TAXONOMIC STUDY AND FERMENTATION OF PRODUCING ORGANISM AND ANTIMICROBIAL ACTIVITY OF MILDIOMYCIN

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A taxonomic study of strain B-98891, which produced an antibiotic effective against powdery mildew of barley, identified it as *Streptoverticillium rimofaciens*. On agar media the antibiotic, which was named mildiomycin, was only weakly active against most fungi and bacteria tested. However, it inhibited some *Mycobacterium* and *Rhodotorula*, and it showed excellent control of powdery mildew of barley plants in greenhouse tests at concentrations between 31.2 and 62.5 ppm. *Rhodotorula rubra* IFO 0907 was selected as the test organism for *in vitro* assay of mildiomycin.

In the course of our screening program for new agricultural antibiotics, strain B-98891, isolated from soil collected in Papua New Guinea, was found to produce an antibiotic effective against powdery mildew of barley. Though some antibiotics such as gougerotin[1], polyetherin[2], polyoxin[3], A-9145[4] are known to be active against powdery mildew, only polyoxin is in practical use as the mildew eradicant. The antibiotic isolated from the filtered broth of strain B-98891 was found to be a new nucleoside antibiotic[5], and named mildiomycin because of its specific action against powdery mildew.

This report deals with taxonomic studies of strain B-98891, with the production, microbiological assay method, *in vitro* antimicrobial activity and with the effect of mildiomycin against powdery mildew of barley plants in a greenhouse test.

Materials and Methods

Taxonomic Studies

The taxonomic characterization was carried out according to the methods described by WAKSMAN[6], SHIRLING and GOTTLIEB[7], PRIDHAM and GOTTLIEB[8] and M. P. and H. LECHEVALIER[9]. The color names used in this study were based on the Color Harmony Manual[10].

Selection of the Test Organism for *In Vitro* Assay of Mildiomycin

Many strains of yeasts were tested for their susceptibility to mildiomycin on two media, modified PFEFFER's agar[11] and Trypticase Soy agar with 1.0% glucose added. The paper disc dipped in an 800 μg/ml aqueous solution of mildiomycin was placed on an agar plate seeded with a yeast, and after incubation at 28°C for 40 hours strains that showed an inhibition zone were selected.

Fermentation Procedure

Five hundred milliliters of the seed medium consisting of 3.0% glucose, 2.2% soybean flour, 0.3% peptone and 0.4% $CaCO_3$ (pH 7) in a 2-liter flask was inoculated with a loopful of spores from a slant culture of strain B-98891, incubated on a reciprocating shaker (120 strokes per minute) at 28°C for 48 hours and was transferred to 30 liters of a medium of the same composition in a 50-liter fermentor. After incubation at 28°C for 48 hours, 10 liters of the culture were added to a 200-liter fermentor con-

taining 100 liters of the following production medium: 5.0% glucose, 3.5% soybean flour, 1.0% Pharmamedia, 0.5% NaCl, 0.5% $CaCO_3$, 0.05% Antifroth (Dai-ichi Kogyo Seiyaku Co.) in tap water. The medium was adjusted to pH 7 before autoclaving. Cultivation was carried out at 28°C for 114 hours with an air-flow rate of 50 liters per minute and an agitation at 100 rpm.

Antimicrobial Activity *In Vitro*

Antimicrobial activity of mildiomycin against various microorganisms was examined by the agar dilution and disc-plate agar diffusion methods. In the latter case, a paper disc (8-mm diameter) dipped in a 5,000 μg/ml aqueous solution of mildiomycin (purity, 97%) was used.

Greenhouse Test

Barley plants (*Hordeum vulgare* L. c. v. Shiga Hakkoku No. 5) of 5- to 6-leaf stage, grown in pots (12cm in diameter) in a greenhouse at 20°C, were inoculated with conidia of *Erysiphe graminis* DE CANDOLLE f. sp. *hordei* EM. MARCHAL by placing them adjacent to the mildewed barley plants. An adequate volume of the fungicidal solution was sprayed on the leaves three times at 10 days intervals. Percent areas mildewed on 20 leaves (taking 10 of the 6th leaves that had been unfolded and 10 of the 9th that had been folded, at the time of first spray) were assessed 10, 20 and 30 days after the first spray, as specified in the official criteria[12].

