ENTEROCIN, A NEW ANTIBIOTIC

TAXONOMY, ISOLATION AND CHARACTERIZATION

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Enterocin is a new antibiotic isolated from cultures of two strains of *Streptomyces*, which were given the names *Streptomyces candidus* var. *enterostaticus* WS-8096 and variant M-127 of *Streptomyces viridochromogenes*[1]. Its elementary analysis and mass spectroscopic measurement suggest the molecular formula is $C_{22}H_{20}O_{10}$. The ultraviolet absorption gave two maximal peaks at 250 nm and 283 nm in methanol. Enterocin has static activities against gram-positive and gram-negative bacteria and no activity against fungi and yeast.

In the course of our antibiotics screening program, a new antibiotic was isolated from cultures of two strains of *Streptomyces*, which were designated WS-8096 and M-127 in our culture collection. The antibiotic was effective against gram-positive and gram-negative bacteria, especially against the Enterobacteriaceae and was therfore named enterocin.

In this paper, described are the characteristics of strains of WS-8096 and M-127, the fermentation and isolation procedures and the physico-chemical and biological properties of enterocin.

Taxonomic Studies on the Producing Organisms

Enterocin is produced by two strains of *Streptomyces* isolated from soil samples (collected in Osaka Prefecture, Japan), which were designated WS-8096 and M-127 in our collection. According to the taxonomic studies described below these organisms were designated as *S. candidus* var. *enterostaticus* and variant M-127 of *S. viridochromogenes*, respectively.

Unless otherwise stated, the experiments to determine the cultural characteristics were carried out at 30°C for 13~15 days. The gelatin stab culture was observed after incubation at room temperature for 20 days.

1. Characteristics of Strain WS-8096

Microscopic examination of the culture on CZAPK's agar revealed thick and straight, so-called tuft aerial mycelium (Plate 1). The electron micrographs showed spores with smooth surfaces (Plate 2). The WS-8096 strain generally grew well at about 30°C and pH 7~8.

The cultural characteristics and physiological properties of strain WS-8096 are shown in Tables 1 and 2, respectively. The ability of the organism to utilize different carbon sources was also investigated according to the method described by PRIDHAM and GOTTLIEB (Table 3)[2]. On the most media, yellow substrate mycelium develops moderately and the aerial mass is white with yellowish tinge. No soluble pigment is produced on the media including tyrosine

Plate 1. Aerial mycelia of strain WS-8096.

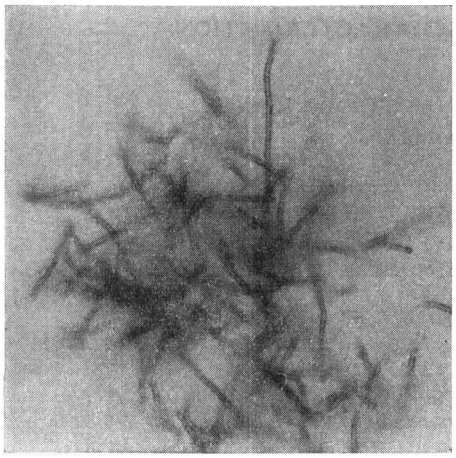
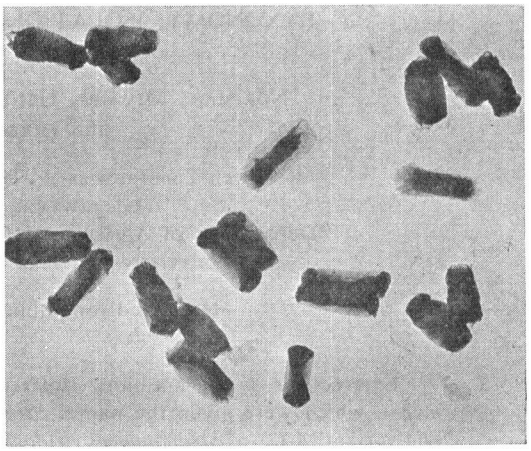


Plate 2. Electronmicrograph of the spores of strain WS-8096.



agar, nutrient agar, yeast malt agar and other proteinous media such as gelatin, milk and potato. Accordingly, strain WS-8096 is considered to be non-chromogenic. The hydrolytic activity on starch, gelatin and milk is strong.

