MILBEMYCINS, A NEW FAMILY OF MACROLIDE ANTIBIOTICS: PRODUCING ORGANISM AND ITS MUTANTS

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In the course of screening for insecticidal substances, strain B-41-146 was found to produce antibiotics having an acaricidal activity. The strain was found to belong to genus Streptomyces and the antibiotics produced by the strain were new antibiotics α_1, α_2, α_3, α_4, α_5, α_6, α_7, α_8, α_9, α_{10}, β_1, β_2, β_3, and small amounts of milbemycins D, E, F, G and H as shown in previous report[1].

In order to obtain strains producing new members of the milbemycin family or high-yield strains, various mutants were isolated from strain B-41-146. Some of these did not produce any milbemycins at all, but others produced milbemycins D, E, F, G and H mainly or new milbemycins J and K. The isolation, physicochemical properties and structure of these antibiotics will be reported in subsequent papers.

In this paper, the taxonomical characteristics and milbemycin productivity of strain B-41-146 and its mutants are described.

Taxonomic Studies of Strain B-41-146

Strain B-41-146 was isolated from soil collected at Kuttian-cho in the Abuta District of Hokkaido, Japan. A water suspension of the soil was plated on an agar medium and the plate was incubated at 28°C for 14 days. The organism was inoculated into ISP medium 1 in a Sakaguchi flask and grown for 3 days at 28°C on a reciprocal shaker. The culture was centrifuged, washed twice with sterile water and then used as an inoculum for various studies. Washed cells were inoculated into International Streptomyces Project (ISP) media described by SHIRLING and GOTTLIEB[2] and those recommended by WAKSMAN[3].

Morphological Characteristics: The morphological characteristics of the spore chains and hyphae of strain B-41-146 grown on various agar media at 28°C for 7 to 21 days were studied under a light microscope. The spores prepared with a critical point dryer (HCP-1, Hitachi Co., Ltd.) were observed under a MSM-6 scanning electron microscope (Akashi Seisakusho Co., Ltd.). Vegetative hyphae of the strain fully develop with branching. They do not fragment into coccoid or bacillary elements. Sporophores arrange singly, in pairs, or occasionally in tufts along straight or flexous main aerial hyphae, terminating in coil (Spirals) of two or more volution (Plate 1), but with no evidence of true verticillate branching. Spores are covered with a capsule like membrane with a fairly irregular or rugose, possibly warty surface (Plate 2). Special organs, such as sporangia, zoo-

Plate 1. Photomicrograph of strain B-41-146 (on yeast extract-malt extract agar, 28°C, 14 days ×300).

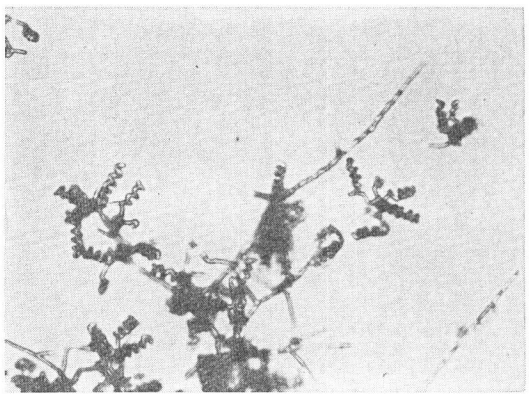

Plate 2. Scanning electron micrograph of spores of strain B-41-146 (on yeast extract-malt extract agar, 28°C, 14 days ×5,000).

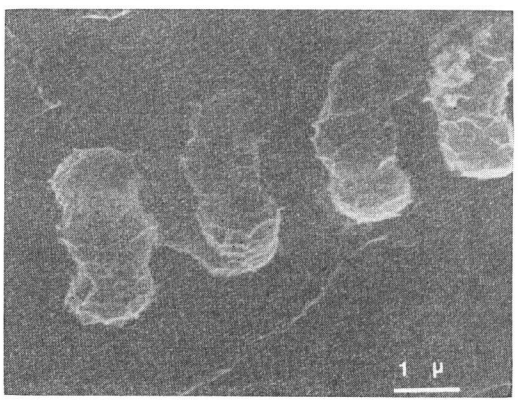

Table 1. Cultural characteristics of strain B-41-146.

