PRODUCTION OF CINERUBINS BY A *STREPTOMYCES GRISEORUBIGINOSUS* STRAIN

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Streptomyces No. 4915 was isolated and revealed to produce cinerubins A and B. This strain was different from other cinerubins-producing strains. Production of cinerubins is reported. Assignments of the signals of the ^{13}C-NMR spectrum of cinerubin A, the major product, have been made.

A new cinerubins-producing strain, No. 4915, was isolated from French soil and found to differ from the other known cinerubins-producing species. It was identified as a strain of *Streptomyces griseorubiginosus*.

This report deals with taxonomic studies of the organism, and production, isolation and purification of cinerubins.

Taxonomic Studies

Strain No. 4915 exhibits good sporulation and formation of diffusible pigment with all studied media. Morphological characteristics were observed with both optical and electron microscopes (Figs. 1 and 2). Aerial mycelium bears short, straight and thick spore chains. The spore surface is smooth; the same chain exhibits both oval and cylindrical spores, varying in their diameter.

Table 1 gives the cultural characteristics of strain No. 4915. The experiments to determine them were carried out at 27°C for 10~15 days. Media were prepared according to recommendations in

Fig. 1. Aerial mycelium of No. 4915 strain (×400).

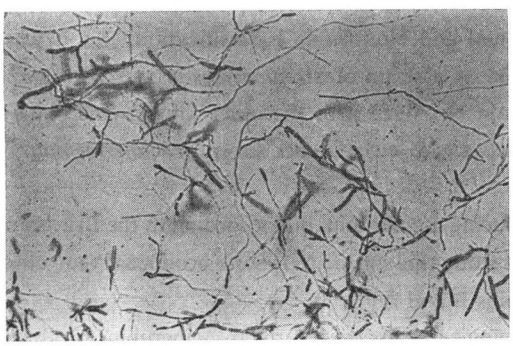


Fig. 2. Electronmicrograph of the spores of strain No. 4915 (×3,000).

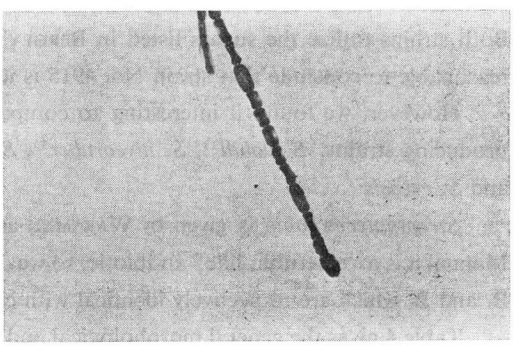


Table 1. Cultural characteristics of strain No. 4915.

Medium	Growth and color of vegetative mycelium	Aerial mycelium	Soluble pigment
CZAPEK's agar	very good brown violet	rosy reddish	pinkish brown
EMERSON agar	very good	pinkish gray	brown
BENNETT agar	very good brown	thick pinkish purple	brown
Yeast-malt agar	very good dark brown	thick pinkish gray	dark brown
Oatmeal agar	good brown	thin grayish	pale brown
Potato corn-steep glucose	very good red-brown	thin reddish purple	brown

Table 2. Physiological properties of strain No. 4915.

Temperature for growth	21°~37°C
Temperature optima	27°C
pH range for growth	6~8.5
Melanin formation	+
H_2S production	+
Tyrosinase reaction	+
Nitrate reduction	+
Liquefaction of gelatin	+
Coagulation of milk	+
Peptonization of milk	+
Cellulose decomposition	−

Table 3. Utilization of carbon sources by strain No. 4915.

Carbon source	Growth
Sucrose, D-Glucose, L-Arabinose, Raffinose, D-Mannitol, L-Rhamnose, D-Galactose, D-Fructose.	very good
Sorbitol, Lactose, Salicine, DL-Inositol, Starch, D-Xylose, Na-Succinate.	moderate
Na-Citrate	none

WAKSMAN[1] and SHIRLING & GOTTLIEB[2]. Melanin formation was determined on agar slants of ISP-6 and -7 media[2].

