THE ANTIBIOTIC XK-41 COMPLEX. I

PRODUCTION, ISOLATION AND CHARACTERIZATION

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(Received for publication December 27, 1973)

An antibiotic XK-41 complex consisting of five components (A_1, A_2, B_1, B_2 and C) produced by a new species named *Micromonospora inositola* MK-41 was isolated. The components, A_1, A_2, B_1 and C, were found to be macrolide-type antibiotics similar to megalomicins, while one component XK-41-B_2 was considered to be a new antibiotic probably belonging to megalomicin-group antibiotic. Erythronolide B was also found to be produced as a byproduct by this *Micromonospora* sp.

In the course of our search for new antibiotics, a thus far undescribed *Micromonospora* sp. MK-41 was isolated from a forest soil found in Hokkaido Jingu, Sapporo, Hokkaido, Japan. The culture later designated as *Micromonospora inositola* nov. sp. was found to produce an antibiotic complex consisting of five components XK-41-A_1, A_2, B_1, B_2 and C which were all active against gram-positive bacteria. This report presents the taxonomy of this novel *Micromonospora* species, fermentative production, isolation and characterization of XK-41 antibiotics and biological activities of one new antibiotic XK-41-B_2. The detection of erythronolide B, an aglycone of these antibiotics, in this *Micromonospora* sp. fermentation is also reported here.

XK41-Producing Organism

Most of the procedures used in the taxonomic study of MK 41 were carried out in accordance with methods adopted by the International Streptomyces Project (ISP)[1]. Additional media recommended by WAKSMAN[2] were also used. The various media were inoculated with washed mycelial suspension grown for 72 hours in a liquid medium (dextrin 1.0%, glucose 1.0%, yeast extract 0.5%, Polypeptone 0.5% and $CaCO_3$ 0.1%).

Morphological Characteristics

Well-developed, branching, non-septate substrate mycelia having a diameter of 0.5~0.8 μ are formed. Spores are generally difficult to find on various media, borne singly on short sporophores (2~3 μ in length), 0.8~1.0 μ in diameter, oval-shaped or round with a smooth surface under an electron microscope.

Appearance on Various Media

This strain is slow in growth as compared with ordinary strains of the genus *Streptomyces*. The following characteristics were observed during cultivation on various media for two weeks. The number in parentheses corresponds to the hue number used in "Color Harmony Manual".[3]

Sucrose-nitrate agar: Poor growth.
Glucose-asparagine agar: Poor growth.

Glycerol-calcium malate agar: Poor growth.
Inorganic salts-starch agar: Poor growth.
Egg albumin agar: Poor to moderate growth; shell pink (5 ca); no soluble pigment.
Oatmeal agar: Poor to moderate growth, flat; light melon yellow (3 ea); no soluble pigment.
Potato: Moderate growth, plicate; apricot (4 ga); no soluble pigment.
Nutrient agar; Good growth, plicate; orange (4 la); no soluble pigment.
Yeast extract-malt extract agar: moderate growth, flat and wet; orange (4 la); no soluble pigment.
BENNETT's agar: Good growth, plicate; bright orange (4 na); no soluble pigment.
EMERSON's agar: Moderate growth, plicate; bright melon yellow (3 ia); no soluble pigment.
Peptone-iron agar: Moderate growth, flat; bright yellow (3 na); no soluble pigment.
Tyrosine agar[4]: Good growth; orange (4 la); no soluble pigment; tyrosine crystals disappeared.

Utilization of Carbon Sources

The test was performed using the basal medium consisted of 0.5% yeast extract and 0.1% reagent grade $CaCO_3$, because the strain grows poorly on the medium according to SHIRLING and GOTTLIEB.

The results are the following: Strong positive utilization; D-galactose, D-glucose, D-mannose, D-raffinose, starch and D-xylose. Positive utilization; D-fructose, L-inositol and sucrose. Negative utilization; D-arabinose, glycerol, D-mannitol, L-rhamnose, salicin, and D-sorbitol.

