A NEW ANTIBIOTIC XK-90

I. TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION, ISOLATION AND PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES

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(Received for publication July 5, 1976)

A new antibiotic XK-90 is produced by *Streptomyces* sp. MK-90 and is active against Gram-positive and Gram-negative bacteria. The taxonomy of the organism, fermentation, isolation and physicochemical and biological properties are described.

From a field soil from Chiba city, Chiba Prefecture, Japan, the authors isolated a strain (MK-90) capable of producing a new antibiotic XK-90. This antibiotic is active against Gram-positive and Gram-negative bacteria.

This report deals with the taxonomy of the producing organism, fermentation, isolation, and physicochemical and biological properties of XK-90. The elucidation of the structure of this antibiotic will be reported separately.[2]

Characteristics of Strain MK-90

The strain designated MK-90 has been deposited at American Type Culture Collection as ATCC 31017. The taxonomical studies of this culture are carried out in accordance with the method adopted

Table 1. Cultural characteristics of *Streptomyces* sp. MK-90.

Medium	Growth	Substrate mycelium	Aerial mycelium		Soluble pigment
			Formation	Color	
Sucrose-nitrate agar	good	bamboo chamois (2 gc)	good	white (a)	none
Glucose-asparagine agar	poor		none		none
Glycerol-asparagine agar	moderate	colorless-light ivory eggshell (2 ca)	poor	white (a)	none
Starch-inorganic salt agar	moderate	bamboo chamois (2 gc)-cinnamon yellow maple (3 le)	moderate	white (a) fresh pink (4 ca)	none
Tyrosine agar	moderate	colorless-light ivory eggshell (2 ca)	moderate	white (a)	none
Nutrient agar	poor	colorless-light ivory eggshell (2 ca)	poor	white (a)	none
Yeast extract-malt extract agar	good	yellow maple (3 ng)	good	shell pink (5 ba)	none
Oat meal agar	poor	bamboo chamois (2 gc)	poor	white (a)	none

Streptomyces sp. MK-90 was cultured at 30°C for 2 weeks.

The indications in the parentheses are in accordance with the color classification of Color Harmony Manual (Container Corporation of America).

by the International Streptomyces Project (ISP).[11]

The aerial mycelia of MK-90 are developed from the substrate mycelia. The sporophores occur on aerial mycelia, are straight or little flexuous and possess more than 10 spores. The spore size is about 1×1.5 μm and its surface is smooth. Zoospores and sporangia are not observed. From these observations, it is thought that the strain MK-90 belongs to the genus *Streptomyces*. Its cultural characteristics on various media after culturing for 14 days at 30°C are described in Table 1. The substrate mycelia are pale yellow or brown and the aerial mycelia are white. The soluble pigment is not observed in all media tested.

The strain utilizes D-glucose, D-fructose and saccharose but does not utilize D-raffinose, D-arabinose, D-mannitol, *i*-inositol, L-rhamnose and D-xylose. The temperature for growth is 20~40°C, with optimal growth at around 30°C. Some other physiological properties are as follows: the liquefaction of gelatin and hydrolysis of starch are positive, peptonization and coagulation of milk, and chromogenic action are negative.

Fermentation and Isolation of XK-90

The strain was first inoculated into 10 ml of seed medium comprising 1% glucose, 1% soluble starch, 0.1% yeast extract, 0.5% peptone and 0.1% $CaCO_3$ (pH 7.2 before sterilization) in 50-ml test tube and cultured at 30°C for 3 days. The volume of the seed medium was increased from 10 ml to 90 ml, from 90 ml to 900 ml and finally from 900 ml of 15 liters of the fermentation medium in a 30-liter jar fermentor. The fermentation medium consisted of 2% oat meal, 2% soy-bean meal and 0.1% $CaCO_3$ (pH 7.2 before sterilization) and fermented at 30°C for 60 hours with 15 liters/minute aeration and 300 rpm agitation.

