

STUDIES ON VALIDAMYCINS, NEW ANTIBIOTICS. I
STREPTOMYCES HYGROSCOPICUS VAR. *LIMONEUS* NOV.
VAR., VALIDAMYCIN-PRODUCING ORGANISM

TAKASHI IWASA, HIROICHI YAMAMOTO and MOTOO SHIBATA

Microbiological Research Laboratories, Research and Development Division,
Takeda Chemical Industries, Ltd., Osaka, Japan

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Streptomyces sp., strain No. T-7545 was found to produce new antibiotics, validamycins A and B that are effective for the control of sheath blight of rice plants. The validamycins exhibit no *in vitro* activity. A taxonomic study of strain No. T-7545 was carried out and it was characterized as follows: It forms gray to yellow aerial mycelium, bright yellow to yellow ochre vegetative mycelium and faint, brownish yellow diffusible pigment, good growth and development of abundant aerial mycelium with the formation of coiled chains of spores at 25~45°C, and black moist areas in the aerial mycelium on certain media. As a result of comparison of strain No. T-7545 with known species, the name *Streptomyces hygrosopicus* WAKSMAN *et* HENRICI, 1948 var. *limoneus* nov. var. is proposed.

In the course of the screening for new antibiotics effective in the control of sheath blight, a destructive disease of rice plants caused by *Pellicularia sasakii* (SHIRAI) S. ITO, *Streptomyces* sp., strain No. T-7545, was found to produce antibiotic substances showing a curative effect against the disease. Later, these substances were also found to prevent damping-off of cucumber seedlings caused by *Rhizoctonia solani* KÜHN.

As a result of the isolation, purification and characterization of the active substances, they were found to be new, weakly basic, water-soluble antibiotics, and were named validamycins A and B respectively¹⁾. They have quite specific properties in that they exhibit remarkable therapeutic effects against the plant diseases above mentioned despite their lack of antimicrobial activities against common bacteria and fungi, including *P. sasakii* and *R. solani*, in ordinary *in vitro* tests.²⁾

An examination of the morphological and cultural characteristics of strain No. T-7545 revealed that its properties were similar to those of *Streptomyces hygrosopicus*. However, it is different from a typical strain of *S. hygrosopicus* in some respects, such as its abundant growth and development of aerial mycelium at 45°C and its formation of a bright yellow vegetative mycelium on certain media. Therefore, it is considered to represent a variety of *S. hygrosopicus*, and *Streptomyces hygrosopicus* WAKSMAN *et* HENRICI, 1948 var. *limoneus* nov. var. is proposed as its name.

In the course of the screening for strains with high productivity of the validamycins, a number of mutants were obtained. The most common ones isolated were those with yellow aerial mycelium. The original strain and one of the yellow mutant

strains have been deposited in the Institute for Fermentation, Osaka under the accession numbers of IFO-12703 and IFO-12704 respectively.

In this report the morphological and cultural characteristics of strain No. T-7545 are described.

Materials and Methods

Streptomyces sp. strain No. T-7545: Isolated from a soil sample collected in Akashi City, Hyogo Prefecture, Japan.

Yellow mutant: Obtained through spontaneous mutation of the original strain.

Morphological observations: The culture of strain No. T-7545 incubated on glucose asparagine agar at 28°C for about 10 days, was observed with a light microscope and an electronmicroscope (JEM-SS, Japan Electron Optics Laboratory Co., Ltd.).

Cultural characteristics: Each of the media used in this study was prepared according to recommendations in WAKSMAN³⁾. Spores were collected from a 7-day culture on glucose asparagine agar, suspended in sterilized water and a loopful of the suspension was used to inoculate each medium studied. Unless otherwise stated, all cultures were incubated at 28°C for 21 days and observations were carried out every 7 days after inoculation. The color names used in this study and appearing in the tables were based on RIDGWAY⁴⁾.

Utilization of carbon sources: Utilization of carbon sources by strain No. T-7545 was investigated with the method of PRIDHAM and GOTTLIEB⁵⁾.