From the observations described above, the distinctive characters of strain WS-8096 are as follows:

(1) The aerial mass color is white and the color of substrate mycelium on most media is yellow.

(2) The aerial mycelium is in tufts and the surfaces of spores are smooth.

(3) No soluble pigment is produced on either synthetic or organic media, suggesting the strain is non-chromogenic.

(4) Proteolytic activity on gelatin or milk is strong.

As a result of the comparisons of the characteristics of those *Streptomyces* species in non-chromogenic series described in "The Actinomycetes Vol. 2." by WAKSMAN,[3] the ISP reports by SHIRLING and GOTTLIEB[4,5,6,7] and the 8th edition of "BERGEY's Manual of Determinative Bacteriology"[8] strain WS-8096 was found to be closely related to *Streptomyces candidus*. However, strain WS-8096 differs from standard strains of *S. candidus* in its ability to utilize raffinose, its strongly proteolytic activity on gelatin or milk and its ability to produce a new antibiotic, enterocin.

From these considerations, strain WS-8096 can reasonably be classified as a variant of *S. candidus* and designated as *Streptomyces candidus* var *enterostaticus*.

2. Characteristics of Strain M-127

The aerial mycelium of strain M-127 branches simply and mature spore chains generally form small spirals (Plate 3). The special morphological features such as sclertia, sporangia, and flagellated spores and so on are not observed. As shown in the electron micrographs of strain M-127 (Plate 4), the spores have spiny surfaces. M-127 strain could grow at 15~42°C and at pH 4.5~9.0. It generally grew well at 30°C under the condition of pH 7.

The cultural characteristics and physiological properties of M-127, including utilization of carbon sources are shown in Tables 4, 5 and 6, respectively.

Table 1. Cultural characteristics of strain WS-8096.

Medium	Substrate mycelium	Aerial mycelium	Soluble pigment
CZAPEK's agar	White, flat spreading growth	Thick, white powdery	None
Starch ammonium agar	Bright yellow, weak growth	White powdery	None
Starch-inorganic salt agar	Bright yellow growth	White powdery	None Moderate hydrolysis
Glucose asparagine agar	Bright yellow growth	Thick, white powdery	None
Glycerine asparagine agar	Yellow growth	White powdery	None
Calcium-malate agar	Yellow growth with halo	Thin, white powdery	None
Tyrosine agar	Pale yellowish brown growth	None	None
Bouillon agar	Yellow, flat and wrinkled surface	None	None
BENNETT's agar	Yellow growth	Thick, pale yellow-white powdery	More growing at 30°C than at 37°C
Yeast malt agar	Yellowish brown growth	White powdery	None
Oat meal agar	Yellowish brown growth	White powdery	None
Peptone-yeast iron agar	Yellow growth	White powdery	None
Glucose bouillon	Creamy growth	None	None Becoming weak acidic
Glucose CZAPEK's solution	Brown small colonies	None	None Reduction of nitrate: positive
Milk	Pale brownish ring	None	None Peptonization: strong Coagulation: probable
Gelatin stab (15~20°C, 20 days)	Faint growth yellow	None	Liquefaction: strong
Potato-plug	Yellow growth, wrinkled surface	White powdery	None
Cellulose	No growth		
Nutrient agar	Yellow growth, flat and wrinkled surface	None	None
LOEFFLER's blood serum	Yellow growth wrinkled surface	None	None
Peptone-glucose agar	Yellow growth	Yellowish white powdery	None
Egg	Yellow growth	None	

On most media, a brown substrate mycelium develops moderately and the aerial mass is white or green with bluish tinge. Brown soluble pigment is produced on the media tested including tyrosine agar, nutrient agar, yeast malt agar and other proteinous media such as gelatin, milk and potato. Accordingly, strain M-127 is considered to be chromogenic. The hydrolytic activity was positive on gelatin, and negative on starch or milk.

