ANTIVIRAL ANTIBIOTIC S15-1

TAXONOMY OF THE PRODUCING STRAIN AND STUDY OF CONDITIONS FOR PRODUCTION OF THE ANTIBIOTIC

TAKESHI KAWAMURA*, KUNIHIKO TAGO**, TERUHIKO BEPPU and KEI ARIMA

Laboratory of Microbiology, Department of Argicultural Chemistry,
The University of Tokyo, Bunkyo-ku, Tokyo, Japan

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Strain S15-1 which produces antiviral antibiotic S15-1 belonging to the streptothricin group of antibiotics was isolated from a soil sample. Cell analysis, colony morphology, the absence of sporangia-like vesicles, the formation of spores in chains of the *Rectus Flexibilis* type, and the ability to produce melanoid pigment, indicate that this strain belongs to the genus *Streptomyces* WAKSMAN and HENRICI. A comparison of the characteristics of strain S15-1 with related *Streptomyces* show that it should be identified as *Streptomyces purpeofuscus* YAMAGUCHI and SABURI.

The investigation of cultural conditions show that soluble starch and meat extract are the most suitable carbon and organic nitrogen sources for the production of antibiotic S15-1. Strain S15-1 grew poorly on media with no organic nitrogen sources, and did not produce the antibiotic. Antiviral antibiotic S15-1 is accumulated at the highest level after 3 or 4 days growth of the producing organism.

In the course of screening for antiviral antibiotics, a strain of microorganism was isolated from soil which produced the streptothricin group antibiotic S15-1.[1,2] Strain S15-1 belongs to the family *Streptomycetaceae*, and according to the classification of *Streptomyces* in the publications resulting from the International *Streptomyces* Project[4~7] and BERGEY's Manual of Determinative Bacteriology[8] is classified in the genus *Streptomyces*. The characteristics of strain S15-1 and the production of antiviral antibiotic S15-1 by the original strain and mutant substrains are described in this paper.

Characteristics of Strain S15-1

Streptomyces strain S15-1 was isolated from a soil sample obtained at Sendai, Miyagi, Japan.

1. Morphological characteristics.

Strain S15-1 grows well on various media such as yeast extract-malt agar, inorganic salts-starch agar, BENNETT's agar and some chemically defined agar media. The morphology of cultures was observed with the microscope after growth on BENNETT's agar at 27°C for 7 days. Spores on BENNETT's agar were studied with an electron microscope (Nihondenshi Co., Ltd.). The results are shown in Fig. 1 and Fig. 2. Colonies were velvety and grayish-white colored in earlier stages of growth, and turned grayish-pink on yeast extract-malt agar and BENNETT's agar. Aerial mycelia were long, filamentous and branched. No spirals or whorls were observed. Spore chains were straight and spores were observed after about 8-day culture on BENNETT's

Present address: *Asahi Chemical Industry Co., Ltd. 2-5, 3-Chome, Kasumigaseki, Chiyoda-ku, Tokyo, Japan.
**Asahi Chemical Industry Co., Ltd. 2-1, Samejima, Fuji, Shizuoka, Japan.

Fig. 1. Photomicrograph of strain S15-1 on BENNETT's agar (7 days culture). (×90)

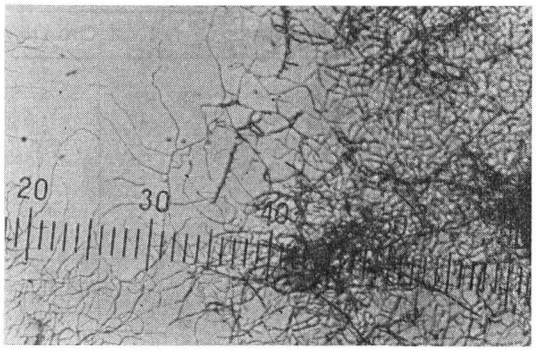
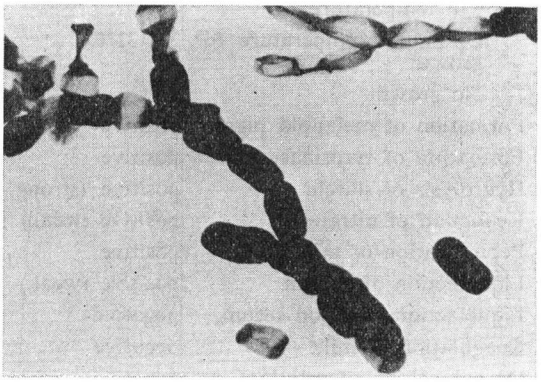


Fig. 2. Electron micrograph of the spores of strain S15-1. (×3,750)



agar (Fig. 2). The spores are ellipsoidal with smooth surfaces (size: 0.8×1.7μ).