Results and Discussion

1. Morphological characteristics

The aerial mycelium of strain No. T-7545 is simply branched and terminates in coils of 3 to 5 volutions (Fig. 1). The spores are oval or cylindrical and measure $1.0\sim 1.3\times 1.0\sim 1.5\mu$. Their surfaces are smooth (Fig. 2).

2. Cultural characteristics

The color of aerial mycelium is gray to gray and yellow, and black moist areas form in the aerial mycelium on glucose asparagine agar and starch agar. The color of the vegetative mycelium on most of the media is bright yellow to ochre with, in

Fig. 1. Morphology of *Streptomyces hygroscopicus* var. *limoneus* strain No. T-7545
×950 ×1,900

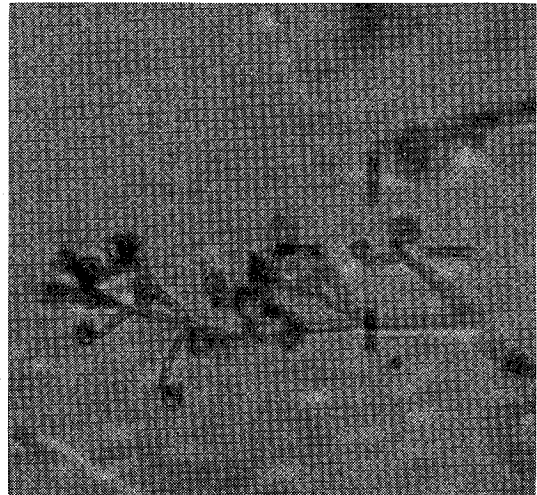
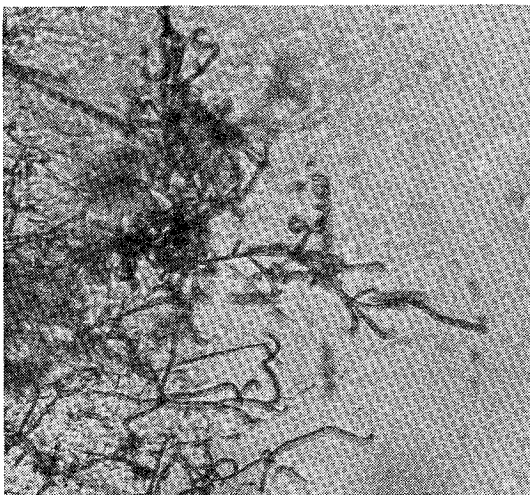