From the observations described above, the distinctive characters of strain M-127 are as follows:

(1) The aerial mass color is in the Green color series when adequate aerial mycelium is

Table 2. Physiological properties of strain WS-8096.

Optimum temperature for growth	30°C
Optimum pH range for growth	7~8
Tyrosinase reaction	negative
Melanoid pigment	negative
Reduction of nitrate	positive
Liquefaction of gelatin	strong
Coagulation of milk	probable
Peptonization of milk	strong
Hydrolysis of starch	moderate
Cellulose decomposition	negative
Product	enterocin

Table 3. Carbon utilization pattern for strain WS-8096.

Source of carbon	Growth
D-Xylose	+
L-Arabinose	−
D-Fructose	±
D-Glucose	+
L-Rhamose	±
Sucrose	−
Lactose	−
Trehalose	+
Raffinose	+
D-Mannitol	+
Inositol	−
Salicin	±
Negative control	−

(+) utilization, (±) probable utilization, (−) no utilization.

Plate 3. Aerial mycelia of strain M-127.

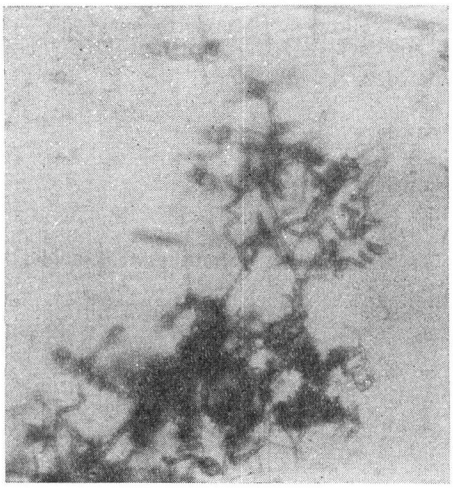
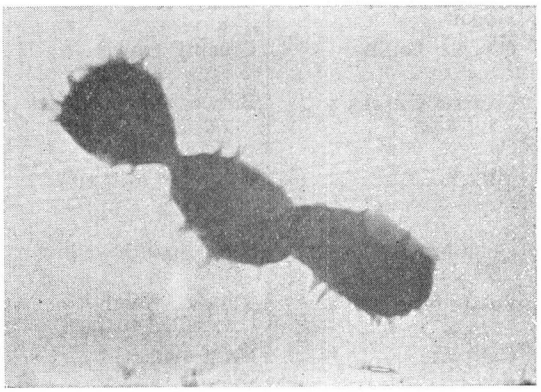


Plate 4. Electronmicrograph of the spores of strain M-127.



produced.

(2) The aerial mycelium branches finely and its mature spore chains form small spirals.

(3) The surfaces of spores are spiny.

(4) The strain shows chromogenic character and produces brownish soluble pigment in the proteinous media.

(5) The carbon utilization pattern is broad.

According to WAKSMAN's classification system[3], the ISP reports by SHIRLING and GOTTLIEB[4,5,6,7] and the 8th edition of "BERGEY's Manual of Determinative Bacteriology"[8] this organism belongs to the viridochromogenes group and is to be closely related to *S. viridochromogenes*. However, strain M-127 differs from standard strains of *S. viridochromogenes* in its negative milk coagulation, its negative milk peptonization, its poor ability to utilize arabinose, inositol, mannitol and rhamnose, its lack of ability to hydrolyze starch and its ability to produce a new antibiotic, enterocin.

From these considerations, strain M-127 can reasonably be classified as a variant of *S. viridochromogenes*.