Physiological Characteristics

Liquefaction of gelatin (27°C for 2 weeks): positive.
Action upon milk (27°C for 3 weeks): slight coagulation, no peptonization.
Decomposition of cellulose (27°C for 4 weeks); slightly positive.
Hydrolysis of starch (27°C for 2 weeks); positive.
Reduction of nitrate (27°C for 2 weeks): negative.
Tyrosinase reaction (27°C for 2 weeks): negative.
Melanine formation (27°C for 2 weeks): negative.
Temperature for growth: 25°~40°C (opt. Temp. 30~35°C).
pH for growth: 5.5~8.5 (opt. pH 7.3).

The microscopic and cultural studies on strain MK 41 indicate that this isolate belongs to the genus *Micromonospora* ØRSKOV (1923).

Differentiation of many species of the genus *Micromonospora* has been discussed by several workers,[5,6,7] but there is no systematic method enough to classify them. Therefore strain MK 41 was compared with all the species of *Micromonospora* with regard to mycelial and sporulation pattern, surface of spore, mycelial pigments, diffusible pigments, utilization of carbohydrate and other physiological properties.

Most *Micromonospora* produce orange mycelial pigments, and dark green, dark brown or black spores. Other mycelial pigments might be a criterion for characterization of species. In this respect SVESHNIKOVA[5] divided species of *Micromonospora* into the following four groups: *fusca-chalcea* group (no diagnostic color), *purpurea* group (red-violet or red), *coerulea* group (bluish green and green) and *brunnea* group (dark brown and brown). Strain MK 41 belongs to *fusca-chalcea* group according to SVESHNIKOVA.

The surface of spores is considered a stable character for description of *Streptomyces*. Neither spiny surface recognizable in *Micromonospora echinospora*,[8] *M. grisea*[9] and *M. inyoensis*[10], nor warty one in *M. brunnea*[11], *M. fulvopurpurea*[11], *M. lilacina*[11] and *M. rubra*[11], was

Table 1. Comparison of strain MK 41 with other species of *Micromonospora* producing spores with smooth-surface and only orange mycelial pigment.

		aurantica	*carbonacea*	*chalcea*	*fusca*	*globosa*	*halophytica*	*megalomicea*	*melanosporea*	*narashino*	*parva*	MK 41
Shape of spore	spherical	+	+	+	+	+	+	+		+	−	+
	oval	−	+	−	−	−	−	−		+	+	+
Sporulation morphology	monopodial		−	+		+	+			+		+
	sympodial		+	−		−	−			−		−
	cluster type	+	−	−	+	+	−			+		−
	open type	+	+	+	−	−	+			−		+
Carbohydrate utilization	D-arabinose		−	−	−	+	−	−	−	−	−	−
	D-galactose		+	+	+	+	+	−	+	+	+	+
	D-lactose		+	+	+	+	+	−	+	+	−	±
	D-fructose	+	+	+	+	+	+	−	+	±	+	+
	L-inositol		−	−	−	−	−	−	−	−	−	+
	D-mannitol	+	−	−	−	−	−	−	−	−	−	−
	D-raffinose	+	−	+	−	+	±	−	−	−	−	+
	L-rhamnose	+	−	−	−	−	−	−	−	−	−	−
	D-xylose	+	+	+	+	+	+	+	+	+	−	+
Action on milk	coagulation			+		+	+	−	+	+		±
	peptonization		+	+	+	+	+	−	+	+	±	−
Liquefaction of gelatin			+	+	+	+	+	−	+	+	−	+
Decomposition of cellulose		+	±	+	−	+	+	−	±		−	+
Reduction of nitrate		±	−	−	+	+	+	−		−	−	−
Formation of melanoid		−	+	+	−		−	−	±	+	−	−

observed on spore of strain MK 41.