Results and Discussion

1. Taxonomic Characteristics of Strain B-98891

Good formation of aerial mycelium and whorls with secondary verticils were observed on glucose-asparagine agar, yeast-malt agar, oatmeal agar and inorganic salts-starch agar (Fig. 1). Loop or hook spore chains, not in verticils, were observed occasionally on sucrose-nitrate agar and glucose-asparagine agar. Spores occurred in chains of 3 to 16, oval or cylindrical in shape, and $0.6 \sim 0.8 \times 0.7 \sim 1.3$ μ in size. The surface of the spores was usually smooth, but occasionally it showed some irregularity (Fig. 2). Sporangia, flagellated spores and sclerotia were not observed on the media commonly used in taxonomic studies of *Actinomycetes*.

Cultural characteristics of strain B-98891 are shown in Table 1. The surface of the vegetative mycelium was colorless to pale yellow, and its reverse side was pale yellow to yellowish brown to brown. The aerial mycelium was yellowish white or, in the cottony part, slightly grayish yellow to beige. Pale yellowish brown to brown soluble pigment was produced on most of the media. It was noticeable that bluish green pigment appeared near the surface of glucose-peptone gelatin after about 10 days' incubation, later changing to dark bluish green, diffusing downward. This pigment was also observed on gelatin medium but not on nutrient gelatin.

Fig. 1. Aerial hyphae of *Streptoverticillium rimofaciens* B-98891 on inorganic salts-starch agar, 10 days (×370)

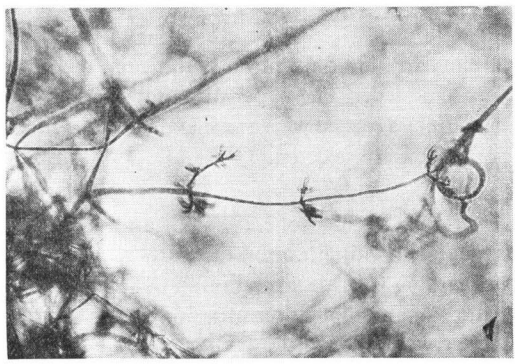


Fig. 2. Spores of *Streptoverticillium rimofaciens* B-98891; electron micrograph from 8-day culture of inorganic salts-starch agar (×6240)

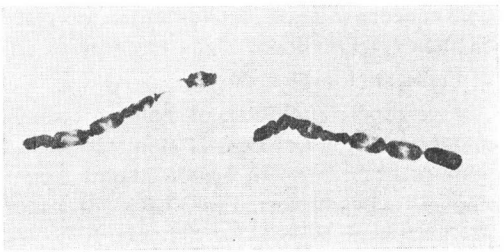


Table 1. Cultural characteristics of strain B-98891 on various media

Medium	*Characteristics
Sucrose nitrate agar	G: Colorless, penetrating into medium R: White to light ivory (2 ca) AM: Scant, cottony, shell (3 ca) SP: None
Glucose asparagine agar	G: Colorless, penetrating into medium R: Light ivory (2 ca) to chamois (2 gc) to cinnamon (3 le) AM: Thin, white with a tinge of yellow, or moderate, cottony shell (3 ca) to pearl (3 ba) SP: None or very faint, yellowish brown
Glycerol asparagine agar	G: Chamois (2 gc) to tan (3 ie), penetrating into medium R: Cinnamon (3 le) AM: Thin, white to light ivory (2 ca) or moderate, cottony, white with a tinge of pinkish yellow SP: Pale brown
Ca-Malate agar	G: Cream (1 1/2 ca), later chamois (2 gc) R: Honey gold (2 ic) to cinnamon (3 le) AM: None or thin, light ivory (2 ca) SP: Pale brown
Inorganic salts-starch agar	G: Colorless, penetrating into medium R: Cream (1 1/2 ca) to light ivory (2 ca) AM: Abundant, cottony, white to pearl (2 ba), later pearl (3 ba) to shell (3 ca) to light beige (3 ec) SP: Pale yellow
Yeast malt agar	G: Good, colorless, penetrating into medium R: Light ivory (2 ca) to golden brown (3 pi) AM: Moderate, light ivory (2 ca) to pastel yellow (1 db) to chamois (2 gc) or cottony, white to pearl (3 ba) SP: Pale brown
Oatmeal agar	G: Good, colorless R: Yellow maple (3 ng) AM: Abundant, cottony, white to pearl (3ba) to light beige (3 ec) SP: Pale yellowish brown
Nutrient agar	G: Colorless R: Light maize (2 ea) AM: Thin, white SP: Pale brown
Glucose peptone gelatin	G: Surface growth good, colorless. Growth in the medium, scant, colorless AM: Moderate, cream (1 1/2 ca) SP: Brown pigment, only around the growth. After about 10 days incubation, bluish green pigment appeared near the surface, later changing to dark bluish green, and diffusing downward. Gelatin, not liquefied.

