellow	None
Nutrient broth	Surface growth, colorless, later producing sediment	Moderate, powdery, white		None
Nutrient agar	Moderate, spreading, colorless	Moderate, powdery, white	Colorless	None
Glucose nutrient agar	Abundant, spreading, wrinkled, colorless	Moderate, powdery, white to Cream Color (Rdg. XVI, 19′-f)	Pale brown	None
Glycerin nutrient agar	Abundant, spreading, wrinkled, colorless	Moderate, powdery, white	Pale brown	None
Starch agar	Abundant, spreading, Mustard Yellow (Rdg. XVI, 19′-b)	Abundant, powdery, Pale Ochraceous-Buff (Rdg. XV, 15′-f) to Cream Color (Rdg. XVI, 19′-f)	Naples Yellow (Rdg. XVI, 19′-d) to Light Ochraceous-Salmon (Rdg. XV, 13′-d)	None or faint yellow
Egg (37°C)	Moderate, spreading, Naples Yellow (Rdg. XVI, 19′-d)	None or very scant, white to Cream Color (Rdg. XVI, 19′-f)		None
Potato plug	Abundant, spreading, wrinkled, Antimony Yellow (Rdg. XV, 17′-b)	Abundant, powdery, white to Light Ochraceous-Buff (Rdg. XV, 15′-d)		None, later becoming brown
Carrot plug	Abundant, spreading, Cream Color (Rdg. XVI, 19′-f) to Ochraceous-Buff (Rdg. XV, 15′-b)	White or Pale Ochraceous-Buff (Rdg. XV, 15′-f) to Light Ochraceous-Salmon (Rdg. XV, 13′-d)		None
Cellulose	Moderate, spreading, colorless to Cream Color (Rdg. XVI, 19′-f)	Moderate, powdery, white to Pale Ochraceous-Salmon (Rdg. XV, 13′-f)		

(To be continued)

Table 2 (Continued)

	Vegetative mycelium	Aerial mycelium	Reverse	Soluble pigment	
Calcium malate agar	Abundant, spreading, Cream Color (Rdg. XVI, 19′-f) to Ochraceous-Buff (Rdg. XV, 15′-b)	Scant, white to Pale Ochraceous-Salmon (Rdg. XV, 13′-f)	Cream Color (Rdg. XVI, 19′-f) to Light Ochraceous-Salmon (Rdg. XV, 13′-d)	None	
Tyrosine agar	Scant, thin, colorless	None	Colorless	None	
Peptone agar	Moderate, spreading, colorless	Moderate, powdery, white to Pale Ochraceous-Buff (Rdg. XV, 15′-f)	Colorless to Cream Color (Rdg. XVI, 19′-f)	None	
Yeast extract agar	Abundant, spreading, wrinkled, colorless, to faint brown	Abundant, powdery, Cream Color (Rdg. XVI, 19′-d) to Naples Yellow (Rdg. XVI, 19′-d)	Antimony Yellow (Rdg. XV, 17′-b) to Light Ochraceous-Salmon (Rdg. XV, 13′-d)	None	
Gelatin (24°C, one month)	Scant, colorless	Scant, white to Cream Color (Rdg. XVI, 19′-f)		None	Slow liquefaction
Nutrient gelatin (24°C, one month)	Abundant, wrinkled, Ochraceous-Buff (Rdg. XV, 15′-b)	Moderate, white to Cream Color (Rdg. XVI, 19′-f)		None	Rapid liquefaction
Litmus milk (37°C)	Pale yellow ring at surface, producing Pinkish Buff (Rdg. XXIX, 17″-d) sediment	None			Peptonization without coagulation Reaction unchanges or becomes faintly acid
LÖFFLER'S medium (37°C)	Moderate, colorless to pale yellow	None or scant, yellow	Yellowish brown or pale yellow	None	Liquefaction

Nitrate reduction in CZAPEK'S solution : Positive.
Starch hydrolysis : Enzymatic zone/Growth zone=22 mm/11 mm, 26 mm/13 mm

* Rdg. : R. RIDGWAY, Color Standard and Color Nomenclature.