Medium	Growth	Aerial mycelium	Soluble pigment
Yeast extract - malt extract agar (ISP-2)	Abundant, dark yellowish brown	Abundant, brownish gray with yellowish patches and with black moistened patches	Yellow
Oatmeal agar (ISP-3)	Abundant, colorless to dark olive gray	Abundant, brownish gray with yellowish patches and with black moistened patches	Yellow
Inorganic salts - starch agar (ISP-4)	Abundant, colorless to yellowish brown	Abundant, brownish gray with yellowish patches and with black moistened patches	Light olive gray
Glycerol - asparagine agar (ISP-5)	Good, colorless to yellow	Thin, white to light brownish gray with yellowish patches	Pale yellowish brown
Tyrosine agar (ISP-7)	Good, dark yellowish brown	Good, light brownish gray with yellowish patches	Pale yellow
Nutrient agar (Difco)	Poor, colorless to pale yellow	Scant, white	None
Sucrose - nitrate agar	Good, colorless	Thin, grayish white	Pale yellow
Glucose - asparagine agar	Good, colorless to pale yellowish	Good, grayish white	Pale yellowish brown

spores, ball-like bodies or sclerotia were not observed on the media employed.

Cultural Characteristics: Reading of the results of the growth on various agar media were made at 3 to 21 days. The mass colors of the mycelium were described in common terminology, but exact color were determined by comparing the mycelial color with color-tips from the Japan Color Standard[4]. The cultural characteristics of the strain on various media are presented in Table 1. The aerial mycelium is abundant and varies from white to brownish gray in mass color. Within 3 to 5 days, it forms a golden yellow globose accumulation on liquid exudate in various media, and subsequent yellowish patches on the aerial mycelium. Sometimes, moist black, liquefied (hygroscopic) areas are also found in the aerial mycelium of older cultures. These are especially common in ISP medium 2, ISP medium 3 and ISP medium 4. The color of the vegetative mycelium is pale yellow to dark yellowish brown. Soluble pigment is yellow to light olive gray.

Physiological Characteristics: The media used for each test were as follow; ISP media 1, 6 and 7 for melanoid pigment formation, nitrate broth (Difco) for nitrate reduction, ISP medium 4 for starch hydrolysis, gelatin stab for gelatin liquefaction, and dehydrated skim milk for coagulation and peptonization. The cultures on all of the media tested were incubated at 28°C for 14 days except for those on milk (37°C, 10 days) and gelatin (25°C, 21 days) media. Physiological characteristics of the strain are shown in Table 2. As shown in the Table, nitrate reduction, starch hydrolysis, milk peptonization and coagulation and gelatin liquefaction tests are positive, whereas melanoid pigment is negative. Carbohydrate utilization was examined by using the Pridham-Gottlieb basal medium (ISP medium 9). The results are shown in Table 3. The effect of temperature on growth was investigated by streaking the inoculum over the surface of ISP medium 2 with a temperature gradient incubator TN-3 (Toyo Kagaku Sangyo Co., Ltd.). Optimum temperature for growth on ISP medium 2 appeared to be about 28°C from the following results.

Table 2. Physiological characteristics of strain B-41-146.

Test	Result
Starch hydrolysis	Positive
Melanoid pigment	Negative
Gelatin liquefaction	Positive
Milk coagulation	Weakly positive
Milk peptonization	Weakly positive
Nitrate reduction	Positive

Table 3. Carbohydrate utilization pattern of strain B-41-146.

Carbohydrate	Response
D-Glucose	++
L-Arabinose	+
Sucrose	++
D-Xylose	+
Inositol	+
D-Mannitol	++
D-Fructose	++
D-Rhamnose	++
Raffinose	+

++: Strongly positive utilization.
+: Positive utilization.

Temperature (°C)	10	15	18	22	25
Growth*	−	±	+	+	++
Temperature (°C)	28	30	34	37	42
Growth*	+++	++	+	+	−

* − no growth, ± faint growth, + fair growth, ++ good growth, +++ excellent growth.

Sodium chloride tolerance was examined by streaking the inoculum onto the same medium as used for the temperature study, except containing sodium chloride at 1.0, 2.0, 3.0, 5.0, 7.0, 10.0 and 15.0% and incubating at 28°C for 14 days. The growth was observed at 5.0% or below, but not above 7.0% of sodium chloride concentration.

Analyses of Cell Wall and Whole Cell: Cell wall and whole cell were analyzed by the procedure of BECKER *et al.*[5] and of LECHEVALIER[6], respectively. The cell wall contained LL-diaminopimelic acid (LL-DAP) and glycine as major constituents. The whole cell sugar pattern was not characteristic. The strain can be considered to be a Cell Wall Type I.

Summarizing the above, strain B-41-146 belongs to the genus Streptomyces Waksman and Henrici 1943. Among known species of Streptomyces, *Streptomyces hygroscopicus* (Jensen 1931) Waksman and Henrici 1948 most resembles strain B-41-146 in morphological, cultural and physiological characteristics. However, strain B-41-146 differs from *S. hygroscopicus* as follow: (1) strain B-41-146 forms golden yellow drops of exudate on aerial mycelium, whereas *S. hygroscopicus* usually does not, (2) the growth color of strain B-41-146 is dark yellowish brown, whereas that of *S. hygroscopicus* is colorless to pale yellowish brown, (3) strain B-41-146 utilizes sucrose and raffinose but *S. hygroscopicus* probably does not, (4) strain B-41-146 produces antibiotics of the milbemycin family, whereas no other microorganism belonging to streptomycetes produced milbemycins.