Carbon utilization tests were performed with ISP-9 medium. The physiological properties and utilization of carbon sources of strain No. 4915 are summarized in Tables 2 and 3, respectively.

As the result of our observations, the following characteristics were used to key out strain No. 4915: aerial mycelium is gray; melanin production is positive; color of substrate mycelium is brown; spore chains belong to section *rectiflexibilis* type; surface of spores is smooth.

A comparison of strain No. 4915 with *Streptomyces* species described in BERGEY's Manual[3] and NONOMURA's classification[4] shows that the species *S. griseorubiginosus* resembles our organism with respect to the gray mass color, spore chains belonging to section *rectiflexibilis* type with smooth surfaced spores, color of substrate mycelium, presence of diffusible pigment and production of melanin. Both strains utilize the sugars listed in BERGEY's Manual and NONOMURA's classification. So it was reasonable to conclude that strain No. 4915 is identified as a strain of *Streptomyces griseorubiginosus*.

However, we found it interesting to compare strain No. 4915 with the other known cinerubins-producing strains, *S. bobili*[1], *S. niveoruber*[5], *S. galilaeus*[5], *S. cinereoruber* subsp. *fructofermentans*[6] and *S. ryensis*[7].

Streptomyces bobili is given by WAKSMAN as producing a cinerubin, but according to the BERGEY's Manual it is a "cinerubin-like" antibiotic, so we did not study this strain. *S. ryensis* produces ryemycins B_1 and B_2 which are respectively identical with cinerubins B and A[7,8].

Table 4 gives the general morphological and physiological properties of the five cinerubins-produc-

Table 4. Morphological and physiological properties of strains included in the study.

	Aerial mass color	Melanoid pigment	Reverse side pigment	Soluble pigment	Spore chain	Spore surface	Arabinose	Xylose	Inositol	Mannitol	Fructose	Rhamnose	Sucrose	Raffinose
S. cinereoruber subsp. *fructofermentans*	GyR	1	1	0	RF	sm	+	+	−	−	+	+	−	−
S. niveoruber	R	0			S	sm	+	+				+		
S. galilaeus	Gy	1	0	0	S	sm	+	+	+	+	+	−	−	−
S. ryensis	R	1	1	0	S	sm	−	−	+	+		+	+	+
S. griseorubiginosus	Gy	1	1	1	RF	sm	+	+	+	+	+	+	+	+
Strain No. 4915	Gy	1	1	1	RF	sm	+	±	±	+	+	+	+	+

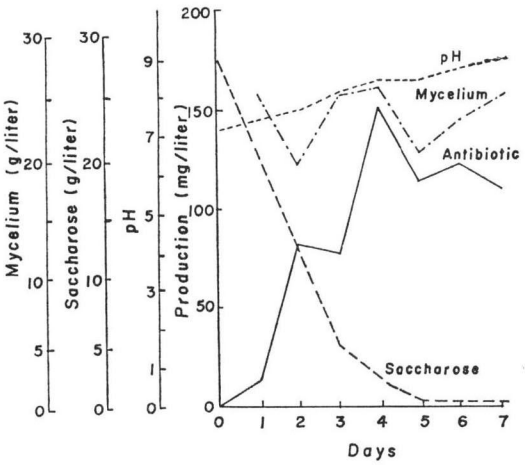
Symbols. Gy: gray series; R: red series; 1 or 0: presence or absence; RF: *Rectiflexibilis*; S: *Spirales*; sm: smooth.
Carbon utilization: +: positive; −: negative; ±: doubtful.

ing strains and of strain *S. griseorubiginosus* according to NONOMURA's classification.

Streptomyces niveoruber, S. galilaeus and *S. ryensis* differ from our organism with respect to morphological characters. *Streptomyces cinereoruber* subsp. *fructofermentans* has similarities but it produces no soluble pigment and does not utilize inositol, mannitol, sucrose, or raffinose.