Table 3. Carbon source utilization of *Streptomyces fungicidicus* No. B5477 and the yellow mutant

Carbon sources	No. B5477	Yellow mutant	Carbon sources	No. B5477	Yellow mutant
Erythritol	±	+	D-Maltose	+++	+++
Adonitol	±	+	Sucrose	+	+
D-Sorbitol	+	+	Lactose	+++	+++
i-Inositol	+++	+++	Raffinose	+	+
D-Mannitol	+++	+++	Trehalose	+++	++
Dulcitol	+	+	Salicin	+++	+++
D-Xylose	+++	+++	Esculin	+	++
L-Arabinose	+++	+++	Inulin	±	+
L-Sorbose	+	++	Dextran	+++	+++
D-Galactose	+++	+++	Starch	+++	+++
D-Glucose	++	++	Glycerin	+++	+++
D-Mannose	++	++	Na-Acetate	++	+++
D-Fructose	+++	+++	Na-Succinate	+	++
Rhamnose	+++	+++	Na-Citrate	+	++
Melibiose	++	++	Control	±	+

+++ : Abundant growth. ++ : Moderate growth. + : Growth.
± : Poor growth. − : No growth.

Inositol, D-mannitol, D-xylose, D-galactose, D-glucose, D-mannose, glycerin, D-fructose, D-maltose, lactose and salicin support good growth. Erythritol, adonitol, D-sorbitol, dulcitol, L-sorbose, melibiose, sucrose, raffinose and inulin are carbon sources that support no growth or poor growth. The yellow mutant attains good growth on the medium containing sodium acetate, sodium succinate and sodium citrate, while the original culture shows poor growth on the medium containing sodium succinate and sodium citrate.

4. Comparison between Strain No. B 5477 and Related Species

Based on the morphological and cultural properties, strain No. B 5477 belongs to Section Spira and Series Gray proposed by PRIDHAM *et al.*[4] Comparison of the strain with known species, reveals that the strain resembles *Streptomyces fungicidicus* OKAMI *et al.* group G[1].

The differences between the cultural properties of strain No. B 5477 and *Streptomyces fungicidicus* are as follows:

Streptomyces fungicidicus produces a pink soluble pigment only at an early age when grown on calcium malate agar, grows on milk medium with coagulation and peptonization and on LOEFFLER's serum medium with no or doubtful liquefaction, while strain No. B 5477 produces no soluble pigment or only a faint yellow soluble pigment on calcium malate agar, does not coagulate milk and liquefies LOEFFLER's serum medium.

The morphological properties of strain No. B 5477 are the same as those of *Streptomyces fungicidicus*. The cultural and physiological characteristics are also similar, except as indicated above. Therefore, the present strain is believed to be a strain of *Streptomyces fungicidicus*, and has been designated *Streptomyces fungicidicus* No. B 5477.

II. Antibiotic Activity

1. Antibacterial Spectrum by the Cross-streak Method

Strain No. B 5477 was found to have antibiotic activity against Gram-positive bacteria by the spot inoculation test[5]. The antibiotic activity by the cross-streak method and cross streak-agar disk method[5] was therefore examined.

Bouillon and glycerin bouillon agar plates were incubated with strain B 5477. After 4 days incubation at 28°C, the test organisms were cross struk against strain No. B 5477. After incubation at 37°C for 18 hours for general bacteria and for 40 hours for acid-fast bacteria, the inhibition of the test organisms was measured.

As shown in Table 4, antibiotic activity was not demonstrated on bouillon agar, but activity was found against *Staphylococcus aureus*, *Bacillus subtilis*, and *Bacillus cereus*, *etc.* on glycerin bouillon agar. It was also found that the antibiotic activity was stronger on basic agar than on acidic agar by the cross streak-agar disc method. The antibiotic from strain No. B 5477 was thus shown to be a basic substance. The antibacterial spectrum of the yellow mutant was investigated by this method and found to be the same as for the original strain.

2. Antibacterial Spectrum of Enduracidin

A liquid medium was inoculated with strain No. B 5477 and fermented on a rotary shaker. The antibacterial activity from the filtrate of the fermented broth and acetone extracts of the mycelium was investigated. As shown in Table 5, antibiotic activity was found against Gram-positive and acid-fast bacteria. The activity was found to be stronger in the extract from the mycelium than in the filtrate.

The agar dilution method was used to investigate the antibacterial spectrum of enduracidin. Enduracidin was obtained from the fermented broth of strain No. B 5477 by the method described in a subsequent report[2]. Tests were carried out on bouillon agar for Gram-positive and Gram-negative bacteria, on glycerin bouillon agar for acid-fast bacteria, and on glucose bouillon agar for fungi, yeast and phytopathogenic bacteria.