Table 4. Cultural characteristics of strain M-127.

Medium	Substrate mycelium	Aerial mycelium	Soluble pigment
CZAPEK's agar	Pale brown, small colonies	White powdery, not much abundant	None
Starch ammonium	Pale brown growth	Green powdery with a bluish tinge	None
Starch-inorganic salt agar	Pale greenish brown growth	Green powdery with a bluish tinge	None
Glucose asparagine agar	White, spreading growth	White powdery not much abundant	None
Glycerine asparagine agar	Pale brown small colonies	None	None
Calcium-malate agar	White growth turned to brown	White powdery not much abundant	None
Tyrosine agar	Brown growth	White powdery	Faint brown. Production of tyrosinase: probable
Bouillon agar	Grey growth, granular surface	White powdery in traces	Deep brown
BENNETT's agar	Dark brown growth	Green powdery with a bluish tinge	Deep brown
Yeast malt agar	Dark brown colonies	Green powdery, with a bluish tinge	Deep brown
Oat meal agar	Pale yellow growth	Bluish green and white powdery	None
Peptone-yeast iron agar	Dark brown growth	Bluish green powdery	Deep brown
Glucose bouillon	Dark brownish ring	Bluish green powdery	Dark brown
Glucose CZAPEK's solution	Brown, wrinkled surface	White powdery	Brown Reduction of nitrate: negative
Milk	Creamy ring	Absent	Pale brown. Peptonization and coagulation: negative
Gelatin stab (15~20°C, 20 days)	Brown colonies	Thin, white powdery	Brown Liquefaction: positive
Potato-plug	Brown, raised and wrinkled surface	Grayish white powdery	Brown
Cellulose	No growth		
Nutrient agar	Gray growth	Thin, white powdery	Brown
LOEFFLER's blood serum	Brown growth	Thin, white powdery	Brown
Peptone-glucose agar	Dark brown growth	Bluish green powdery	Deep brown
Egg	Dark brown	Thin, white powdery	Deep brown

Fermentation and Isolation of Enterocin

For the production of the antibiotic, slant cultures of the strain *S. viridochromogenes* var. M-127 were grown for 5 days at 30°C on BENNETT's agar. An aqueous suspension made from the slant culture was used to inoculate the shake culture. A 48-hour shake flask culture at 30°C was transferred to a 500-liter stainless steel fermentor containing 150 liters of the medium composed of 2% potato-starch, 2% Pharmamedia (Trader Oil Mill Co.), 1% corn steep liquor, 0.3% $CaCO_3$, 2.18% KH_2PO_4 and 1.43% $Na_2HPO_4h12H_2O$, which was incubated at 30°C and used as a seed tank. The tank was agitated at 250 r.p.m. and aerated with 1.0 v/v/min. After 24 hours the broth in the seed tank was transferred to 3 m^3 of the same medium in a 4 m^3-stainless steel tank. Fermentation was run at 30°C, agitated at 130 r.p.m.

Table 5. Physiological properties of strain M-127.

Optimum temperature for growth	30°C
Optimum pH range for growth	7
Tyrosinase reaction	probable
Melanoid pigment	positive
Reduction of nitrate	negative
Liquefaction of gelatin	positive
Coagulation of milk	negative
Peptonization of milk	negative
Hydrolysis of starch	negative
Cellulose decomposition	negative
Product	enterocin

Table. 6. Carbon utilization pattern for strain M-127.