Therefore, the strain B-41-146 represents a new subspecies of *S. hygroscopicus* whose name is proposed as *Streptomyces hygroscopicus* subsp. *aureolacrimosus*. Etymology: derived from *L.* aureo=golden yellow + *L.* lacrimosus=lachrymal, referring to golden yellow tear-bearing. Progeny of the type strain SANK 60576 of *S. hygroscopicus* subsp. *aureolacrimosus* subsp. nov. have been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Ibaragi, Japan, with an accession number of FERM P-1438.

Taxonomical Features and Milbemycin Productivity of the Mutants

Mutagenesis: Strain B-41-146 was cultivated for 12 days on ISP medium 2. Spores on the slant were suspended in saline and mutagenized for 2 minutes with ultraviolet light using a Toshiba ultraviolet lamp, located at a distance of 60 cm from the surface of the spore suspension (98~99.9% lethality). Spores suspended in 1/15 M phosphate buffer (pH 7.0) were also treated with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) at 5,000 μg/ml at 28°C for 1 hour (90~99% lethality). The mutagenized spores were spread on ISP medium 2 plates and incubated at 28°C for 12 days. Each colony on the plates was inoculated into 100-ml Erlenmeyer flasks containing 20 ml of BM-1 medium. After 10 days incubation at 28°C on a rotary shaker, their milbemycin productivity was examined by thin-layer chromatography assay[1].

In order to obtain strains producing new members of the milbemycin family or high-yield strains, many mutants of strain B-41-146 were isolated. Among these, 3 mutants designated as Nt-15, Au-3 and Rf-107 showed different features from the original strain in both cultural characteristics and milbemycin productivity as shown in Table 4. Strain Nt-15 showed a very strong hygroscopic character in all media employed and produced a dark brown soluble pigment in tyrosine agar, but did not produce any milbemycins. Strain Au-3 had poor aerial mycelium, sometimes showed pock or plaque in the aerial mass and the growth color was a red-

Table 4. Taxonomical features and milbemycin productivity of mutant strains.

	B-41-146	Nt-15	Au-3	Rf-107
Growth[a)]	Abundant, dark yellowish brown	Good, dark brown	Abundant, reddish brown	Abundant, olive gray
Aerial mycelium[a)]	Abundant, brownish gray with yellowish patches and with black moistened patches	Good, brownish gray, becomes moist and exhibits dark brownish gray	Poor, white to brownish gray with pock or plaque patches	Good, brownish gray with yellowish patches
Soluble pigment[a)]	Yellow	None	Pale reddish brown	Olive gray
Melanoid pigment[b)]	—	+	+	+
Milbemycins[c)]	α_1, α_2, α_3, α_4, α_5, α_6, α_7, α_8, α_9, α_{10}, β_1, β_2, β_3, D, E, F, G and H	None	α_1, α_2, α_3, α_4, α_9, α_{10}, β_1, β_2, D, E, F, G, and H	J and K

a) Yeast extract - malt extract agar
b) Tyrosine agar
c) BM-1 medium

dish tinge when compared with the original strain B-41-146. A dark brown soluble pigment was also produced in tyrosine agar. The strain produced milbemycins D, E, F, G and H mainly together with α_1, α_2, α_3, α_4, α_9, α_{10}, β_1 and β_2 which were produced by the parent strain. Strain Rf-107 had brownish gray mycelium and sometimes showed white or yellowish patches in the aerial mycelium. The growth color was somewhat greenish tinge comparing with original strain. A dark brown soluble pigment was produced in tyrosine agar. This strain produced new milbemycins J and K.

References

1) TAKIGUCHI, Y.; H. MISHIMA, M. OKUDA, M. TERAO, A. AOKI & R. FUKUDA: Milbemycins, a new family of macrolide antibiotics: Fermentation, isolation and physico-chemical properties. J. Antibiotics 33: 1120~1127, 1980
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization Streptomyces species. Intern. J. Syst. Bact. 16: 313~340, 1966
3) WAKSMAN, S. A.: "The Actinomycetes. Vol. 2". The Williams & Wilkins Co., 1961
4) "Guide to Color Standard". Nippon Shikisai Kenkyusho, Tokyo, 1954
5) BECKER, B.; M. P. LECHEVALIER & H. LECHEVALIER: Chemical composition of cell wall preparation from strains of various form genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
6) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. & Clin. Med. 71: 934~944, 1968
7) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strains of Streptomyces. Intern. J. Syst. Bact. 22: 265~394, 1972