As shown in Table 6, enduracidin was essentially inactive against the Gram-negative bacteria, fungi and yeast examined, and strongly active against some Gram-positive bacteria with a minimum inhibitory concentration of 0.1~2.0 mcg/ml. Moderate antibiotic activity was demonstrated against acid-fast bacteria at 5 mcg/ml and against *Xanthomonas oryzae* at 2 mcg/ml. Enduracidin showed no cross resistance with chlortetracycline, novobiocin, chloramphenicol, erythromycin, mikamycin[6], glumamycin[7], chromomycin, xanthomycin, streptomycin and neomycin. The activity was enhanced to some degree on basic medium.

Table 4. Antibacterial spectra of *Streptomyces fungicidicus* No. B5477 and the yellow mutant by cross-streak method

Test organisms	Inhibition zone (mm)			
	No. B5477		Yellow mutant	
	Bouillon agar	Glycerin bouillon agar	Bouillon agar	Glycerin bouillon agar
Escherichia coli	0	0	0	0
Proteus vulgaris	0	0	0	0
Staphylococcus aureus	0	17	0	15
Bacillus subtilis	0	17	0	15
Bacillus cereus	0	14	0	12
Bacillus brevis	0	0	0	0
Sarcina lutea	0	0	0	0
Micrococcus flavus	0	0	0	0
Mycobact. avium	0	0	0	0
Bacillus subtilis pH 6	13.0		12.5	
pH 8	16.5		15.5	
Mycobact. avium pH 6		0		0
pH 8		0		0

Table 5. Antibiotic activity of the fermented broth of *Streptomyces fungicidicus* No. B5477 and the yellow mutant

Test organisms	Inhibition zone (mm)			
	No. B5477		Yellow mutant	
	Filt. broth	Mycel. ext.	Filt. broth	Mycel. ext.
E. coli	0	0	0	0
Proteus vulgaris	0	0	0	0
Staph. aureus	13	20	11	16
〃 OM & EM-R	13	20	11	16
〃 CTC-R	13	20	11	16
〃 CP-R	13	20	11	16
〃 CHM-R	13	20	11	16
〃 MM-R	13	20	11	16
〃 GM-R	12	17	10.5	14
B. subtilis	12.5	22	11	17
B. cereus	10	21	10	16
B. brevis	10.5	21	10.5	16
Mycob. avium pH 6	0	13	0	12
〃 pH 8	0	16	0	14
Candida albicans	0	0	0	0

OM & EM-R : Oleandomycin and erythromycin resistant.
CTC-R : Chlortetracycline resistant.
CP-R : Chloramphenicol resistant.
CHM-R : Chromomycin resistant.
MM-R : Mikamycin resistant.
GM-R : Glumamycin resistant.

Table 6. Antimicrobial spectra of enduracidin

Test organisms	M. I. C. (mcg/ml)			Test organisms	M. I. C. (mcg/ml)		
	pH 6	pH 7	pH 8		pH 6	pH 7	pH 8
E. coli IFO 3044	>100	>100	>100	*Mycobacterium avium* IFO 3153	10	5.0	5.0
Proteus vulgaris IFO 3045	>100	>100	>100	〃 SM-R	10	5.0	5.0
Staph. aureus FDA 209P	0.1~0.2	0.1	0.1	〃 DM-R	10	5.0	5.0
〃 OM & EM-R	0.2	0.2	0.1	*Mycob. smegmatis* IFO 3083	2.0	2.0	1~2
〃 CTC-R	0.1~0.2	0.1	0.1	*Mycob. phlei* IFO 3158	10	5.0	5.0
〃 CP-R	0.1~0.2	0.1	0.1	*Mycob.* sp. ATCC 607	10	5.0	5.0
〃 CHM-R	0.1~0.2	0.1	0.1	*Piricularia oryzae*	>100	>100	>100
〃 MM-R	0.1~0.2	0.1	0.1	*Asp. niger* IFO 4066	>100	>100	>100
〃 GM-R	0.5	0.2	0.2	*Pen. chrysogenum* IFO 4626	>100	>100	>100
〃 NV-R	0.1~0.2	0.1	0.1	*Candida albicans* IFO 0583	>100	>100	>100
B. subtilis PCI 219	0.2	0.1	0.1~0.07	*Sacch. cerevisiae*	>100	>100	>100
B. cereus IFO 3466	2.0	2.0	1.0	*Xanthomonas oryzae*	5.0	2.0	2.0
B. brevis IFO 3331	2.0	2.0	1~2				
Sarcina lutea IFO 3232	0.2~0.5	0.2	0.2				
Micrococcus flavus IFO 3242	0.5	0.5	0.2~0.5				

OM & EM-R : Oleandomycin and erythromycin resistant. CTC-R : Chlortetracycline resistant. CP-R : Chloramphenicol resistant. CHM-R : Chromomycin resistant. MM-R : Mikamycin resistant. GM-R : Glumamycin resistant. NV-R : Novobiocin resistant. SM-R : Streptomycin resistant. NM-R : Neomycin resistant.