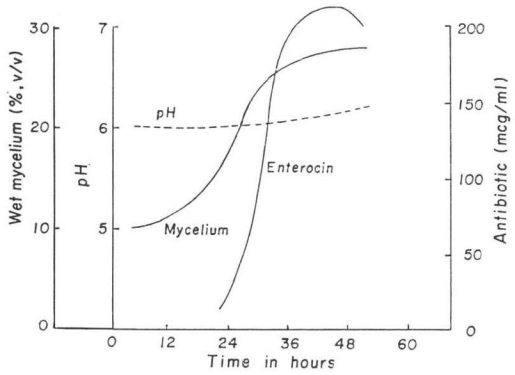
Source of carbon	Growth
D-Xylose	+
L-Arabinose	±
D-Fructose	+
D-Glucose	+
L-Rhamnose	±
Sucrose	±
Lactose	+
Trehalose	+
Raffinose	+
D-Mannitol	±
Inositol	±
Salicin	−
Negative control	−

(+) utilization, (±) probable utilization, (−) no utilization.

Fig. 1. Fermentation of enterocin.

Three cubic meters of medium were placed in a 4m³-stainless steel fermenter.

Aeration rate : 3 m³/minute. Agitation rate: 130 r.p.m.



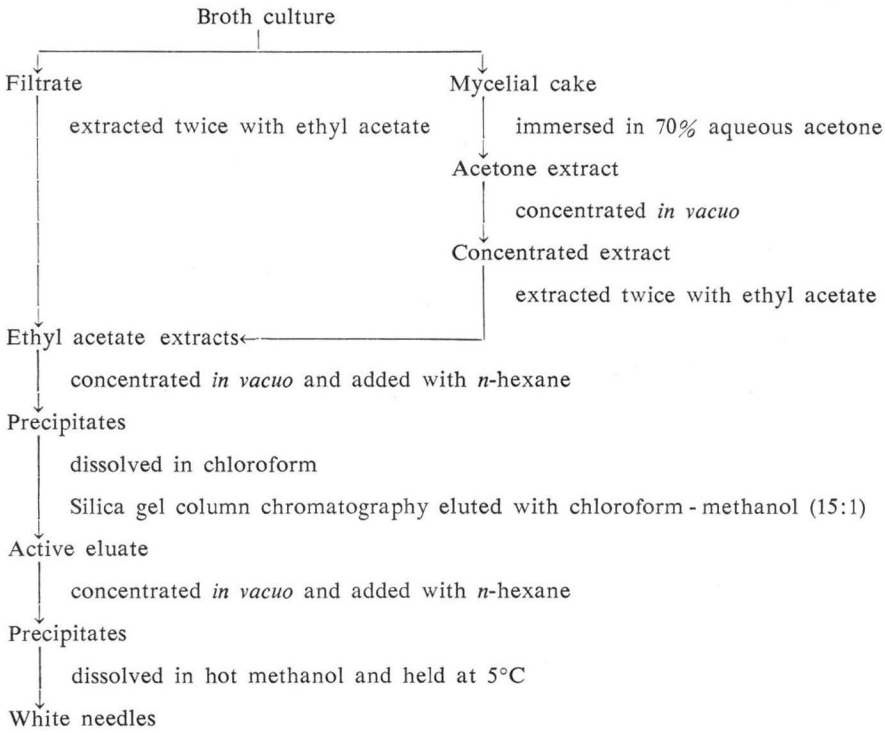
and aerated with 1.0 v/v/min. for 48 hours. A typical fermentation process is shown in Fig. 1.

During the fermentation and isolation process, antibiotic activity was assayed by an agar diffusion method with *Escherichia coli* NIHJ JC-2 as the test organism using the standard samples of pure enterocin.

Enterocin was produced mostly in the filtrate, but was also contained in the mycelium. A flow sheet for the isolation and purification of the antibiotic is shown in Fig. 2. The mycelial cake was extracted with 70 % aqueous acetone and concentrated *in vacuo*. The concentrate was extracted twice with ethyl acetate. The ethyl acetate extracts of the mycelial extract were combined with those of the filtrate and concentrated *in vacuo*. The concentrate was treated with 5 volumes of *n*-hexane to precipitate the active factor. The crude precipitate with brownish tinge was further purified by chromatography on a silica gel column, which was packed in *n*-hexane. By developing the column with a mixture of chloroform and methanol (15:1), active fractions were eluted. These fractions were combined, concentrated and precipitated with *n*-hexane. The precipitate was dissolved in hot methanol and held at 5°C overnight to obtain enterocin as white crystalline needles. For further purification a mixture solvent of methanol and ethanol was used.