Table 1. Cultural characteristics of *Streptomyces hygrosopicus* var. *limoneus*

Medium	Cultural characteristics	Medium	Cultural characteristics
CZAPEK'S agar	Growth (G): Moderate, colorless, folded. Reverse (R): Raw Sienna (Rdg., III 17-i) to Sudan Brown (Rdg., III 15-k). Aerial Mycelium (AM): Tilleul-Buffer (Rdg., XL 17'''-f) to Light Buff (Rdg., XV 17'-f), partially Mouse Gray (Rdg., LI 15''''-d) along the periphery of the colony. Soluble Pigment (SP): Yellow with a faint brownish tinge.	Tyrosine agar	G: Colorless to Strontian Yellow (Rdg., XVI 23'). R: Pale Ochraceous Buff (Rdg., XV 15'-f) to Light Ochraceous-Buff (Rdg., XV 15'-d). AM: None. SP: None.
		Yeast extract agar	G: Colorless, folded. R: Cream Color (Rdg., XVI 19'-f). AM: White. SP: Light brown.
Glucose CZAPEK'S agar	G: Moderate, colorless to Sulphin Yellow (Rdg., IV 21-i), folded. R: Raw Sienna. AM: Tilleul-Buffer to Massicot Yellow (Rdg., XVI 21'-f), partially Light Olive-Gray (Rdg., LI 23''''-d) along the periphery of the colony. SP: Yellow with a faint brownish tinge.	Nutrient agar (37°C)	G: Colorless. R: Colorless. AM: None. SP: None.
		Glucose nutrient agar (37°C)	G: Colorless wrinkled. R: Cartridge Buff to Pale Ochraceous-Buff. AM: None. SP: None.
Glycerol CZAPEK'S agar	G: Moderate, colorless to Orange-Citrine (Rdg., IV 19-k), folded. R: Raw Sienna. AM: Tilleul-Buffer to Massicot Yellow, partially Light Olive-Gray. SP: Yellow with a faint brownish tinge.	Peptone glucose agar	G: Chartreuse Yellow. R: Honey Yellow (Rdg., XXX 19''). AM: Thin, Cream Buff. SP: Yellow with a brownish tinge.
		Nutrient broth (37°C)	G: Surface growth colorless, and colorless flocculent growth at bottom of tubes. AM: None. SP: None.
Glucose asparagine agar	G: Colorless. R: Old Gold (Rdg., XVI 19'-i) to Antimony Yellow (Rdg., XV 17'-b) to Cinnamon-Brown (Rdg., XV 15'-k). AM: Light Olive-Gray to Mouse Gray, with yellow patches and black moist areas. SP: Light brown.	Glucose nutrient broth	G: Surface growth Cartridge Buff, and colorless flocculent growth at bottom of tubes. AM: None. SP: None.
		Potato plug	G: Colorless to Pale Ochraceous-Buff. AM: Tilleul-Buffer to Mouse Gray. Color of the plug turned to Sayal Brown (Rdg., XXIX 15''-i).
Calcium malate agar	G: Primuline Yellow (Rdg., XVI 19'). R: Primuline Yellow. AM: Sparse at first, but later Tilleul-Buffer to Light Olive-Gray. SP: Pale Yellow.	Carrot plug	G: Colorless. AM: White to Mouse Gray. Color of the plug turned to Cinnamon-Rufous (Rdg., XIV 11'-i) to Cinnamon-Brown.
Starch agar**	G: Colorless to Barium Yellow (Rdg., XVI 23'-d). R: Deep Colonial Buff (Rdg., XXX 21''-b) to Snuff Brown (Rdg., XXIX 15''-k). AM: Cartridge Buff (Rdg., XXX 19''-f) to Mouse Gray, with black moist areas. SP: Light Brown. Hydrolysis of starch was observed.	Cellulose	G: Poor growth, Chartreuse Yellow (Rdg., XXXI 25''-d) to Reed Yellow (Rdg., XXX 23''-b). AM: Mouse Gray. SP: Pale Yellow.

(to be continued)

(continued)

Medium	Cultural characteristics	Medium	Cultural characteristics
Gelatin (25°C)	G: Very poor. AM: None. SP: None. Liquefaction, slow.	Litmus milk (37°C)	G: Surface growth Cream Color to Seashell Pink (Rdg., XIV 11'-f). AM: None. SP: Army Brown (Rdg., XL 13'''-i). Peptonization with or without weak coagulation. Reaction of the medium, weakly acidic.
Nutrient gelatin (25°C)	Same results as with gelatin.		
Whole egg (37°C)	G: Colorless. AM: None. SP: None.	LÖFFLER'S medium (37°C)	G: Naples Yellow (Rdg., XVI 19'-d) becoming Light Buff. AM: None. SP: None. No liquefaction.

* Rdg. : RIDGWAY

** Soluble starch 1%, potassium monohydrogen phosphate 0.3%, calcium carbonate 0.3%, magnesium sulfate 0.1%, ammonium sulfate 0.2%, sodium chloride 0.05%, agar 2%.

Table 2. Physiological properties of *Streptomyces hygroscopicus* var. *limoneus*

	Properties
Temperature and pH ranges*	Growth occurs at 15~45°C, better growth at 37~45°C, no growth at 10°C and 50°C. Growth occurs at pH 5~pH 10, no or poor growth at pH 4, optimum range pH 6~pH 7.
Gelatin	Slow liquefaction.
Starch	Hydrolysis. Diameter of hydrolyzed area/diameter of colony=33 mm/8 mm.
Tyrosinase reaction	Negative.
Litmus milk	Peptonization. Coagulation, doubtful. Reaction, weakly acidic.
Reduction of nitrate to nitrite	Negative (in peptone solution and CZAPEK'S solution).
Cellulose decomposition	Negative.
Chromogenicity	Negative.
Liquefaction of serum	Negative.
Products	Validamycins A and B.