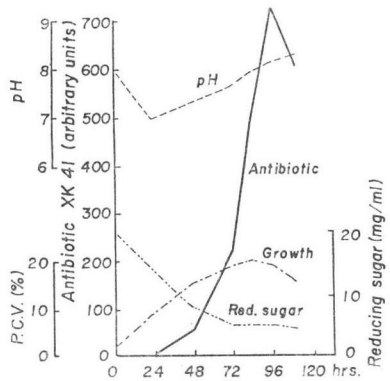
Table 1 shows the comparison of strain MK 41 with the species of *Micromonospora* which produce spores with smooth surface and only orange mycelial pigment. Strain MK 41 appeared similar to *M. chalcea* and *M. halophytica*[12]. LUEDEMANN[6] described that the significant characteristics separating *M. halophytica* from broad *M. chalcea* species were not readily recognized. *Micromonospora chalcea* (FOULERTON 1905) ØRSKOV 1923 was observed to produce fair growth on glucose-asparagine agar, to diffuse brown pigments in tyrosine agar and to peptonize milk, while strain MK 41 showed the poor growth on glucose-asparagine agar, no diffusible brown pigment in tyrosine agar, and no peptonization of milk. Consequently there is no species identical with the strain MK 41. Of known microorganisms of the genus *Micromonospora*, no strain utilizes L-inositol, while strain MK 41 slightly utilized the sugar-alcohol. In view of this characteristic, the present strain is considered to be a new species of *Micromonospora* and was designated as *Micromonospora inositola* nov. sp. MK 41.

Fermentation

A loopful of *M. inositola* MK 41 was inoculated into 30 ml of a seed medium containing

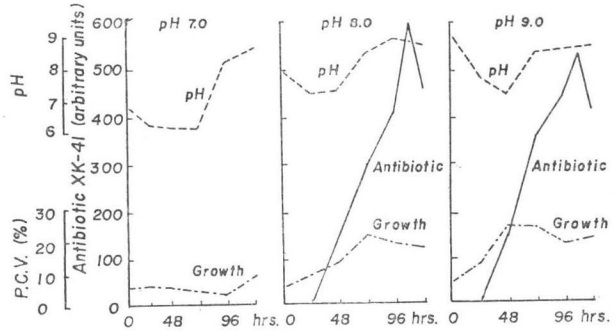
1% dextrin, 1% glucose, 0.5% Polypeptone, 0.5% yeast extract and 0.1% calcium carbonate (pH 7.2 before autoclaving) in a 250-ml Erlenmeyer flask. The seed was grown by shaking at 30°C for 4 days. Thirty ml of the first seed was inoculated into 300 ml of the same medium in a 2-liter indented Erlenmeyer flask. After shaking at 30°C for 2 days, 900 ml of the second seed was inoculated into 15 liters of a fermentation medium in a 30-liter glass jar fermentor. The medium comprised: soluble starch 2%, Pharmamedia 0.65%, Polypeptone 0.5%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.03% and calcium carbonate 0.1% (pH was adjusted to 8.0 with sodium hydroxide after sterilization). Fermentation was run at 30°C for 110 hours. Conditions for the jar fermentor were 350 rpm and 15 liters per minute aeration. The typical chemical changes in this fermentation are shown in Fig. 1. The growth and utilization of carbohydrate were very slow, and the antibiotic came out just before the stationary phase of growth, which was about 70 hours after inoculation.

Fig. 1. Growth, pH change, residual sugar and production of antibiotics in fermentation of strain MK 41
Growth; packed cell volume.
Residual sugar; as D-glucose after acid hydrolysis of broth supernatant.
Antibiotic XK 41; bioassay *vs. B. subtilis* No. 10707.



Nitrogen sources in fermentation medium were examined. They were ammonium sulfate, casamino acids, casein, corn steep liquor, Ebios (dry yeast), meat extract, NZ-amine, peptone, Pharmamedia, Polypeptone, soluble vegetable protein, soy bean meal, yeast extract and so on. Among them Polypeptone and Pharmamedia were good nitrogen sources for production of antibiotic XK-41. On the other hand, among conditions of fermentation such as temperature, agitation and aeration, initial pHs of fermentation medium exhibited most significant effect on the production, as shown in Fig. 2. A neutral or acidic condition resulted in no growth and no production. Initial pH 8.0 seemed to be more favorable for the production than pH 9.0.

Fig. 2. Effect of initial pH on production of antibiotic XK 41 in 5-liter jar fermentation
Growth; packed cell volume.
Antibiotic XK 41; bioassay *vs. B. subtilis* No. 10707.



