A NEW ANTIBIOTIC VICTOMYCIN (XK 49-1-B-2)

I. TAXONOMY AND PRODUCTION OF THE PRODUCING ORGANISM

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A new antibiotic designated as victomycin which belongs to the phleomycin-bleomycin group antibiotics was isolated from a sporangia-forming actinomycete. From taxonomic studies, the producing strain was classified as *Streptosporangium violaceochromogenes* nov., sp. KAWAMOTO *et* NARA 1974. Fermentative production of antibiotic complex XK 49 is described.

In the course of screening for new antibiotics, a complex of antibacterial and antitumor antibiotics was obtained from the culture broth of an actinomycete. This antibiotic complex (XK 49) was then found to belong to the phleomycin[1]-bleomycin[2] group. One of the antibiotics, antibiotic XK 49-1-B-2, was identified as a new antibiotic and designated victomycin. Isolation, characterization and biological activity of this compound will be reported in a subsequent paper.[3]

The producing strain MK 49 was characterized by the formation of spherical sporangia containing numerous non-motile sporangiospores and classified as a new species of *Streptosporangium* COUCH 1955. Only four antibiotics were reported to be produced by the strains of this genus, sporaviridin,[4] selenomycin,[5] sibiromycin[6] and chloramphenicol.[7] Phleomycin,[1] bleomycin[2] and chloramphenicol[8] had previously been reported to be produced by strains of *Streptomyces*. It is interesting that the same or related antibiotics are produced by strains of different genera of actinomycetes.

This report describes the taxonomy of the producing organism and fermentative production of antibiotic complex XK 49.

Taxonomy

Strain MK 49 was isolated from a swamp soil sample collected in Yoshioka village, Kitagunmagun, Gunma, Japan. This strain have been deposited at the American Type Culture Collection, Rockville, Maryland, U.S.A. and have been assigned accession number 21807.

Most of the taxonomic studies of the culture were carried out in accordance with methods adopted by the International Streptomyces Project (ISP).[9] Additional media recommended by WAKSMAN[10] were also used.

Morphological Characteristics

Both aerial and substrate mycelia were well developed, branched, septate and 0.4~0.8 μ in width. Sporangia were abundantly produced and were borne from only on aerial mycelia,

usually on more or less long sporangiophores. The sporangia were spherical, 5~9 μ in diameter, with irregular surface. A rupture of the sporangia membrane occurred when the sporangia were transferred in water. Sporangiospores were numerous in sporangium, formed in coils, oval or cylindrical in shape, 0.8~0.9 by 1.2~1.6 μ, and non-motile. Flagella were not observed on the surface of sporangiospores (Plates 1, 2, 3, 4, 5).

Appearance on Various Media

The cultural characteristics of strain MK 49 shown in Table 1 were observed after two weeks of incubation at 27°C on the designated media. The number in parentheses corresponds to the hue number in "Color Harmony Mannual".[11]

Growth of strain MK 49 was relatively poor on chemically defined media compared to growth on natural nutrient media. The addition of biotin, thiamine and other vitamins, did not stimulate growth on chemically defined media.

Characteristic violet or rose diffusible pigments were produced in natural nutritional agar media, which had antibacterial activity against gram-positive bacteria, such as *Bacillus subtilis*, *Staphylococcus aureus* and *Streptococcus faecalis*, but had no activity against gram-negative bacteria. The pigments were extractable in lower alcohols. The extracts were violet or rose in alkaline and neutral range, but yellow in acidic range.