* On glucose asparagine agar.

some cases, a slight greenish tinge. A light yellow to faint yellowish brown diffusible pigment was noted in various media, but because dark brown soluble pigment on proteinaceous media was not observed, strain No. T-7545 is considered to be non-chromogenic.

Physiological characteristics of strain No. T-7545 are shown in Table 2. As shown in the table, a wide temperature range and rather high optimum temperature for growth are characteristic for this organism. Starch hydrolysis and milk peptonization tests are positive, whereas tyrosinase, nitrate reduction, cellulose decomposition and serum liquefaction are negative. Gelatin is slowly liquefied. A variety of carbon sources

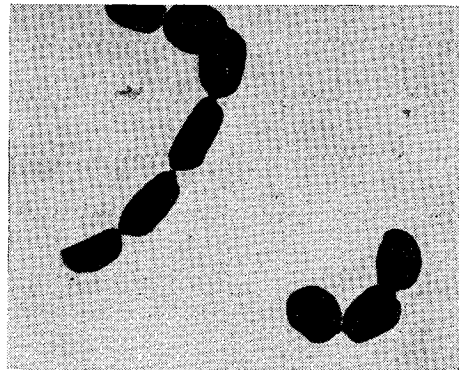
Fig. 2. Spores of *Streptomyces hygroscopicus* var. *limoneus* strain No. T-7545 ($\times 10,000 \times 1/1.5$)

Table 3. Utilization of carbon sources by *Streptomyces hygroscopicus* var. *limoneus* strain No. T-7545 and the yellow mutant, strain No. T-7545Y

Carbon source	No. T-7545	No. T-7545Y	Carbon source	No. T-7545	No. T-7545Y
Erythritol	±	±	Inositol	++	++
Adonitol	±	±	D-Mannitol	++	++
Sorbitol	+	+	Dulcitol	±	±
D-Xylose	++	++	Trehalose	++	++
L-Arabinose	++	++	Salicin	±	±
L-Sorbose	±	±	Esculin	±	±
D-Galactose	++	++	Inulin	++	+
Glucose	++	++	Dextran	+	+
D-Fructose	++	++	Mannose	++	++
L-Rhamnose	++	++	Glycerol	++	++
Melibiose	++	++	Na-acetate	+	+
Maltose	++	++	Na-succinate	+	+
Sucrose	++	++	Na-citrate	+	+
Lactose	++	++	Ca-2-ketogluconate	±	±
Raffinose	++	++	Carbon-free control	±	±

++ : Good growth + : Fair growth ± : No or very poor growth

such as D-xylose, L-arabinose, L-galactose, glucose, D-fructose, L-rhamnose, melibiose, maltose, sucrose, lactose, raffinose, inositol, D-mannitol, trehalose, inulin, mannose and glycerol each are well utilized for growth.

In view of the relatively high optimum temperature for growth of the strain, the cultural characteristics at 45°C were observed and compared with those at 28°C. The characteristics at the higher temperature were found to be almost the same as those at 28°C. A few properties such as sparse formation of white aerial mycelium on CZAPEK's agar, CZAPEK's-glucose agar, CZAPEK's-glycerol agar and filter paper, and a slightly deeper color of the vegetative mycelium on calcium malate agar were noted.

The characteristics of strain No. T-7545 are summarized as follows: Good growth and abundant aerial mycelium and coiled chains of spores form at 25~45°C; on a variety of media, gray to gray and yellow aerial mycelium, bright yellow to ochre vegetative mycelium and faint, brownish yellow diffusible pigment form; on certain media, black moist spots form in the aerial mycelium. Dark brown diffusible pigment is not produced on proteinaceous media, *i. e.* the strain is non-chromogenic.