Strain No. 4915 differs significantly from the other cinerubins-producing species. In our opinion strain No. 4915 is a new cinerubin-producing strain similar to *Streptomyces griseorubiginosus*.

Fig. 3. Changes occuring during fermentation with strain No. 4915 in sucrose, corn-steep medium.



Production of Cinerubins

Several media were used for the fermentation with strain No. 4915 including those recommended for *S. cinereoruber* subsp. *fructofermentans*, strain ETH 6143[9]. The media containing soyameal and mannitol for cinerubin A and soyameal and glycerin for cinerubin B gave only traces of cinerubins with strain No. 4915. On the other hand, when we inoculated strain ETH 6143 into the most appropriate medium for strain No. 4915 no cinerubin at all was obtained. This confirms the fact that the two strains differ biochemically.

For strain No. 4915 the best sources of carbon and nitrogen were saccharose and corn-steep, and we improved remarkably the production of cinerubins by adding K_2HPO_4.

Strains No. 4915 was maintained as frozen mycelia. A stock culture was inoculated into 100 ml seed medium composed of glucose 3%, soyabean flour 0.5%, corn-steep solids 0.5% and $CaCO_3$ 0.5% (pH adjusted to 7.2 before sterilization).

Table 5. ^{13}C NMR chemical shifts[a] superscript of cinerubin A.

Carbon No.		Carbon No.		Carbon No.		Carbon No.	
1	157.6	9	71.7	1′	101.5	5″	66.9
2	129.7	10	57.1	2′	33.5	6″	17
3	130.0	10a	142.4	3′	61.6	1‴	99.3
4	158.4	11	120.3	4′	73.8	2‴	34.0
4a	112.0	11a	131.4	5′	68.4	3‴	27.7
5	190.1	12	185.3	6′	17.9	4‴	210.1
5a	114.5	12a	112.2	$N(CH_3)_2$	42.8	5‴	70.7
6	162.2	13	32.1	1″	100.0	6‴	14.8
6a	132.5	14	6.7	2″	33.5		
7	71.6	15	171.1	3″	65.2		
8	34.1	16	52.5	4″	82.6		

a. In parts per million down field from Me_4Si.

After 24 hours of incubation at 27°C on a rotary shaker, a 2-liter fermentor containing 1 liter of the same medium was inoculated with 100 ml of the shaken culture and incubated 24 hours with agitation at 400 rpm and aeration at 0.5 v/v/min. The resultant culture was transferred to 15 liters of the following medium: sucrose 3%, corn-steep solids 1.5%, NaCl 0.2%, $FeSO_4 \cdot 7H_2O$ 0.01%, $ZnSO_4 \cdot 7H_2O$ 0.01%, K_2HPO_4 0.075% (pH adjusted to 7.2 before sterilization).

Fermentations were run at 27°C with agitation for 3 or 4 days. The level of dissolved O_2 was maintained at 90%. Results obtained during a typical fermentation are shown in Fig. 3.

Isolation and Purification of Cinerubins

Cinerubins were extracted from the mycelial cake obtained by continuous centrifugation. Extraction was carried out with methylene chloride. The extract was concentrated *in vacuo* and the antibiotics precipitated from the concentrated solvent with hexane. A first separation of the crude precipitate was accomplished by column chromatography on silicagel 60 Merck (70~230 mesh ASTM) with the solvent system CH_2Cl_2 - acetone - hexane - CH_3OH (30: 10: 2: 2).

Further purification was performed by preparative thin-layer chromatography on silicagel 60 $PF_{254+366}$ Merck with the same solvent. Identification of cinerubins A and B was made by comparison of their ^1H NMR spectra with the published spectra[10,11].

The ^{13}C NMR spectrum of cinerubin A (given in Table 5) can be useful for identification. The assignments of the carbon have been made using off resonance decoupling and comparison of the spectrum with known compounds of the same family[12]. It can be noted that cinerubin A has the same aglycone as marcellomycin and the same carbohydrate sequence as aclacinomycin A[12].

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