Physiological Characteristics

The physiological properties of strain MK 49 are shown in Table 2. Temperature and pH

Table 1. Cultural characteristics of strain MK 49 on various agar media

Medium	Growth, color of substrate mycelium	Aerial mycelium	Soluble pigment
CZAPEK's agar	poor colorless	fair, powdery shell pink (5ba)	none
Glucose asparagine agar	poor colorless	fair, powdery shell pink (5ba)	none
Glycerol asparagine agar	poor colonial yellow maize (2ga)	poor white (a)	none
Inorganic salts starch agar	poor colorless	poor shell pink (5ba)	none
Egg albumin agar	poor colorless	fair, powdery shell pink (5ba)	none
Nutrient agar	good orange (41a)	good white (a)	raspberry (9nc)
Yeast extr.-malt extr. agar	poor gold (21c)	poor white (a)	none
Oatmeal agar	moderate gold (21c)	good shell pink (5ba)	dusty yellow (1/2ge)
BENNETT's agar	good melon yellow (3ge)	good white(a)→peach pink (5ea)	raspberry (9nc)
EMERSON's agar	good amber (3nc)	good white (a)	raspberry (9nc)
Glucose yeast extr. agar	good colonial yellow maize (2ga)	good white (a)	none
Peptone iron agar	poor mustard gold (2ne)	poor white (a)	none
Tyrosin agar	poor colonial yellow maize (2ga)	fair, powdery shell pink (5ba)	none

Plate 1. Photomicrograph of *Streptosporangium violaceochromogenes* MK 49 on egg albumin agar.

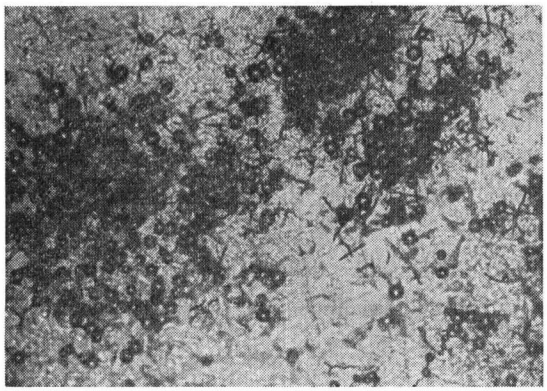


Plate 2. Photomicrograph of *Streptosporangium violaceochromogenes* MK 49 on egg albumin agar.

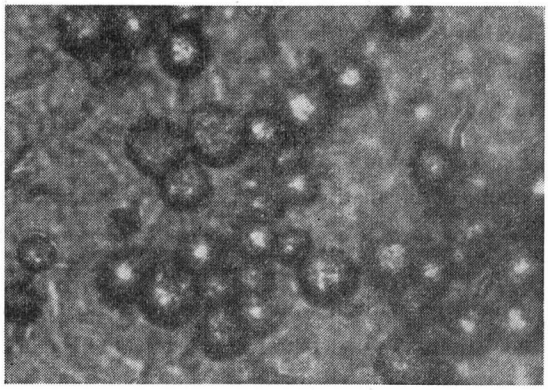


Plate 3. Photomicrograph of ruptured sporangia.

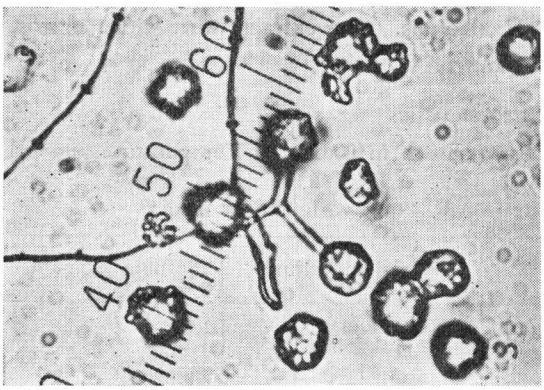


Plate 4. Electronmicrograph of sporangia on egg albumin agar.

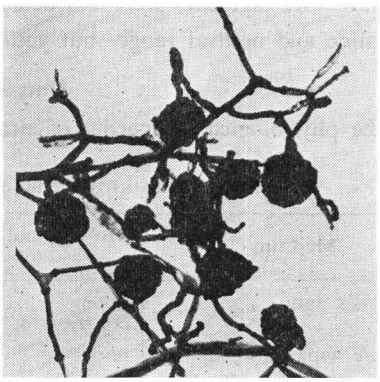
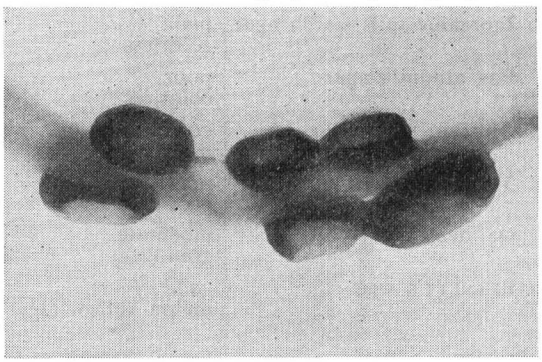