2. Cultural and physiological characteristics.

The cultural characteristics and physiological properties of strain S15-1 are shown in Tables 1 and 2. Each of the media used was prepared according to recommendations by SHIRLING and GOTTLIEB,[8] and WAKSMAN.[9] Mature spores and mycelia on BENNETT's agar were used to inoculate each medium. All cultures were incubated at 27°C for 2 weeks except for gelatin

Table 1. Cultural characteristics of strain S15-1.*

Medium	Vegetative growth and color	Aerial mycelium amount and color	Soluble pigment color
Yeast extract-malt extract agar	Good, velvety. Brown	Abundant. Gray	Light brown
Oatmeal agar	Good, velvety. Brown	Abundant. Gray	None
Inorganic salts-starch agar	Good, velvety. Light brown	Abundant. Grayish-white	None
Glycerol-asparagine agar	Moderate, velvety. Light brown	Moderate. Grayish-white	None
CZAPEK's agar	Poor. Colorless	Moderate, powdery. White	None
BENNETT's agar	Good, velvety. Brown	Abundant. Gray to grayish-pink	None
Glucose-asparagine agar	Poor. Colorless	Moderate, powdery. White	None
Nutrient agar	Good, wrinkled. Grayish-yellow	None.	None
LOEFFLER's coagulated serum	Moderate, smooth. Grayish-yellow	None.	Gray to black
Tyrosine agar	Moderate, smooth. Grayish-brown	None.	Dark brown
Potato	Good, wrinkled. Grayish-yellow	Scant. White	Grayish-brown to black
Milk	Moderate, surface, ring. Light brown	None.	Faint brown
Gelatin**	Scant, velvety. Light brown	Moderate. Grayish-white	Brown

* Two-week incubation at 27°C.

** 20-day incubation at 15°C, stab cultures.

Table 2. Physiological properties of strain S15-1.

Growth temperature*	
Optimum temperature for growth	25~32°C
No growth	37°C
Formation of melanoid pigment	positive
Formation of tyrosinase	positive
Hydrolysis of starch	positive (strong)
Reduction of nitrate	positive (weak)
Peptonization of milk	positive
Liquefaction of gelatin	positive (weak)
Liquefaction of blood serum	negative
Coagulation of milk	negative
Decomposition of cellulose	negative

* On BENNETT's agar after 7 days' incubation.

Table 3. Utilization of carbohydrate by strain S15-1.

Carbohydrates	Growth
L-Arabinose	+
D-Fructose	+
D-Glucose	+
i-Inositol	−
Lactose	±
Maltose	−
D-Mannitol	−
Mannose	+
D-Raffinose	−
L-Rhamnose	−
Salicin	−
Sucrose	±
Trehalose	±
D-Xylose	+
No carbohydrate control	−

+: Growth and positive utilization.
±: Faintly growth, probably no utilization.
−: No growth, no utilization.

stabs (15°C for 20 days). Strain S15-1 grows well in all media with the exception of CZAPEK's agar, glucose-asparagine agar and Ca-malate agar. The utilization of carbon sources by strain S15-1 was studied using the method of SHIRLING and GOTTLIEB[3] (Table 3). Strain S15-1 belongs to the chromogenic type of *Streptomyces*, because deep brown diffusible pigment is produced on organic media.

Amino acids of a cell wall hydrolysate were analyzed according to the method of BOONE and PINE.[10] The results of two-dimentional paper chromatography showed that hydrolysates of strain S15-1 contain L-diaminopimelic acid, glycine, alanine, and traces of other amino acids.

