CLOSTOMICINS, NEW ANTIBIOTICS PRODUCED BY *MICROMONOSPORA ECHINOSPORA* SUBSP. *ARMENIACA* SUBSP. NOV.

II. TAXONOMIC STUDY OF THE PRODUCING MICROORGANISM

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Taxonomic properties of actinomycete strain KMR-593, a soil isolate, which produces new anti-anaerobe antibiotics, clostomicins, were investigated. The strain was identified as a new subspecies of the genus *Micromonospora* and designated *Micromonospora echinospora* subsp. *armeniaca* subsp. nov.

In the course of our screening program for new anti-anaerobe antibiotics of actinomycete origin, we found the new antibiotics, clostomicins, which are active against Gram-positive bacteria, especially *Clostridium* sp.

The producing microorganism, strain KMR-593, was isolated from a soil sample collected at a rice field in Mishima-cho, Niigata prefecture, Japan.

Strain KMR-593 exhibited taxonomic properties of the genus *Micromonospora* Orskov 1923. The present paper deals with the taxonomy of the producing microorganism. The isolation and characterization of clostomicin is described in the accompanying paper[1].

Materials and Methods

Bacterial Strain

Strain KMR-593 was isolated by plating a soil suspension on an organic agar medium and incubating the medium at 27°C for 2 weeks. The culture was maintained on inorganic salts - starch agar.

Microscopy

The morphology of strain KMR-593 was observed with a scanning electron microscope (model S-430, Hitachi).

Cultural and Physiological Characteristics

Most of the procedures used in the study were carried out in accordance with the methods adopted by the International Streptomyces Project (ISP)[2]. Media recommended by WAKSMAN[3] and media mentioned in the catalogue of the American Type Culture Collection (ATCC) were also used. The various media were inoculated with a washed mycelial suspension grown for 5 days at 27°C in a liquid medium (yeast extract 1.0% and glucose 1.0%). The cultures were observed after incubation at 27°C for 3 weeks. Color names and hue numbers indicated are those of the Color Harmony Manual (4th Ed.) published by Container Corporation of America. Utilization of carbon sources was tested by growth on LUEDEMAN's medium (yeast extract 0.5% and $CaCO_3$ 0.1%)[4] containing 1.0% each carbon source.

Chemical Analyses of Whole Cell and Cell Wall

Spores and mycelia of strain KMR-593 were transferred to a 500-ml Sakaguchi flask containing

Fig. 1. Scanning electron micrograph of spores of strain KMR-593 grown on glycerol - asparagine agar for 21 days at 27°C.

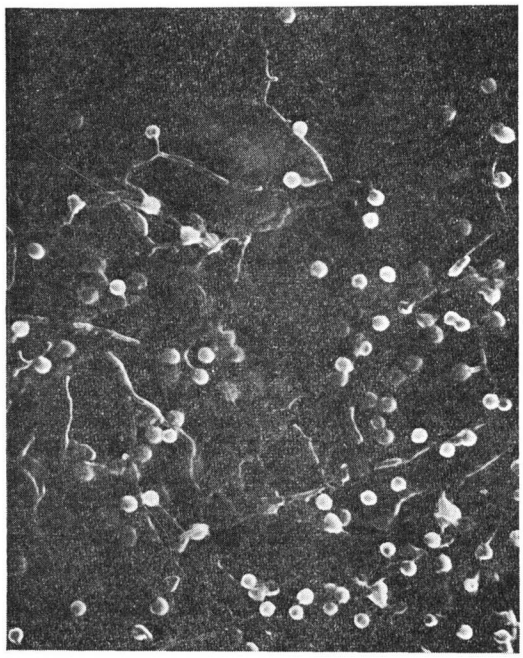
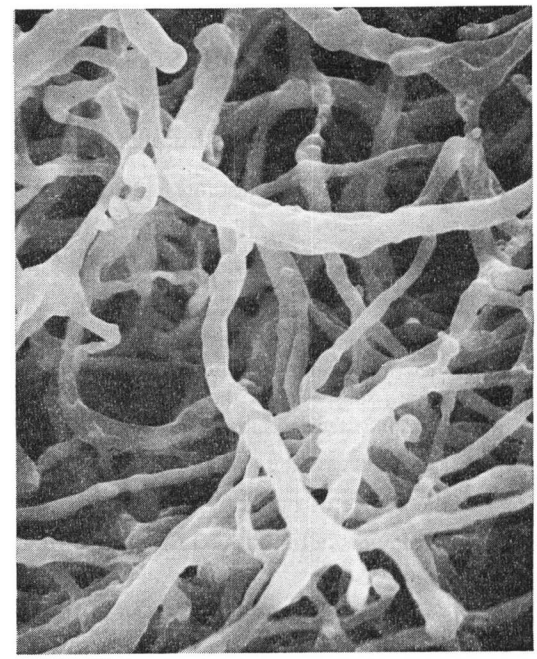


Fig. 2. Scanning electron micrograph of aerial mycelia of strain KMR-593 grown on sucrose - nitrate agar for 21 days at 27°C.