Plate 5. Electronmicrograph of sporangiospores.



range for growth were observed after cultivation for 5 days in a liquid medium consisted of 2% glucose, 0.5% peptone, 0.5% yeast extract and 0.1% $CaCO_3$.

Cell Wall Components

Amino acids from cell wall hydrolysate were analyzed according to the method of BOONE and PINE.[12] The methods used for analysis of DAP-isomer were those of BECKER *et al.*[13] Whole cell sugar analysis was carried out according to the method of LECHEVALIER and LECHEVALIER.[14] Additionally other following solvent systems were also used for paper chromatography.

Phenol solvent (phenol 160 g + water 40 ml) - ethanol - water - ammonia (150 : 40 : 10 : 1) (amino acid analysis)
Ethylacetate - pyridine - water (12 : 5 : 4) (whole cell sugar analysis).

Table 2. Physiological properties of strain MK 49

Liquefaction of gelatin:	weakly positive
Peptonization of milk:	weakly positive
Coagulation of milk:	weakly positive
Decomposition of cellulose:	negative
Hydrolysis of starch:	positive
Reduction of nitrate:	positive
Formation of tyrosinase:	negative
Formation of melanoid pigment:	negative
Utilization of carbohydrate:	Good growth; D-fructose, D-glucose, D-mannose, starch.
	Moderate growth; D-galactose, glycerol, D-xylose, sucrose.
	Poor growth; D-arabinose, L-inositol, D-lactose, D-mannitol, D-raffinose, L-rhamnose.
pH range for growth:*	6.0~8.5 (opt. pH 7.3)
Temperature range:*	25~40°C (opt. Temp. 30~37°C)

* Medium used consisted of 1% glucose, 1% dextrin, 0.5% Polypeptone, 0.5% yeast extract and 0.1% $CaCO_3$.

Results of Experiments

Amino acid: meso-diaminopimelic acid (present), aspartic acid (present), glycine (trace), valine (trace).

Sugar: arabinose (none), xylose (none), galactose (none), madurose (none).

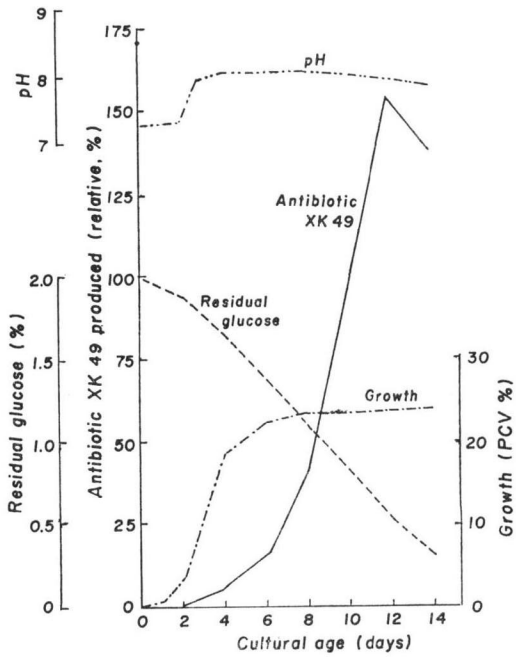
Actinomycetes which form sporangia were reported probably first by SHCHEPKINA[15] in 1940 and then by COUCH in 1949.[16] COUCH[17,18] added the family *Actinoplanaceae* to *Actinomycetales*. Other sporangia-bearing actinomycetes then became to be recognized, and now these actinomycetes were classified into about 13 genera by such morphological characteristics as shape of sporangium, number of sporangiospores in a sporangium and presence of flagella, and by cell