The white crystalline needles, which were obtained from the culture broth of strain WS-8096 by the procedure described above was shown to be enterocin on the basis of its physicochemical properties.

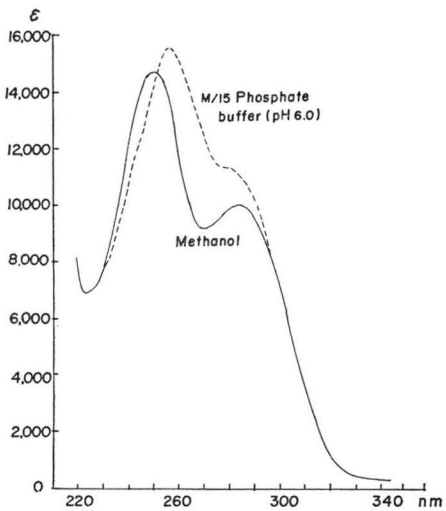
Fig. 2. Isolation and purification procedure of enterocin.



Physico-chemical Properties of Enterocin

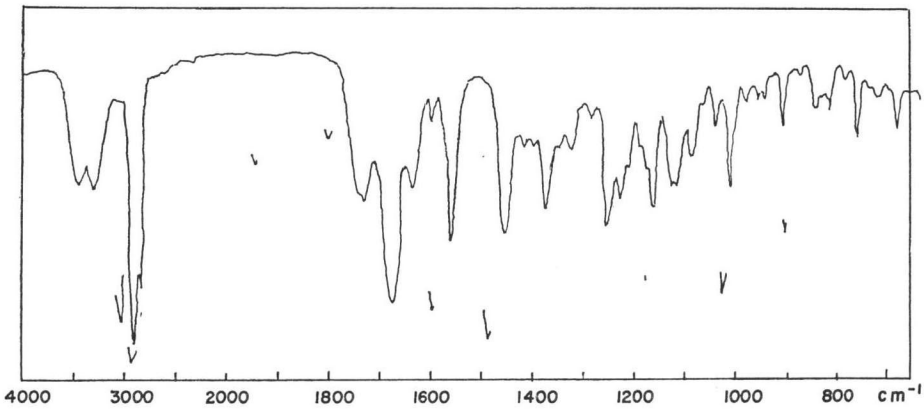
Enterocin is in the form of white crystalline needles which melt at 163~167°C. It is neutral, freely soluble in methanol and pyridine, soluble in water, acetone, ethyl acetate and dimethylcellosolve, sparingly soluble in ether, chloroform and benzene, and insoluble in *n*-hexane and petroleum ether. The following reactions were all positive; FEHLING, TOLLENS and the decolorization of potassium permanganate. The following were all negative; anthrone, MOLISCH and ferric chloride. The optical rotation is $[\alpha]_D^{20}$ −10.5°(*c* 1, methanol). The ultraviolet absorption spectra of enterocin are shown in Fig. 3, with peaks at 250 nm (ε 14,652) and 283 nm (ε 9,945) in methanol. The infrared absorption spectrum is shown in Fig. 4.

Fig. 3. Ultraviolet spectra of enterocin.



Elementary analysis of enterocin gave: C 59.48, H 4.69, O 36.16 (%). Nitrogen, phosphorus, halogen and heavy metals were not present. The composition was calculated for $C_{22}H_{20}O_{10}$ (M.W. 444.38): C 59.46, H 4.54,

Fig. 4. Infrared absorption spectrum of enterocin (Nujol).



O 36.01 (%). The empirical formula $C_{22}H_{20}O_{10}$ was established by the parent peak in the mass spectrum at *m/e* 444 and the elementary analysis.