3. Effect of Serum on the Antibiotic Activity of Enduracidin

The effect of horse serum on the antibiotic activity of enduracidin was compared with other antibiotics. Activity was tested by the dilution method using bouillon agar and bouillon with or without 10 % horse serum.

The test samples used were enduracidin free base, erythromycin lactobionate, glumamycin calcium-salt and mikamycin complex. *Staphylococcus aureus* FDA 209 P, *Bacillus subtilis* PCI 219, *Bacillus cereus* and *Bacillus brevis* were tested by the agar dilution method and *Staphylococcus aureus* FDA 209P by the liquid dilution method.

As shown in Table 7, the antibiotic activities of mikamycin were the same in both the medium containing serum or the control medium, while glumamycin showed decreased activity in the medium containing serum. Those of erythromycin and enduracidin were the same in both media or slightly higher in the medium containing the serum.

Table 7. Effect of horse serum on the antibacterial activity of enduracidin and other antibiotics

Test organisms	M. I. C. (mcg/ml)							
	Enduracidin		Erythromycin		Mikamycin		Glumamycin	
	C	S	C	S	C	S	C	S
*Staph. aureus**	0.1	0.1	0.2	0.1~0.2	0.2	0.2~0.5	2.0	2~5
*B. subtilis**	0.1	0.1	0.1	0.1~0.05	1.0	1~2	0.5	1.0
*B. cereus**	2.0	0.5	0.5	0.2	1.0	1.0	1.0	2.0
*B. brevis**	2.0	1.0	1.0	0.5	2.0	2.0	5.0	10.0
*Staph. aureus***	0.125	0.125	0.5	0.5	0.25	0.25	12.5	12.5

* : Agar dilution method. ** : Broth dilution method.
C : Control. S : Medium containing the horse serum.

4. Diffusibility of Enduracidin

Although enduracidin showed strong activity against Gram-positive bacteria by the dilution method, its inhibition zone was found to be small. The diffusion of enduracidin free base on bouillon agar plate was compared with other antibiotics, such as mikamycin complex, glumamycin calcium-salt, erythromycin lactobionate, streptomycin sulfate and neomycin sulfate.

These antibiotics were tested by the paper disk method using *Staphylococcus aureus* FDA 209P as the test organism and an assay medium composed of 0.5% EHRLICH meat extract, 0.5% Polypeptone, 0.5% NaCl and 1.5% agar (pH 7.0). Tests were carried out on agar plates consisting of 5 ml of medium as the base layer and 4 ml of medium as the seed layer. The diameter of the inhibition zone was measured after incubation for 18 hours at 37°C. The standard curve of each antibiotic is shown in Fig. 1.

The inclination of the standard curve of enduracidin was found to be lower than those of other antibiotics. The interrelation between the concentration and the diameter of the inhibition zone of those antibiotics was studied and the regression coefficients (b) between the concentration and the inhibition diameters were calculated as follows:

Mikamycin	b=8.46	Erythromycin	b=7.64	Neomycin	b=5.42
Glumamycin	b=7.82	Streptomycin	b=6.82	Enduracidin	b=4.23

The regression coefficient of enduracidin was found to be the smallest among these antibiotics. Therefore, the diffusibility of enduracidin on bouillon agar plate was found to be the smallest of the antibiotics examined.

Fig. 1. Comparison of diffusibility of enduracidin and other antibiotics.