3. Characteristics of yellow mutant, strain IFO-12704

As a result of the investigation of the morphological and cultural characteristics of the yellow mutant, it was found to resemble the original strain except for the following differences: 1) The tip of the aerial hyphae had a tendency to form loops or open coils of spores; 2) color of the aerial mycelium was yellow on glucose asparagine agar, calcium malate agar, potato plug and filter paper. Both strains showed quite similar physiological properties except for minor differences in utilization of carbon sources as shown in Table 3.

4. Comparison of strain No. T-7545 with other species

Although the present strain showed a high optimum temperature for growth, it could not grow at 50°C. Therefore, its characteristics were compared with those of known mesophilic species^{3,6,7)} belonging to the genus *Streptomyces*. Among these it seemed to resemble *Streptomyces ambofaciens* PINNERT-SINDICO³⁾, *Streptomyces platensis*

Table 4. Cultural characteristics of strain No. T-7545Y, a yellow mutant of *Streptomyces hygroscopicus* var. *limoneus*

Medium	Cultural characteristics	Medium	Cultural characteristics
CZAJEK'S agar	G: Yellow Ocher (Rdg., XV 17'). R: Yellow Ocher. AM: None. SP: Faint yellow.	Peptone glucose agar	G: Colorless. R: Light Ochraceous-Buff. AM: White. SP: Light brown.
Glucose CZAJEK'S agar	G: Yellow Ocher. R: Yellow Ocher. AM: None. SP: Faint yellow.	Nutrient broth (37°C)	G: Surface growth Tilleul-Buff, colorless flocculent growth at bottom of tubes. AM: None. SP: None.
Glycerol CZAJEK'S agar	G: Yellow Ocher. R: Yellow Ocher. AM: None. SP: Faint yellow.	Glucose nutrient broth (37°C)	G: Surface growth Tilleul-Buff, colorless, flocculent growth at bottom of tubes. AM: None. SP: None.
Glucose asparagine agar	G: Colorless. R: Primuline Yellow. AM: Massicot Yellow. SP: Faint, brownish yellow.	Potato plug	G: Seashell Pink. AM: Pale Vinaceous-Fawn (Rdg., XL 13'''-f) to Pale Ochraceous-Buff. Color of the plug turned to Sayal Brown.
Calcium malate agar	G: Tilleul-Buff to Barium Yellow. R: Massicot Yellow to Primuline Yellow. AM: Tilleul-Buff to Massicot Yellow. SP: Faint yellow.	Carrot plug	G: Colorless. AM: White to Cream Color. Color of the plug turned to Ochraceous-Orange (Rdg., XV 15').
Starch agar	G: Colorless. R: Strontian Yellow. AM: Massicot Yellow to Light Mouse Gray (Rdg. LI 15''''-b). SP: Faint yellow. Hydrolysis of starch was observed.	Cellulose	G: Poor, colorless. AM: Poor, Massicot Yellow. SP: None.
Tyrosine agar	G: Colorless to Strontian Yellow. R: Pale Ochraceous-Buff to Light Ochraceous-Buff. AM: None. SP: None.	Gelatin (25°C)	G: Very poor. AM: None. SP: None. Liquefaction, slow.
Yeast extract agar	G: Colorless. R: Cream Color. AM: White. SP: Light brown.	Nutrient gelatin	Same results as with gelatin.
Nutrient agar (37°C)	G: Colorless. R: Colorless. AM: None. SP: None.	Whole egg (37°C)	G: Colorless AM: Thin, white. SP: None.
Glucose nutrient agar (37°C)	G: Colorless. R: Pale Ochraceous-Buff. AM: Thin, white. SP: None.	Litmus milk (37°C)	G: Surface growth, Seashell Pink. AM: None. SP: Army Brown. Peptonization with or without weak coagulation. Reaction of the medium, weakly acidic.
		LÖFFLER'S medium (37°C)	G: Light Buff. AM: None. SP: None. No liquefaction.

TRESNER and BACKUS⁹⁾ and *Streptomyces hygrosopicus* (JENSEN) WAKSMAN and HENRICI⁹⁾.