100 ml of a medium consisting of yeast extract 1.0% and glucose 1.0%. The flask was incubated on a reciprocal shaker at 27°C for 5 days. The mycelia were harvested by centrifugation and thoroughly washed with distilled water. The washed mycelia were used for whole cell preparation. The cell walls were prepared according to the method of YAMAGUCHI[5] with some modification[6]. Amino acids and sugars in cell wall and whole cell preparations were analyzed as described by BECKER *et al.*[7]. Analyses of the cellular phospholipids and glycolic acid in the cell wall were carried out by the methods of LECHEVALIER *et al.*[8] and of UCHIDA and AIDA[9], respectively.

Results

Morphology

Vegetative mycelia were well developed and branched on inorganic salts - starch agar and sucrose - nitrate agar, but poorly developed on other media. Poor aerial mycelia were produced on sucrose - nitrate agar.

Single spores were produced on short sporophores of the vegetative mycelia. Spores were not observed on aerial mycelia (Figs. 1 and 2). Spores were 0.8~1.0 μm in diameter and spherical in shape. The sporulation morphology was open-web type.

Cultural and Physiological Characteristics

The cultural characteristics of strain KMR-593 are given in Table 1. Bright orange vegetative mycelia were formed on most media. This color remained even after 4 weeks and did not change to dark brown or brownish black. Although mycelial pigment of known *Micromonospora* strains

Table 1. Cultural characteristics of strain KMR-593.

Yeast extract - malt extract agar*	G:	Poor, bright orange (5na)
	R:	Bright orange (5na)
	AM:	None
	SP:	None
Oatmeal agar*	No growth	
Inorganic salts - starch agar*	G:	Moderate, bright orange (5na)
	R:	Bright orange (5na)
	AM:	None
	SP:	None
Glycerol - asparagine agar*	G:	Poor, apricot (4ia)
	R:	Apricot (4ia)
	AM:	None
	SP:	None
Glucose - asparagine agar	No growth	
Peptone - yeast extract - iron agar*	G:	Poor, bright orange (5na)
	R:	Bright orange (5na)
	AM:	None
	SP:	None
Tyrosine agar*	G:	Poor, apricot (4ia)
	R:	Apricot (4ia)
	AM:	None
	SP:	Light apricot (4ea)
Sucrose - nitrate agar*	G:	Good, bright orange (5na)
	R:	Bright orange (5na)
	AM:	Moderate, powdery, light apricot (4ea)
	SP:	None
Glucose - nitrate agar**	G:	Very poor, bright orange (5na)
	R:	Bright orange (5na)
	AM:	Poor, powdery, light apricot (4ea)
	SP:	None
Glycerol - calcium malate agar**	G:	Poor, light apricot (4ea)
	R:	Light apricot (4ea)
	AM:	None
	SP:	None
Glucose - peptone agar**	G:	Very poor, bright orange (5na)
	R:	Bright orange (5na)
	AM:	None
	SP:	None
Nutrient agar**	G:	Moderate, bright orange (5na)
	R:	Bright orange (5na)
	AM:	Poor, powdery, light apricot (4ea)
	SP:	None
Sporulation agar ATCC No. 5	G:	Poor, bright orange (5na)
	R:	Bright orange (5na)
	AM:	None
	SP:	None
NZ Amine with soluble starch and glucose agar ATCC No. 172	G:	Good, bright orange (5na)
	R:	Bright orange (5na)
	AM:	None
	SP:	None
Emerson agar ATCC No. 199	G:	Poor, bright orange (5na)
	R:	Bright orange (5na)
	AM:	Poor, powdery, light apricot (4ea)
	SP:	None

* Medium recommended by ISP.

** Medium recommended by S. A. WAKSMAN.

Abbreviations: G; Growth of vegetative mycelium, R; reverse, AM; aerial mycelium, SP; soluble pigment.

Table 2. Physiological properties of strain KMR-593.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Nitrate reduction	+
Hydrolysis of starch	+
Liquefaction of gelatin (21°C)	−
Peptonization of milk	+
Coagulation of milk	−
Temperature range for growth	20~35°C
Growth on potato	−
Growth on potato+$CaCO_3$	+
NaCl tolerance	2.0%

+; Positive, −; negative.

Table 3. Utilization of carbon sources by strain KMR-593.