According to the classification systems in publications resulting from the International *Streptomyces* Project and BERGEY's Manual, strain S15-1 is categorized in the Gray color series with *Rectus Flexibilis* spore-chain morphology. Melanoid pigment were produced, and spores were glabrous (smooth). In addition, it was found that the physiological properties of strain S15-1 are very similar to those of *Streptomyces purpeofuscus*. Yet there are some differences in physiological properties of this organism and different antibiotics are produced, that is: strain S15-1 produces moderate amounts of melanoid pigment on tyrosine agar, utilizes D-fructose, and produces the streptothricin group antibiotic designated S15-1. On the other hand, the type strain of *Streptomyces purpeofuscus* (strain H-5080) produces only a trace of melanoid pigment on tyrosine agar, does not utilize D-fructose, and produces negamycin, a non-streptothricin group antibiotic.

From the above results it is believed that strain S15-1 does not represent a new species, but is most closely related to *Streptomyces purpeofuscus* even though there are some differences in physiological properties. Strain S15-1 therefore was identified as *Streptomyces purpeofuscus*.

Production of Antiviral Antibiotic S15-1

Thirty six different kinds of media were used in the evaluation of various carbon sources and organic nitrogen sources for the production of antibiotic S15-1. One hundred mililiters of

Table 4. Effect of various carbon sources and organic nitrogen sources on the production of antibiotic S15-1 with wild-type strain.*

Organic nitrogen source (%)		Carbon source					
		Glucose		Dextrin		Soluble starch	
		pH	S15-1** (mm)	pH	S15-1 (mm)	pH	S15-1 (mm)
Peptone	1.0	4.6	+***	6.0	13.5	7.7	15.6
	2.0	4.4	+	7.0	13.0	7.7	15.4
Yeast extract	1.0	4.4	+	7.2	13.2	8.0	14.3
	2.0	7.2	+	6.5	14.7	7.8	14.6
Soybean meal	1.0	7.0	15.4	7.2	15.4	7.6	16.9
	2.0	7.8	15.6	7.2	14.9	7.7	17.1
Meat extract	1.0	5.0	15.3	7.6	13.8	7.8	14.6
	2.0	5.0	15.8	7.2	+	7.8	19.5
Proflo	1.0	7.4	15.5	7.6	14.5	7.2	13.6
	2.0	6.8	15.0	7.7	15.4	7.2	13.7
Corn steep liquor	1.0	4.6	+	7.2	14.6	7.6	16.8
	2.0	4.6	+	7.7	14.9	7.7	16.7
None		6.0	−	6.0	−	6.0	−

* Basal medium: $NaNO_3$ 0.4%, K_2HPO_4 0.2%, KCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, and $FeSO_4 \cdot 7H_2O$ 0.002%.
Carbon sources: Three per cent of glucose, dextrin or soluble starch.
Fermentation conditions: 27°C, reciprocal shaker, 115 strokes per minute; 100 ml of medium (initial pH, 7.0) in 500-ml flask; inoculum size, 5.0% of seed broth.
Bacillus subtilis strain PCI 219 bioassays and pH determinations made after 4 days' incubation. Samples for assays were prepared with 5-fold dilution with saline solution.
** Inhibition zone diameter (8 mm diameter paper discs).
*** +: Diameter of inhibition zone is about 8.5~9.0 mm.

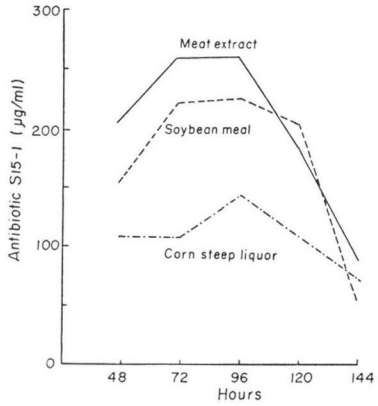
each medium in 500-ml shaking flasks was sterilized at 120°C for 20 minutes. Five mililiters of seed broth (48-hour shaken cultures in the same medium) was added to each flask. Inoculated media were incubated at 27°C on a reciprocal shaker at 115 stokes per minutes. Antibiotic

Table 5. Summary of production of antibiotic S15-1 by all mutant substrains obtained by ultraviolet irradiation.*

Irradiation time (sec.)	Number of mutant substrains				
	0~500 μg/ml	501~1,000 μg/ml	1,001~1,500 μg/ml	1,501~2,000 μg/ml	2,001~ μg/ml
60	680	32	18	7	2
90	490	0	1	0	0
120	550	1	0	1	0

* Medium: starch 3.0%, soybean meal 2.0%, $NaNO_3$ 0.4%, K_2HPO_4 0.2%, KCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, and $FeSO_4 \cdot 7H_2O$ 0.002% (pH 7.0).
Fermentation conditions: 26.5°C, 6 days, reciprocal shaker 115 strokes per minute; 100 ml of medium in 500-ml flask; inoculum size, 5.0% of seed broth.
Bioassay using *Bacillus subtilis* strain PCI 219.