The cultural broth (15 liters) was adjusted to pH 4.0 with HCl and filtered *in vacuo*. The filtrate was passed through one liter column of Diaion HP-10. After washing with 3 liters of water, XK-90 was eluted with 50% aqueous methanol. The elution was monitored by the pulp disc-plate method using *Escherichia coli* ATCC 26 as test organism. The active fractions were collected and concentrated to dryness. The concentrate was then put on 300 ml of silica gel column and eluted with ethylacetate. The active fractions were collected, concentrated and stored overnight in a refrigerator. Compound XK-90 was separated in crystalline form. The antibiotic was recrystallized from ethylacetate to yield about 180 mg of yellow crystalline substance.

Physicochemical Properties of XK-90

The antibiotic XK-90 is isolated as yellow crystals and is soluble in methanol, ethanol, butanol, acetone, chloroform and ethylacetate, slightly soluble in water and ethylether, and insoluble in *n*-hexane and petroleum ether. As seen in Fig. 1, XK-90 has UV maxima at 236 nm ($E^{1\%}_{1cm}$ 1170), 260 nm (shoulder, $E^{1\%}_{1cm}$ 425) and 377 nm ($E^{1\%}_{1cm}$ 175) in methanol, 236 nm ($E^{1\%}_{1cm}$ 1050), 260 nm (shoulder $E^{1\%}_{1cm}$ 425) and 374 nm ($E^{1\%}_{1cm}$ 150) in 0.1 N HCl-methanol, and 232 nm ($E^{1\%}_{1cm}$ 875),

Fig. 1. The UV spectrum of XK-90

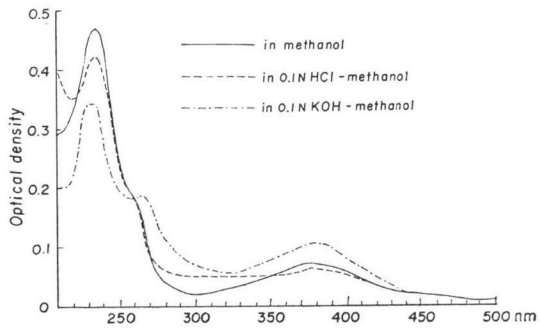


Fig. 2. The IR spectrum of XK-90.

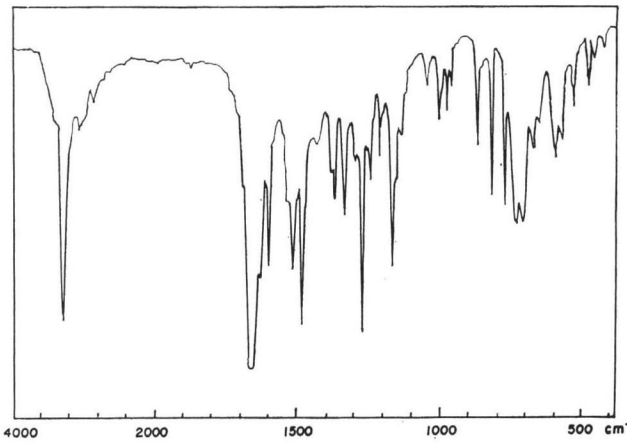


Table 2. Stability of XK-90.

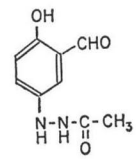
Temp.	pH	Activity remaining		
		0 hr.	3 hrs.	5 hrs.
30°C	2.0	100	100	100
	6.9	100*	100	100
	9.5	100	81	75
60°C	2.0		100	100
	6.9		100	100
	9.5		35	trace

* Relative activity: pH 6.9—0 hour's solution =100.

265 nm ($E^{1\%}_{1cm}$ 425) and 381 nm ($E^{1\%}_{1cm}$ 275) in 0.1 N KOH-methanol.

The IR spectrum in KBr tablet is shown in Fig. 2. The melting point of XK-90 is 128~129°C.

Fig. 3 The structure of XK-90.