Enterocin is stable at room temperature in neutral or acidic solution, but is labile in alkaline solution.

Biological Properties of Enterocin

Enterocin is a bacteriostatic antibiotic and it was not possible to determine the minimal inhibitory concentration of the antibiotic by the broth dilution method and the agar dilution method. Table 7 shows the antimicrobial spectrum of enterocin, which was determined by the zone diameter of inhibition that appeared when the paper disc (8-mm diameter), was dipped in 4 mg/ml solution of the antibiotic and dried, then incubated on the agar plate containing the test organism. Enterocin is bacteriostatic against gram-positive and gram-negative bacteria, including *Escherichia coli* and species of *Proteus*, *Sarcina*, *Staphylococcus*, and *Corynebacterium*. It has no activity against fungi and yeast.

Table 7. Antimicrobial spectrum of enterocin.

Organism	Inhibition zone diameter, mm. Enterocin 4 mg/ml
Escherichia coli NIHJ JC-2	28
Proteus vulgaris IAM-1095	30
Sarcina lutea PCI-1001	26
Staphylococcus aureus 209P JC-1	17
Corynebacterium xerosis BO-404	16
Bacillus subtilis ATCC 6633	0
Bacillus megatherium BB-105	0
Pseudomonas aeruginosa BP-145	0
Candida albicans YC-109	0
Penicillium chrysogenum Q 176	0

The acute toxicity of enterocin seems to be low. Mice injected with 500 mg/kg intraperitoneally or administered with 1,000 mg/kg orally of enterocin did not result in death after 14 days.

The above doses were limited for injection due to the solubility.

Discussion

Enterocin is a bacteriostatic antibiotic. Its molecular formula is $C_{22}H_{20}O_{10}$ as shown by the parent peak at *m/e* 444 and the elementary analysis. Among known antibiotics produced by *Streptomyces* sp., granaticin[9] is the only substance which has the same molecular formula

as enterocin. However, granaticin, which exhibits activities against gram-positive bacteria, differs from enterocin in physicochemical and biological properties.

On the basis of the investigation described above, it seems reasonable to conclude that enterocin is a new antibiotic.

References

1) MIYOSHI, T.; N. MIYAIRI, K. SHIMIZU, H. IMANAKA & H. SAKAI: Process for the production of an antibiotic, WS-8096 substance. Japan Patent 47-15750
2) PRIDHAM, T.G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bact. 56: 107~114, 1948
3) WAKSMAN, S.A.: The Actinomycetes. Vol. 2. Classification, Identification and Description Genera and Species. The Williams and Wilkins Co., Baltimore, 1961
4) SHIRLING, E.B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. II. Species descriptions from first study. Intern. J. Syst. Bact. 18: 69~189, 1968
5) SHIRLING, E.B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. III. Additional species descriptions from first and second studies. Intern. J. Syst. Bact. 18: 279~392, 1968
6) SHIRLING, E.B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species descriptions from the second, third and fourth studies. Intern. J. Syst. Bact. 19: 391~512, 1969
7) SHIRLING, E.B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. V. Additional descriptions. Intern. J. Syst. Bact. 22: 265~394, 1972
8) BUCHANAN, R.E.; N.E. GIBBONS, S.T. COWAN, J.G. HOLT, J. LISTON, R.G.E. MURRAY, C.F. NIVEN, A.W. RAVIN & R.Y. STANIER: BERGEY's Manual of Determinative Bacteriology. 8 ed., 1974
9) CORBAZ, R.; L. ETTLINGER, E. GÄUMANN, J. KALVODA, W. KELLER-SCHIERLEIN, F. KRADOLFER, B.K. MANUKIAN, L. NEIPP, V. PRELOG, P. REUSSER & H. ZÄHNER: Stoffwechselprodukte von Actinomyceten. Granaticin. Helv. Chim. Acta 40: 1262~1269, 1957