Table 3. Comparison of strain MK 49 with the related species of genus *Streptosporangium*

	S. album NONOMURA *et* OHARA	*S. roseum* COUCH	*S. vulgare* NONOMURA *et* OHARA	*S. violaceo-chromogenes* MK 49
Sporangium	6~8 μ	7~9 μ	7~10 μ	5~9 μ
Sporangiospore	oval	oval	oval	oval
Color of aerial mycelium	white	pink pale pink	pink pale pink	white pale yellow
Color of substrate mycelium	pale yellow	reddish orange, pale yellow, yellowish brown	yellowish orange, orange, yellow, pale rose	pale yellow
Soluble pigment	pale yellow	purple brown	pale yellow	violet
Starch hydrolysis	−	+	+	+
Nitrate reduction	−	+	−	+
Requirement:				
Thiamine	+	+	+	−
Biotin	+	±	−	−
Carbon utilization:				
L-inositol	±	+	+	±
L-rhamnose	−	+	+	−

wall components. The taxonomical studies of strain MK 49 indicate that this isolate belongs to the genus *Streptosporangium* COUCH 1955.[17] Compared with known species of *Streptosporagium* according to classification by NONOMURA,[19] strain MK 49 was mostly related to *S. album* NONOMURA *et* OHARA,[20] *S. roseum* COUCH[17] and *S. vulgare* NONOMURA *et* OHARA[20] from the viewpoint of length of sporangiophores, color of aerial and substrate mycelia, and production of iodinin. As shown in Table 3, however, there are distinct differences from these cultures in a number of characteristics, especially in produced soluble pigments, thus permitting to put strain MK 49 into a new species. Therefore strain MK 49 was named *Streptosporangium violaceochromogenes* nov., sp. KAWAMOTO *et* NARA, because this strain produces characteristic and brilliant violet pigments.

Fig. 1. Chemical change in fermentation of *Streptosporangium violaceochromogenes*.



Fermentation

Productivity of victomycin by *Streptosporangium violaceochromogenes* MK 49 was not enough for further investigation when the strain was first found. Therefore some cultural conditions for growth and antibiotic XK 49 production were studied.

A seed medium used is consisted of 1% dextrin, 1% glucose, 0.5% Polypeptone, 0.5% yeast extract and 0.1% $CaCO_3$ (pH 7.2 before autoclaving). Thirty ml medium in a 250-ml Erlenmeyer flask was incubated on a rotary

Table 4. Effect of carbohydrate sources on production of antibiotic XK 49 complex

Carbohydrates	pH		Growth (PCV, %)		Antibiotic XK 49* (relative, %)	
	7 day	11 day	7 day	11 day	7 day	11 day
Gycerol	9.1	9.0	6.5	6.5	29	20
D-Xylose	9.0	8.9	7.5	8.0	48	44
D-Galactose	9.0	8.9	11.0	12.5	100	150
D-Glucose	8.3	8.8	16.5	17.5	100	130
D-Fructose	9.0	9.0	9.0	10.0	54	38
D-Maltose	9.0	9.0	8.0	7.5	56	50
Sucrose	9.1	9.0	9.0	9.0	53	42
Dextrin	9.1	9.0	8.5	8.5	58	42
Soluble starch	9.1	9.0	7.0	7.0	50	28

Basal medium; 2% corn steep liquor and 0.1% $CaCO_3$ (pH 7.4).
All carbohydrates (2% final concentration) were sterilized separately, and added to the basal medium just before inoculation.
* Antibiotic XK 49 bioassay using *Bacillus subtilis* KY 4273 (cup, agar diffusion assay). Eluates of IRC 50 (H^+) from each broth were used as sample for bioassay.

shaker at 220 rpm at 30°C for 5 days after inoculation of a loopful of strain MK 49 spores. Then fermentation medium (30 ml in a 250-ml Erlenmeyer flask, into which 3 ml of seed culture was transferred) was incubated on a rotary shaker at 230 rpm at 30°C for 11 days.