The molecular weight of this antibiotic is 194 (determined by mass spectrum) and it has the molecular formula $C_9H_{10}N_2O_3$ (195.2); C 55.64%, H 5.19% and N 14.15% (calcd. C 55.66%, H 5.19% and N 14.43%). XK-90 is not optically active. XK-90 gives positive ferric chloride, PAULI, TOLLENS and FEHLING reactions, and negative SAKAGUCHI, ninhydrin and RYDON-SMITH reactions.

Table 3. The antibacterial spectrum of XK-90.

Organism	MIC* (μg/ml)
Streptococcus faecalis ATCC 10541	20.9
Staphylococcus aureus ATCC 6538 P	5.3
S. aureus KY 8942 (R-SM, KM, PM)	2.7
S. aureus KY 8950 (R-SM, TC, PC, SA)	5.3
S. aureus KY 8953 (R-SM, KM, NM, TC, EM)	1.4
Bacillus subtilis KY 4273	1.4
B. cereus ATCC 9634	1.4
B. cereus var. *mycoides* ATCC 9463	1.4
Klebsiella pneumoniae ATCC 10031	83.3
Escherichia coli ATCC 26	0.35
E. coli KY 8310 (R-SM, KM, TC, GM, CP)	0.7
E. coli KY 8302 (R-SM, KM, TC, CP)	0.7
E. coli KY 8315 (R-SM, KM, PM, NM)	0.35
Proteus vulgaris ATCC 6897	10.5
P. vulgaris KY 4296 (R-Na)	10.5
P. mirabilis KY 4293	0.7
P. rettgeri KY 4288	5.3
P. morgani KY 4298	10.5
Shigella sonnei ATCC 9290	0.7
Salmonella typhosa ATCC 9992	0.35

* Assayed with agar dilution method at pH 8.0.
R: Resistant, SM: Streptomycin, KM: Kanamycin, PM: Paromomycin, NM: Neomycin, GM: Gentamicin, TC: Tetracycline, CP: Chloramphenicol, SA: Sulfonamide, PC: Penicillin, EM: Erythromycin and Na: Nalidixic acid.

The 50% aqueous methanol eluate of Diaion HP-10 was adjusted to pH 2.0, 6.9 and 9.5 and kept

at 30°C and 60°C in a sealed test tube. After storage for the specified hours, an aliquot of each solution was adjusted to pH 7.0 and measured for activity against *E. coli* ATCC 26. In this experiment, the titer of antibiotic remained is shown as a relative activity to the titer of pH 6.9 - 0 hour's solution. As seen in Table 2, XK-90 is stable in acidic and neutral solution but unstable in alkaline solution.

On paper chromatograms, the following Rf values were observed: 0.80 with 20% aqueous ammonium chloride, 0.71 with water-saturated *n*-butyl alcohol, 0.37 with *n*-butyl alcohol - acetic acid - water (3:1:1) and 0.84 with water-saturated ethylacetate.

On thin-layer chromatograms with silica gel plates the Rf values of XK-90 are: 0.0 with chloroform, 0.13 with chloroform - acetone (6:1), 0.20 with ethylacetate, 0.42 with ethylacetate - acetone (6:1) and 0.38 benzene - methanol (6:1).

From these data described above, XK-90 is considered to be a new antibiotic. The structure of XK-90 was determined as 1-acetyl-2-(3-formyl-4-hydroxyphenyl)-hydrazine (Fig. 3). Details of the structure work on XK-90 will be reported in a subsequent paper.[2]

Biological Properties

As seen in Table 3, XK-90 shows broad spectral activity against Gram-positive and Gram-negative bacteria. This antibiotic also has a weak activity against *Mycobacterium tuberculosis* H37Rv (MIC, 50~100 μg/ml). The acute toxicity of this antibiotic is not found at 400 mg/kg in mice after intravenous injection. XK-90 does not show any antibacterial activity in mice at 200 mg/kg after intravenous injection.

Acknowledgement

The authors are very grateful to Dr. A. C. SINCLAIR and his associates of Abbott Laboratories, North Chicago, Illinois, U. S. A., for their kind advice and encouragement.

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