L-Arabinose	+
D-Cellobiose	+
β-Cellobiose	++
Dextrin	+
D-Fructose	++
D-Galactose	+
D-Glucose	++
iso-Inositol	−
Mannitol	+
D-Mannose	++
Melibiose	−
D-Raffinose	−
L-Rhamnose	++
D-Ribose	−
Salicin	−
L-Sorbose	−
D-Trehalose	++
D-Xylose	++
Cellulose	−

++; Utilized, +; weakly utilized, −; not utilized.

change from orange to dark brown or brownish black during incubation term. Light apricot aerial mycelia were formed on sucrose - nitrate agar and nutrient agar.

Physiological characteristics and utilization of carbon sources of strain KMR-593 are given in Tables 2 and 3, respectively. Melanoid pigment was not produced. The culture grew at 20~35°C. Strain KMR-593 did not grow on natural potato plug, but grew on $CaCO_3$ treated potato plug (Table 4).

Chemical Analyses

The cell wall contained 3-hydroxy-diaminopimelic acid, glycine, arabinose, galactose and xylose.

Table 4. Comparison of *Micromonospora* sp. KMR-593 with *M. aurantiaca* JCM 3232.

	Micromonospora sp. KMR-593	*M. aurantiaca* JCM 3232
Oatmeal agar	G: No growth	Moderate, orange (4la)
	AM: None	None
Sucrose - nitrate agar	G: Good, bright orange (5na)	Poor, apricot (4ga)
	AM: Moderate, light apricot (4ea)	None
Potato plug	G: No growth	Good
Potato plug+$CaCO_3$	G: Good	Good
Utilization of carbon sources		
β-Cellobiose	++	+
D-Fructose	++	+
D-Glucose	++	+
iso-Inositol	−	++
D-Mannose	++	+
Melibiose	−	++
D-Raffinose	−	++
L-Rhamnose	++	+
Mannitol	+	+
D-Xylose	++	+
Cellulose	−	+

++; Utilized, +; weakly utilized, −; not utilized.
Abbreviations: G; Growth of vegetative mycelium, AM; aerial mycelium.

The glycolate test was negative. Phosphatidylethanolamine was detected in the phospholipid.

Identification and Classification

The morphology and cell wall analyses described above indicate that strain KMR-593 belongs to the genus *Micromonospora* Orskov 1923.

According to LUEDEMANN's classification described in BERGEY's Manual[10] for the genus *Micromonospora* which places emphasis on carbon utilization, strain KMR-593 resembles *M. echinospora i.e.* α-melibiose (−) and L-rhamnose (+). However, strain KMR-593 was not identical with three subspecies of *M. echinospora* in mycelial pigment, as follows. Strain KMR-593 produces bright orange color pigment. *M. echinospora* subsp. *echinospora*[4] and *M. echinospora* subsp. *ferruginea*[4] produce dark purple, purplish black or iron rust pigments, and *M. echinospora* subsp. *pallida*[4] produces dark brown to black pigments. Therefore, it is concluded that strain KMR-593 is a new subspecies of *M. echinospora*. It is designated as *Micromonospora echinospora* subsp. *armeniaca* subsp. nov. (ar men i a'ca, L. adj. *armeniaca* apricot). Type strain is strain KMR-593 (=FERM P-7934).

Discussion

Today, two methods, LUEDEMANN's classification[11,12] and SVESHNIKOVA's one[13], are generally used for species classification within the genus *Micromonospora*. The former places emphasis on carbohydrate utilization. However, this method is not always satisfactory criteria in that growth responses of carbon sources change by the basal media employed[14~17], and in that other taxonomic keys for species classification are not fully systematized yet. Since vegetative mycelia of *Micromonospora* produce many kinds of pigments, the latter based on mycelial pigments is convenient. Nevertheless, the SVESHNIKOVA's classification is not necessarily reliable, because reference strains include many unapproved species, and the standard colors are not defind. Taking the above situation into consideration, we think it better to adopt LUEDEMANN's method which gives more reproducible results and which is more widely used by describing in BERGEY's manual (8th Ed.). Therefore, strain KMR-593 was classified according to LUEDEMANN's method, which suggested that this strain is a new subspecies of *Micromonospora echinospora*.

On the other hand, the orange color of mycelia of strain KMR-593 did not change at all even at a later stage of incubation, when sporulation occurred. This property of strain KMR-593 is characteristic, because all known 47 species of *Micromonospora* were reported to change in their mycelial pigments to black, brown or green color. To our knowledge, strain KMR-593 is the first example of a *Micromonospora* strain whose mycelial pigment does not change during an incubation period. Therefore, according to SVESHNIKOVA's classification, strain KMR-593 can be regarded as a new species of the genus *Micromonospora*.

We wish that new criteria are developed and these are systematically used together with carbon utilization and mycelial pigment for species classification of the genus *Micromonospora* in the near future. From such taxonomic advancement, strain KMR-593 may be reclassified.

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