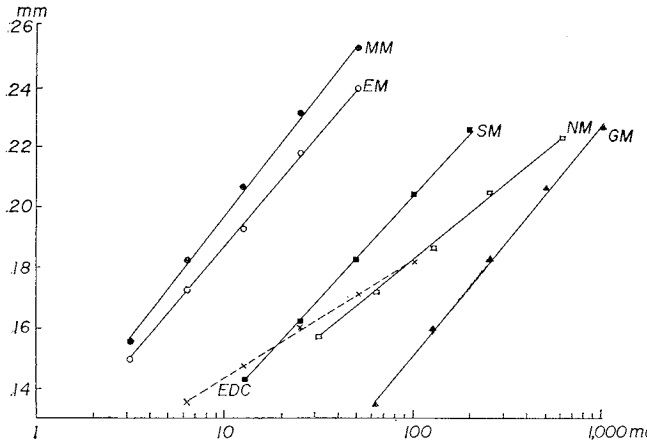
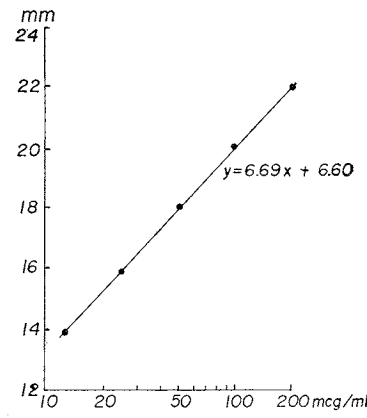


Fig. 2. Standard curve of enduracidin.



III. Production of Enduracidin

1. Assay Method of the Antibiotic Activity

Enduracidin was subjected to microbioassay by the paper disc method using *Sarcina variabilis* IFO 3067 as the test organism. The agar plate was prepared by pouring 5 ml of assay medium as the base layer and 4 ml as the seed layer.

The assay medium was composed of 0.5% EHRLICH meat extract, 0.5% Polypeptone, 0.2% K_2HPO_4, and 1.5% agar, and the pH adjusted to 8.0 before autoclaving. The standard sample enduracidin free base was dissolved in 0.5 M Tris buffer (20% MeOH, pH 8.0) in concentrations of 200, 100, 50, 25 and 12.5 mcg/ml. Ten paper discs were soaked in the stardard samples of each concentration and dried. The paper discs were placed on the plates of the assay medium and incubated for 18 hours at 37°C. After incubation, the diameters of the inhibition zones were measured. The average values of these data are shown in Fig. 2 as black spots. The correlation coefficient (r) was 0.994 and regression coefficient (b) was 6.69 between the logarithmic value (x) of the concentration of enduracidin and the diameter (y) of the inhibition zone. The regression linear line could be calculated for $y=6.69x+6.60$ in the range of 200~12.5 mcg/ml of enduracidin. From these results, the antibiotic activity of enduracidin can be assayed following the penicillin assay method by using the solution of 100 mcg/ml and 25 mcg/ml of endulacidin as the standard sample.

The following method was used for preparing samples of the fermented broth. Fermented broth was centrifuged, and the supernatant assayed. Activity in the mycelium was assayed following extraction with acetone.

2. Production of Enduracidin

Investigation was made on the production of enduracidin with shaking flasks. Two hundred ml of Erlenmyer flasks containing 50 ml of the medium were fermented for 5 days at 28°C on a rotary shaker.

The effect of nitrogen source on the production of enduracidin was studied using a basal medium composed of 5% soluble starch, 0.5% NaCl and 1% $CaCO_3$ (adjusted to pH 7.0). As shown in Table 8, corn steep liquor, corn gluten meal and casein from milk were good nitrogen sources for antibiotic production. The effect of the carbon source on the production of enduracidin was investigated using a basal medium composed of 3% corn steep liquor, 1% soy bean flour, 0.5% NaCl and 1% $CaCO_3$ (adjusted to pH 7.0). Soluble starch and dextrin gave high yields when used as the sole carbon source, however higher yields were obtained in combination with glucose.

Table 8. Effect of nitrogen and carbon sources on the production of enduracidin

			Potency mcg/ml		
			Broth	Mycelium extract	Total
Nitrogen sources	Corn steep liquor	3%	15	70	85
	Soy bean flour	3	10	65	75
	Meat extract	3	<5	<5	7
	Pharmamedia	3	10	50	60
	Corn gluten meal	3	10	75	80
	Milk casein	3	15	70	85
Carbon sources	Glucose	5%	<5	20	23
	Soluble starch	5	15	73	88
	Dextrin	5	16	70	86
	Lactose	5	<5	10	12
	Maltose	5	<5	10	12
	Glycerin	5	<5	15	20
	Glucose 2 / Soluble starch 3		20	150	170
	Glucose 2 / Dextrin 3		15	100	115

Also as shown in Table 8, enduracidin is found mainly in the mycelium with only about 20% or less of the total antibiotic in the filtrate.

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