Isolation and Characterization

The antibiotic complex was isolated by adjusting the filtrate of fermentation broth to pH 9.5 with 2 N NaOH, adding 7.5 liters of chloroform and extracting to the chloroform layer. Such extraction was repeated twice. The 15-liter chloroform solution was concentrated to 5 liters *in vacuo*. By adding 5 liters of distilled water, adjusted to pH 4.0 with 2 N HCl, the antibiotics were extracted to the water layer. The water layer was separated, adjusted to pH 9.5 with 2 N NaOH and again subjected to extraction with 5 liters of chloroform. By concentrating the chloroform layer *in vacuo*, partially purified preparations of XK-41 complex were obtained.

The partially purified antibiotic complex was dissolved into 5 ml of ethyl acetate saturated with 1/15 M phosphate buffer solution having a pH adjusted to 6.0. The solution was poured into a tube of a countercurrent distributor. Countercurrent distribution was carried out between 1/15 M phosphate buffer solution (the stationary phase, the lower layer) and ethyl acetate (the mobile phase, the upper layer) in about 500 transfers. The result was as follows:

Test tubes	Nos. 5~30	Nos. 40~115	Nos. 119~142	Nos. 149~249	Nos. 258~420
Fraction	XK-41-C	XK-41-B_1	XK-41-B_2	XK-41-A_2	XK-41-A_1

Each of the fractions was adjusted to pH 9.5 with 2 N NaOH. After extractions with chloroform, the chloroform layer was concentrated *in vacuo*.

The concentrated chloroform solution was kept at 5°C for 15 hours to precipitate the antibiotic. After separating each component by filtration, the components were recrystallized from ethyl ether solution.

As a result, 103 mg of XK-41-A_1, 83 mg of XK-41-A_2, 129 mg of XK-41-B_1, 29 mg of

Table 2. Comparative paper chromatography of XK 41 complex and other *Micromonospora*-produced antibiotics

	Rf value				
	I	II	III	IV	V
Gentamicin	0.98	0.00	0.05	0.00	0.01
Sisomicin	0.97	0.00	0.04	0.00	0.01
Antibiotic 460	0.95	0.00	0.00	0.00	0.00
Neomycin	0.96	0.00	0.00	0.00	0.00
Halomicin[1]	0.45	0.85	0.75	0.30	0.56
Everninomicin	0.78	0.86	0.83	0.40	0.87
Micromonosporin[2]	0.18	0.75	0.78	0.25	0.70
Megalomicin	0.60	0.85	0.75	0.00	0.92
Rosamicin	0.65	0.85	0.78	0.00,0.90	0.90
Juvenimicin[3]	0.92	0.92	0.85	0.00,0.93	0.90
XK 41 complex	0.60	0.84	0.75	0.00	0.91

System: I 20% Ammonium chloride soln. II *n*-Butanol saturated with water
III *n*-Butanol - acetic acid - water (3:1:1) IV Ethyl acetate saturated with water
V *n*-Butanol saturated with water containing 2% *p*-toluene sulfonic acid and 2% piperidine
Antibiotics were isolated from
1) *Micromonospora halophytica* var. *nigra* NRRL 3097
2) *Micromonospora* sp. ATCC 10026
3) *M. chalcea* var. *izumensis* ATCC 21561
Filter paper: Toyo #51 (20 mm×400 mm) Bioautography *vs. Bacillus subtilis* No. 10707

Table 3. *In vitro* activities of XK 41 complex against macrolide resistant staphylococci

Test organisms	MIC (mcg/ml)		
	XK 41 complex	Mega-lomicin complex	Erythro-mycin A
S. aureus 209P	0.52	0.52	< 0.02
S. aureus 45	1.0	1.0	< 0.02
S. aureus KY8945*	>41.7	>41.7	>41.7
S. aureus KY8946*	>41.7	>41.7	>41.7
S. aureus KY8947*	>41.7	>41.7	5.21
S. aureus KY8949*	>41.7	>41.7	5.21

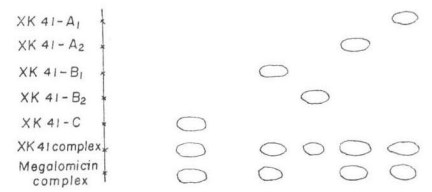
* Clinically isolated macrolide resistant strains. Agar dilution assay at pH 8.0.