S. ambofaciens and strain No. T-7545 are similar in the color of both vegetative and aerial mycelia. However, the former is non-hygrosopic, and forms yellow surface growth and brown-orange pigment on gelatin, whereas strains No. T-7545 forms very poor, colorless growth on gelatin. Both organisms also are different in their utilization of carbon sources and in production of antibiotics.

Several differences were noted between *S. platensis* and strain No. T-7545. The color of the vegetative mycelium of the former is reported as Deep Olive (RIDGWAY) on CZAPEK's agar and orange becoming light brown on calcium malate agar, whereas that of strain No. T-7545 is yellow ocher on CZAPEK's agar and bright yellow on calcium malate agar. In addition, the former reportedly utilizes carbon sources different from those utilized by the latter and produces different antibiotics.

On the other hand, comparison of the characteristics of strain No. T-7545 with those reported for *S. hygrosopicus* reveal that the former is different from the latter in its bright yellow to yellow ocher or light greenish yellow vegetative mycelium on CZAPEK's agar, CZAPEK's-glucose agar, glucose asparagine agar and calcium malate agar and in its white to yellow aerial mycelium on CZAPEK's agar. However, according to TRESNER and BACKUS⁹⁾, brownish gray aerial mycelium, formation of tight spirals and of black moist spots in the aerial mycelium on certain media are the most common and stable features of *S. hygrosopicus*. Other properties such as the color of vegetative mycelium and reverse color of colonies, production of diffusible pigment and liquefaction of gelatin are relatively variable.

Although strain No. T-7545 exhibits the common and stable features of *S. hygrosopicus*, it differs from the latter in the color of both vegetative and aerial mycelia on certain media, in its high optimum temperature for growth and in its production of the validamycins. It is, therefore, considered as a variety of *S. hygrosopicus*. The proposed varietal epithet "limoneus" is the modern Latin adjective meaning lemon yellow, the color of vegetative mycelium of the strain. A detailed characterization of the new taxon follows:

Streptomyces hygrosopicus WAKSMAN and HENRICI, 1948 var. *limoneus* nov. var.

Morphology: Spore bearing hyphae simply branched, terminating in coils of 3 to 5 volutions. Sporangia, flagellated spores or sclerotic granules not observed. Spores 1.0~1.3×1.0~1.5 μ in size arranged in chains; oval or cylindrical with smooth surfaces. Hygrosopic areas are observed in the aerial mycelium.

Color of colonies: Aerial mass color, white (Tilleul-Buff*) to yellow (Light Buff) with gray (Mouse Gray) peripheries on CZAPEK's agar, gray with slight greenish tinge (Light Olive-Gray) with yellow patches and hygrosopic areas on glucose asparagine agar, white (Tilleul-Buff) to gray with slight greenish tinge (Light Olive-Gray) on calcium malate agar. No aerial mycelium on nutrient agar.

Color of reverse of colonies: Bright yellowish brown (Raw Sienna) to brown (Sudan Brown) on CZAPEK's agar, brownish yellow (Old Gold to Antimony Yellow) to brown (Cinnamon-Brown) on glucose asparagine agar, bright yellow (Primuline Yellow) on calcium malate agar, colorless on nutrient agar.

* Color names in parentheses are taken from RIDGWAY's "Color Standards and Color Nomenclature".

Color in medium: Non-chromogenic (negative reaction with glucose nutrient agar, tyrosine agar, and peptone agar). Faint brownish yellow diffusible pigment forms in CZAPEK's agar, glucose asparagine agar. A pale yellow diffusible pigment in nutrient agar.

Physiological properties: Starch hydrolyzed, gelatin slowly liquefied; milk peptonized.

Carbohydrate utilization: D-glucose, D-xylose, D-fructose, rhamnose, L-arabinose, sucrose, *i*-inositol, raffinose and mannitol utilized for growth.

Mesophilic.

Aerobic.

Habitat: Soil.

Antagonistic properties: Produces validamycins A and B.

Type strain: Strain No. T-7545 is designated as the type strain of this variety and has been deposited in the Institute for Fermentation, Osaka and assigned accession number IFO 12703.

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