* Abbreviations: G: Growth, R: Reverse, AM: Aerial mycelium, SP: Soluble pigment.

Table 2. Physiological properties of strain B-98891

Temperature range for growth*	15~38°C, no growth below 10°C and above 40°C, maximum growth at 34~36°C
Liquefaction of gelatin	−
Hydrolysis of starch	+
Production of melanoid pigment	+(peptone yeast-iron agar, tryptone-yeast broth)
	−(tyrosine agar)
Milk coagulation	+
Milk peptonization	+
Nitrate reduction	−(Bacto nitrate broth, CZAPEK's solution)

* Examined on glucose-asparagine agar.

Table 3. Carbon source utilization of strain B-98891

Carbon source	Growth	Carbon source	Growth
Erythritol	±	Sucrose	±
Adonitol	±	Lactose	±
D-Sorbitol	±	Raffinose	±
Inositol	++	Trehalose	+
D-Mannitol	±	Salicin	±
Dulcitol	±	Esculin	±
D-Xylose	±	Inulin	±
L-Arabinose	±	D-Mannose	++
L-Sorbose	±	Starch	++
D-Galactose	++	Glycerol	++
D-Glucose	++	Na-Acetate	++
D-Fructose	+	Na-Succinate	++
Rhamnose	±	Na-Citrate	++
Melibiose	±	Carbon free	
Maltose	++	Control	±

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

Physiological properties and carbon source utilization pattern of strain B-98891 are shown in Tables 2 and 3. It grew at 15~38°C and the optimum temperature range was 34~36°C. It showed positive reaction in starch hydrolysis and in coagulation and peptonization of milk, and negative reaction in liquefaction of gelatin (24°C, 28 days) and in nitrate reduction to nitrite. Formation of melanoid pigment was observed on peptone-yeast-iron agar (ISP No. 6) and in tryptone-yeast broth (ISP No. 1) but not on tyrosine agar (ISP No. 7). In the whole-cell hydrolysate of strain B-98891, L-DAP was detected but arabinose and galactose were not.

Morphological, cultural and physiological characteristics of strain B-98891 were compared with those of known species. As a result, it was found that strain B-98891 closely resembled **Streptoverticillium rimofaciens* (*Streptomyces rimofaciens*[13]). According to the description of *Stv. rimofaciens*, the aerial mycelium on sucrose nitrate agar is cottony, white with a tinge of pink or pinkish yellow, that on glucose-asparagine agar is powdery, white changing gradually to whitish brown and to pale yellowish

Table 4. Differences between strain B-98891 and *Stv. rimofaciens*

	Stv. rimofaciens[13]	Strain B-98891
Nutrient agar and glucose	Cracks in colony	No cracks
Nutrient agar	Soluble pigment, dark brown	Soluble pigment, pale brown
Carrot plug	No growth	Good growth and abundant cottony, white to light yellowish orange aerial mycelium
Utilization of carbon sources	Sorbitol and mannitol are utilized.	Sorbitol and mannitol are not utilized.
Antibiotics produced	Destomycins A and B	Mildiomycin

* *Streptoverticillium* is used as the generic epithet in accordance with BERGEY's Manual of Determinative Bacteriology 8th Edition.

orange. On starch-ammonium sulfate agar the aerial mycelium is cottony, white to pale yellowish orange to brown with colorless drops of exudate. The characteristics of strain B-98891 on these media is quite similar to those of *Stv. rimofaciens* cited above. Besides, almost no difference was found between the colors of the vegetative mycelium and soluble pigment of strain B-98891 and those of *Stv. rimofaciens*. Table 4 shows the differences between the two strains. Differences such as absence of cracks on glucose nutrient agar, growth on carrot plug, failure to utilize sorbitol and mannitol and antibiotic production are too small to regard strain B-98891 as a different species. Therefore, we identified the strain as *Stv. rimofaciens* and designated it *Stv. rimofaciens* B-98891.