Table 4. Comparative paper chromatography of XK 41 and other macrolide antibiotics

Antibiotics	Rf values
Carbomycin	0.00, 0.95
Leucomycin	0.97
Niddamycin	0.95
Tylosin	0.95
Oleandomycin	0.30~0.60
Erythromycin	0.20~0.40
Spiramycin	0.95
Rosamicin	0.00, 0.90
Juvenimicin	0.00, 0.93
Megalomicin	0.00
XK 41 complex	0.00

Filter paper: Toyo #51 (20 mm×400 mm)
Solvent: Ethylacetate saturated with water.
Developed ascendingly for 3 hours at 28°C.
Bioautography *vs. B. subtilis* No. 10707.

Fig. 3. Comparative thin-layer chromatography of XK 41 components and megalomicin
Alumina sheet; Alumina (Type E, Merck & Co., U.S.A.)
Solvent system; Ethylacetate—methanol (9:1)
Developed for 3 hours at room temperature
Bioautography *vs. B. subtilis* No. 10707



XK-41-B_2 and 10 mg of XK-41-C were obtained.

During the above isolation, erythronolide B was incidentally separated from the fermentation beer by the extraction with chloroform at alkaline pH together with the antibiotic XK-41 complex. After removing the XK-41 complex by the extraction with acidic water (pH 4.0) from the chloroform layer, the chloroform solution was concentrated and cooled at 5°C for 15 hours. By filtrating the precipitate and drying *in vacuo* 500 mg of crude erythronolide B was isolated. The crude preparation was recrystallized in ethyl acetate and 300 mg of purified erythronolide B was obtained. The melting point of this substance was 227~228°C. Other chemical data such as elemental analysis, infrared spectrum and nuclear magnetic resonance indicated its identity with erythronolide B. Erythronolide B, an aglycone of erythromycin B[13,14], was reported to be produced by *Streptomyces erythreus* as a precusor in erythromycins biosynthesis[15]. From the structural studies of megalomicin, it was revealed an aglycone of megalomicin A was 12-hydroxy erythronolide B just like in the case of erythromycins A and C[16]. It is therefore expected that the XK-41-producing organism which was later found to produce megalomicin antibiotics produces its aglycone as a byproduct in the fermentation broth.

Thus, the accumulation of erythronolide B by *Micromonospora inositola* is the first instance for its occurrence in *Micromonospora* sp. It is hence suggested here that erythronolide B may also be a precursor for XK-41s biosynthesis as well as for erythromycins.[15]

Earlier studies have revealed various groups of antibiotics produced by *Micromonospora*.[12,17~23] The partially purified XK-41 complex was shown to belong to macrolide group antibiotics on paper chromatogram when compared with other *Micromonospora*-produced antibiotics as seen in Table 2. Furthermore, the antibiotic XK-41 complex showed a cross

resistance with macrolide group antibiotics against *Staphylococcus aureus* which are resistant to macrolide antibiotics as shown in Table 3.

By paper chromatography on Toyo #51 filter paper developed by water-saturated ethyl acetate, XK-41 complex can be readily differentiated from other macrolide antibiotics produced by *Micromonospora* sp. and *Streptomyces* sp. except megalomicin complex, as shown in Table 4.

On thin-layer chromatography coated by Alumina (Type E, Merck & Co., U. S. A.) each component of XK-41 and megalomicin complex showed inhibition zones as seen in Fig. 3.