Fig. 3 Antibiotic S15-1 production with various organic nitrogen sources.



Basal medium: soluble starch 3.0%, $NaNO_3$ 0.4%, K_2HPO_4 0.2%, KCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, and $FeSO_4 \cdot 7H_2O$ 0.002%.

Organic nitrogen sources: Two percent of meat extract, soybean meal or corn steep liquor.

Fermentation conditions: 26.5°C, reciprocal shaker 115 strokes per minute; 100 ml of medium (pH 7.0) in 500-ml flask; inoculum size, 5.0% of seed broth.

Bioassay using *Bacillus subtilis* strain PCI 219.

Table 6. Production of antibiotic S15-1 by mutant substrains giving high yields.*

Mutant substrains	Fermentation time			
	6 days		7 days	
	pH	S15-1 (μg/ml)	pH	S15-1 (μg/ml)
A-60-126	7.7	1,840	7.6	1,780
A-60-128	7.6	1,210	7.7	1,510
A-60-169	7.8	1,820	7.5	1,260
A-60-181	7.6	1,540	7.8	1,000
A-60-207	7.8	1,650	8.0	900
A-60-225	7.6	1,650	7.9	1,350
A-60-227	7.8	1,650	7.6	1,780
A-60-251	8.1	2,100	7.8	980
A-60-262	7.3	1,540	7.6	1,960
A-60-716	7.6	2,030	7.8	2,480
A-60-13	7.8	1,740	7.8	1,100
Control (Parent)	7.6	250	7.6	200

* Medium: starch 3.0%, soybean meal 2.0%, $NaNO_3$ 0.4%, K_2HPO_4 0.2%, KCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, and $FeSO_4 \cdot 7H_2O$ 0.002% (pH 7.0).

Fermentation conditions: 26.5°C, reciprocal shaker, 115, strokes per minute; 100 ml of medium in 500-ml flask; inoculum size, 5.0% of seed broth.

Bioassay using *Bacillus subtilis* strain PCI 219.

activity was found mainly in the broth filtrates. Samples for assay were taken at 4 days, filtered, and diluted 5-fold with saline solution. Assays were done with an agar diffusion method using *Bacillus subtilis* PCI 219 as the test organism. The results are shown in Table 4. Soluble starch was found to be superior to dextrin and glucose as a carbon source. Meat extract, soybean meal, and corn steep liquor were found superior to other organic nitrogen sources tested. The addition of 2% of each nitrogen source was the most suitable concentration for production of antibiotic S15-1. Antibiotic S15-1 was not produced in media in which organic nitrogen sources were omitted.

The time course of production of antibiotic S15-1 was investigated as follows: One hundred mililiter amounts of medium were inoculated with 5 ml of seed broth (48-hour shaken culture with the same medium, incubated in 500-ml flasks at 26.5°C on a reciprocal shaker at 115 stokes per minutes), and incubated at 26.5°C on a reciprocal shaker at 115 stokes per minutes. Samples for assay were diluted appropriately with saline solution. Assays were done with the *Bacillus subtilis* PCI 219 agar diffusion method as before. The amounts of antibiotic produced was determined by comparison of zone diameters with a standard curve obtained with pure antibiotic S15-1. The results are shown in Fig. 3. Maximum production of antibiotic S15-1 occurred after 72~96 hours incubation. The most suitable organic nitrogen source was meat extract. The use of corn steep liquor as an organic nitrogen source did not give as uniform a production rate of antibiotic S15-1 as with other materials (Table 4 and Fig. 3).

Mutant substrains which possessed the capacity for high productivity of antibiotic S15-1 were induced by ultraviolet irradiation of the original strain. Almost all (98 %) of the mutants obtained by ultraviolet irradiation produced antibiotic S15-1 at the same level as the original strain (Table 5). Eleven mutant substrains which possessed the capacity for high productivity of antibiotic S15-1 were tested in the same medium and under the same conditions mentioned above. The results are shown in Table 6. Mutant substrain No. A-60-716 produced 2,480 μg of antibiotic S15-1 per ml, the maximum yield of all mutants tested.

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