2. Microbiological Assay Method for Mildiomycin

Because the powdery mildew fungus is an obligate parasite, *in vitro* bioassay of mildiomycin using this fungus was impossible. In searching for an alternative microorganism 1,400 strains of yeasts were examined. Of these, 29 strains, including 21 strains of *Rhodotorula*, were selected as highly susceptible (the diameter of the inhibition zone was more than 20 mm), and finally *Rhodotorula rubra* IFO 0907 was chosen as the test organism. After experiments to select the composition and pH of the assay medium, conditions for incubation and inoculum size *etc.*, the following assay method was established:

Assay media: (base layer) potato dextrose agar (pH 5)
(seed layer) potato sucrose agar (pH 5)

Inoculum: $2\sim4\times10^9$ cells/ml. The inoculum size is 5% of the seed-layer medium.

Incubation: 28°C for 18~20 hours

The mildiomycin content of samples assayed by the diffusion method should be between 10 and 80 μg/ml. The relationship of zone diameter to mildiomycin concentration is shown in Fig. 3.

Fig. 3. Relation between diameter of inhibition zone and concentration of mildiomycin

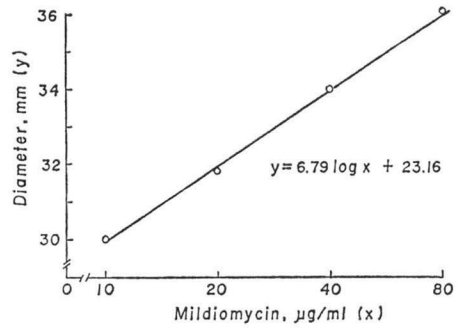
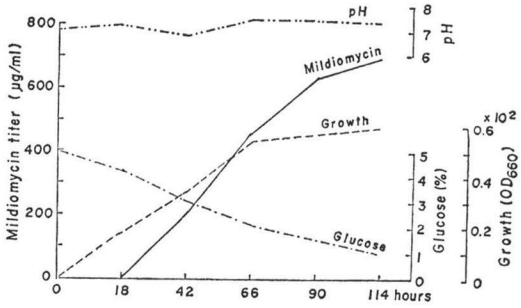


Fig. 4. Time course of mildiomycin production by *Stv. rimofaciens* B-98891
Glucose concentration in broth was determined by the picric acid method. Mycelia in whole broth was collected by sucrose gradient technique, and OD_{660nm} of a suspension in water was measured.



3. Fermentation

Glucose and soybean flour were found to be suitable carbon and nitrogen sources, respectively, for the production of mildiomycin. The effect of C-N ratio, addition of other nitrogen sources and inorganic salts, *etc.*, were tested and the following medium was selected as the production medium; 5.0% glucose, 3.5% soybean flour, 1.0% Pharmamedia, 0.5% NaCl, 0.5% $CaCO_3$ (pH 7). Fig. 4 shows the course of a typical mildiomycin fermentation in a 200-liter fermentor. Both the consumption of glucose and growth took place gradually; the latter reached a maximum at about 66 hours. Production of mildiomycin was

detected after about 20 hours and reached a maximum at 102~114 hours. Little change in pH of the broth occurred throughout the fermentation.

Table 5. Antimicrobial spectrum of mildiomycin

Test organisms	Conditions of assay			Minimal inhibitory concentration (μg/ml)	Diameter of inhibition zone (mm)
	Media*	Temp. (°C)	Time (hour)		
Bacillus subtilis IFO 3513	B	37	20	>500	(14)**
B. cereus IFO 3466	B	37	20	>500	—
B. brevis IFO 3331	B	37	20	>500	(20)
Staphylococcus aureus IFO 3061	B	37	20	>500	(10)
Sarcina lutea IFO 3232	B	37	20	>500	(10)
Escherichia coli IFO 12734	B	37	20	500	(14)
Proteus vulgaris IFO 3045	B	37	20	>500	(11)
Pseudomonas aeruginosa IFO 3449	B	37	20	500	—***
Mycobacterium phlei IFO 3158	C	37	40	50	(27)
M. smegmatis ATCC 607	C	37	40	250	(24)

* B: Nutrient agar; C: Glycerol-nutrient agar.
** Edge of the inhibition zone is obscure.
*** No inhibition zone.