Four components of XK-41 A_1, A_2, B_1 and C which showed the same Rf values as those of megalomicin components were definitely decided to be identical with the megalomicin A and its acyl derivatives in the following paper.[24]

However, from these results described above, XK-41-B_2 was clearly differentiated from the four known megalomicin components, indicating that XK-41-B_2 is a new antibiotic probably belonging to megalomicin group. In fact, further chemical works[24] clearly indicated the

Table 5. Antimicrobial spectra of XK-41-B_2, megalomicin complex and erythromycin A

Test organism	MIC (mcg/ml) (measured at pH 8.0)		
	XK-41-B_2	Megalomicin complex	Erythromycin A
Streptococcus faecalis ATCC 10541	0.83	0.13	< 0.02
Staphylococcus aureus ATCC 6538P	0.83	0.26	< 0.02
S. aureus ATCC 209P	1.65	0.52	< 0.02
S. aureus 45	3.25	1.0	< 0.02
S. aureus KY 8946*	> 41.7	>8.3	>41.7
S. aureus KY 8949*	> 41.7	>8.3	>41.7
S. aureus KY 8947*	105	>8.3	5.21
S. aureus KY 8945*	50.0	>8.3	5.21
Bacillus subtilis No. 10707	0.65	0.13	0.0082
Mycobacterium avium KB 44	0.16	0.14	1.05
M. phlei IFO 3158	0.08	0.009	0.009
M. koda KB 47	0.08	0.14	0.27
M. smegmatis ATCC 10143	1.31	0.27	> 8.3
M. smegmatis ATCC 607	0.16	0.03	2.09
M. smegmatis KB 46	1.31	0.53	> 8.3
Klebsiella pneumoniae ATCC 10031	26.0	>8.3	1.31
Serratia marcescens ATCC 4003	>417	>8.3	41.7
Neisseria catarrhalis ATCC 7900	< 0.21	0.26	0.33
Aerobacter aerogenes ATCC 13048	417	>8.3	>41.7
Escherichia coli ATCC 26	50.0	>8.3	10.5
E. coli ATCC 4352	210	>8.3	2.6
E. coli Juhl KY 4286	50.0	>8.3	10.5
E. coli ATCC 11775	50.0	>8.3	21.0
Paracolon sp. Abbott P-1	> 41.7	>8.3	21.0
Shigella sonnei ATCC 9290	26.0	4.2	10.5
Salmonella typhosa ATCC 9992	105	>8.3	21.0
Candida albicans ATCC 10231	>417	8.3	>41.7
Aspergillus niger	>417	>8.3	>41.7

* Resistant strain to macrolide antibiotics.

Table 6. Mice protection tests of XK-41-B_2 against *Staphylococcus aureus*

Run	Range of LD_{50}* challenge	Route	CD_{50} (mg/kg)	
			XK-41-B_2	Erythromycin A
I	10~100	s.c.	6~12	3
		p.o.	25~50	25~50
II	1~10	s.c.	32	3.1~6.2

* One LD_{50}=challenge dose required to kill 50% of the mice.

XK-41-B_2 is a new component of megalomicin antibiotics. Since the XK-41-B_2 was thus found to be a new antibiotic probably belonging to megalomicin group, this compound has been studied more extensively from the view point of *in vitro* and *in vivo* biological activities.

Biological Properties of XK-41-B_2

Table 5 shows the antimicrobial spectra of XK-41-B_2 in comparison with megalomicin complex and erythromycin A. Results on mice protection tests of XK-41-B_2 against *Staphylococcus aureus* are tabulated in Table 6. As clear from these data, XK-41-B_2 was found to be active against gram-positive organisms such as *Streptococcus faecalis*, *Staphylococcus aureus*, *Bacillus subtilis* and *Mycobacterium* sp., and gram-negative coccus *Neisseria catarrhalis*, but inactive against *S. aureus* resistant to macrolide antibiotics and gram-negative organisms such as *Aerobacter aerogenes*, *Escherichia coli*, and others. The *in vitro* activities of XK-41-B_2 were also found to be inferior to megalomicin complex and erythromycin A, while its *in vivo* activities, though still a little inferior to erythromycin A, approached those of these two antibiotics, as shown in Tables 5 and 6. LD_{50} of XK-41-B_2 to mice was about 150 mg/kg (i.v.).

Acknowledgment

The authors are very grateful for a gift of megalomicin complex and other reference antibiotics to Dr. G.H. WAGMAN, Schering Co. (U.S.A.). They are also thankful to Dr. A.C. SINCLAIR and his associates of Abbott Labs. (U.S.A.) for their kind instruction.

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