Amounts of growth were measured by packed cell volume (centrifugation at 3,000 rpm for 15 minutes). The antibiotic produced was determined by the cup bioassay method using *Bacillus subtilis* KY 4273 as a test organism. The broth was first passed through an IRC50 (H^+) which adsorbed XK 49, but allowed a second antibiotic to pass through. XK 49 was then eluated with 0.5 N hydrochloride and assayed.

Table 4 shows the effect of carbohydrate sources, in which antibiotic produced was compared under a standard condition with the yields presented as a percentage of that obtained

Table 5. Effect of nitrogen sources on production of antibiotic XK 49 complex

Nitrogen sources		pH		Growth* (PCV, %)		Antibiotic XK 49*** (relative, %)	
		7 day	11 day	7 day	11 day	7 day	11 day
Yeast extract	1	8.5	8.6	3.5	5.0	10	10
	2	8.4	8.5	3.5	4.0	10	10
Polypeptone	1	8.7	8.7	7.5	8.0	30	10
	2	8.7	8.7	10.0	9.0	10	10
NZ-amine	1	8.6	8.5	10.0	10.0	40	156
	2	8.6	8.7	9.5	9.5	58	139
Tryptone	1	8.7	8.5	11.0	10.5	58	211
	2	8.7	8.2	7.0	7.0	23	34
Casamino acid	1	8.1	8.2	5.0	5.0	10	48
	2	8.3	8.5	7.0	6.5	10	10
Meat extract	1	8.5	8.3	7.0	7.0	156	334
	2	8.4	8.0	13.5	13.5	400	560
Beef extract	1	8.5	8.5	8.5	7.5	106	368
	2	8.6	8.4	10.0	10.0	250	668
Ebios (dry yeast)	1	8.0	7.8	12.5	14.5	64	100
	2	7.9	8.1	16.5	19.0	55	83
Pharmamedia	1	8.0	8.0	10.0	11.6	117	331
	2	8.1	8.3	15.0	16.5	269	386
SVP**	1	7.9	8.1	10.0	12.0	50	123
	2	8.0	8.2	15.0	16.0	250	223
Soy bean meal	1	8.2	8.3	12.0	12.0	260	345
	2	8.3	8.5	14.5	16.5	131	389
Bacto-liver	1	8.0	7.8	15.0	14.5	543	1,060
	2	8.2	7.9	22.5	22.0	690	3,160
	3	7.0	8.0	18.0	24.0	115	652
Corn steep liquor	1	8.0	8.1	8.0	8.0	100	138
	2	8.0	8.0	10.0	10.5	100	100
	3	8.3	8.4	12.0	13.0	62	58

Basal medium; 2% glucose and 0.1% $CaCO_3$ (pH 7.4).

* Packed Cell Volume when centifuged at 3000 r.p.m. for 15 mimutes.

** Soluble Vegetable Protein.

*** Antibiotic XK 49 bioassay using *Bacillus subtilis* KY 4273 (cup, agar diffusion assay). Eluates of IRC 50 (H^+) from each broth were used as sample for bioassay.

with glucose (7-day beer). Most abundant growth was obtained in medium with glucose, and antibiotic complex was produced at much higher level with glucose and galactose.

With a medium containing glucose as a carbon source, a number of natural nitrogen sources have been tested. As shown in Table 5, Bacto-liver (DIFCO, for liver infusion media) was an excellent source for both growth and production of the antibiotic, while yeast extract, Polypeptone and casamino acids were undesirable sources. Meat extract and beef extract promoted production more than corn steep liquor. It remains to be determined which components of Bacto-liver stimulate the accumulation of antibiotic XK 49.

Fig. 1 shows a typical fermentation time course using glucose and Bacto-liver as carbon and nitrogen sources, respectively. Growth increased rapidly after 2 days and reached to a maximum at 4 days. Glucose was utilized gradually until 14 days, when residual glucose was 0.3%. Antibiotic production began after growth had essentially finished, and the yield increased straightly to a maximum until 12 days and then decreased slightly.

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