Table 6. Antifungal spectrum of mildiomycin

Test organisms	Conditions of assay			Minimal inhibitory concentration (μg/ml)	Diameter of inhibition zone (mm)
	Media*	Temp. (°C)	Time (hour)		
Candida albicans IFO 0583	A	28	40	>500	—
C. tropicalis IFO 0006	A	28	40	>500	(11)
Saccharomyces cerevisiae IFO 0209	A	28	40	>500	(12)
Rhodotorula rubra IFO 0870	A	28	40	50	(80)
Aspergillus niger IFO 4066	A	28	40	>500	+**
Penicillium chrysogenum IFO 4626	A	28	40	>500	(13)
Trichophyton mentagrophytes IFO 5809	D	28	88	500	+
T. rubrum IFO 5467	D	28	88	>500	−
Pyricularia oryzae IFO 5279	A	28	40	>500	−
Cochliobolus miyabeanus LFO 5277	A	28	40	250	(20)
Sclerotinia sclerotiorum IFO 9395	A	28	40	500	(20)
Botrytis cinerea TKF 12	A	20	88	250	(15)
Guignardia laricina IFO 7888	A	28	88	100	(18)
Alternaria kikuchiana IFO 8414	A	28	88	250	(22)
Helminthosporium sigmoideum var. *irregulare* IFO 5273	A	28	40	>500	−
Venturia pirina IFO 6189	A	28	40	>500	(15)
Diaporthe citri IFO 9170	A	28	88	>500	(15)
Elsinoe fawcetti IFO 8417	A	28	136	>500	+
Pythium aphanidermatum IFO 7030	A	28	40	>500	+
Gibberella zeae IFO 7160	A	28	40	>500	(15)

* A: Modified PFEFFER's agar (J. Antibiotics 24: 114, 1971).
D: 1.0% glucose, 0.4% $(NH_4)_2HPO_4$, 0.07% $MgSO_4 7H_2O$, 0.1% KH_2PO_4, 0.1% NaCl, 0.005% $FeSO_4$, and 1.5% agar.
** Very obscure and small inhibition zone.

4. Antimicrobial Spectrum *In Vitro* and Effect Against Powdery Mildew of Barley

The antimicrobial spectrum is shown in Tables 5 and 6. Mildiomycin showed relatively strong activity against *Mycobacterium phlei* and *Rhodotorula rubra*, but little activity against Gram-positive and negative bacteria, yeasts, saprophytic fungi and dermatophytes. Among phytopathogenic fungi tested, *Cochliobolus miyabeanus, Sclerotinia sclerotiorum, Botrytis cinerea, Guignardia laricina* and *Alternaria kikuchiana* were relatively susceptible to mildiomycin, whereas others were almost resistant. With various microorganisms tested the edges of inhibition zones were very obscure. It was concluded that mildiomycin specifically inhibits the growth of *Rhodotorula* and *Mycobacterium* species on agar media.

The activity of mildiomycin in controlling powdery mildew on barley plants grown in pots in the greenhouse is shown in Table 7. The effect at 31.2 ppm (a. i.) was almost the same as that of a 125 ppm (a. i.) solution of Benomyl WP, while the effect of mildiomycin at 62.5 ppm was clearly superior to that of Benomyl WP at 125 ppm. Mildiomycin exhibited specific and excellent control of powdery mildew of various plants besides barley. This will be documented in a future report. Whether or not any correlation exists between the specific activity of mildiomycin on *Rhodotorula* and *Mycobacterium* and on powdery mildew fungus is an interesting problem which has not yet been solved.

Table 7. The effect of mildiomycin against powdery mildew of barley plants

Chemicals	Concentration (a. i., ppm)	Percent area of lesions (%)				
		6th leaf			9th leaf	
		A*	B	C	B	C
Mildiomycin	15.6	0.4	2.1	0.8	2.3	10.5
	31.2	0.3	2.0	1.3	0.3	1.3
	62.5	0.1	0.1	0	0.5	0.4
Benomyl WP**	125	0.3	0.4	0.2	1.3	1.6
Control	—	8.4	100	100	68.5	100

* A: 10 days after the first spray which was carried out on 6-leaf stage barley plants.
B: 10 days after the second spray.
C: 10 days after the third spray.

** Benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate) wettable powder.

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