STUDIES ON NEW PHOSPHONIC ACID ANTIBIOTICS

II. TAXONOMIC STUDIES ON PRODUCING ORGANISMS OF THE PHOSPHONIC ACID AND RELATED COMPOUNDS

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A new species of *Streptomyces* which produces a new cell wall-inhibitory antibiotic, FR-900098[1] containing phosphonic acid in its molecule, is named and described. The species name proposed, *Streptomyces rubellomurinus*, refers to the aerial mass color. *Streptomyces rubellomurinus* subsp. *indigoferus* also produces FR-900098 and the related compound FR-33289[2]. FR-900098 related compounds, FR-32863[2] and FR-31564[2] are produced by *Streptomyces lavendulae*.

In the course of screening for new antibiotics, an interesting cell wall-inhibitory antibiotic, FR-900098 containing phosphonic acid in its molecule, was obtained from culture broth of strain No. 5818 which was newly isolated from a soil sample collected at Mt. Hira, Shiga Prefecture, Japan. Another strain No. 24 was isolated from a different soil sample collected at Koganei City, Tokyo Prefecture, Japan and was found to produce FR-900098 and the related compound, FR-33289. Furthermore, a new isolate, No. 8006 was also found to produce two new phosphonic acids FR-32863 and FR-31564. Strain No. 8006 was isolated from a soil sample collected at Fukue City, Nagasaki Prefecture, Japan. The three organisms are believed to belong to the genus *Streptomyces*, and in this report they are characterized, identified, and named.

Materials and Methods

Isolation of producing organisms

The organisms were selected from a series of soil isolations that were plated at dilutions of $1:10^2$ to $1:10^4$ on media for isolation. Unsterilized Kabicidin (Daigoeiyo Co., Osaka) at 5 mg/liter was added to the melted agar before dilution plates were poured. Inoculated plates were incubated at 28°C for 7 days.

Method

The method and media recommended by the International Streptomyces Project (ISP)[3] were used primarily, along with several supplementary tests. Stock slant cultures were maintained on BENNETT agar. These slants, as well as subsequent cultures used in this study, were incubated at 28°C.

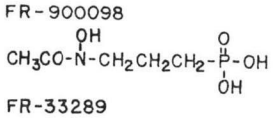
Microscopic observations were made on cultures that were grown for 7~21 days on sucrose-nitrate, ISP No. 5 (glycerin-asparagine), ISP No. 4 (starch-inorganic salts), ISP No. 2 (yeast-malt extract) and ISP No. 3 (oatmeal) agar media. Sporophore morphology was observed on undisturbed plates cultures and carbon shadowing technique was used to obtain electron micrograph.

Colony characteristics were observed on slant cultures using 9 kinds of media after 7 and 21 days of incubation. The formula for BENNETT, glucose-asparagine, sucrose-nitrate and nutrient agars are those in WAKSMAN's book[4]. Temperature requirement for the growth was determined on BENNETT

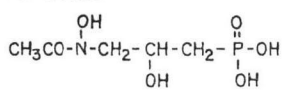
Table 1. Products by strain No. 5818, No. 24 and No. 8006.

Strain No.	No. 5818	No. 24	No. 8006
Products	FR-900098	FR-900098	FR-32863
		FR-33289	FR-31564

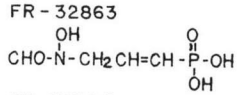
FR-900098



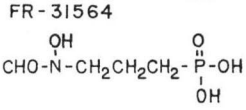
FR-33289



FR-32863



FR-31564



agar slants using temperature gradient incubator (Toyo Kagaku Sangyo Co., Ltd.). Gelatin liquefaction was examined at 21 days on a medium composed of 20% gelatin, 2% glucose and 0.5% peptone. The medium was refrigerated after incubation to detect liquefaction. Starch hydrolysis was observed by the starch-iodine reaction after incubation on ISP No. 4 agar plate for 10 days. Melanin production was determined on agar slants of ISP No. 6 (peptone-yeast iron) and ISP No. 7 (tyrosine), and also other organic media, especially ISP No. 1 (tryptone-yeast extract) broth. Carbon utilization tests were made according to PRIDHAM-GOTTLIEB method[3].

The procedure of BECKER *et al.*[5] was used for preparation of cells and chromatographic detection of the isomers of diaminopimelic acid.

Results and Discussion

Morphology

The aerial mycelia of strains No. 5818 and No. 24, which are monopodially branched, are usually long and straight. The spore chain morphology of these strains is classified in the Rectiflexibiles Section. Mature spore chains are moderately long, with usually 10 to more than 50 spores per chain (Fig. 1). Spores are oblong to cylindrical, averaging 0.4~0.8 by 1.1~1.6 μm in size, with smooth spore surface (Fig. 2). Neither fragmentation nor formation of spores occurred in the vegetative mycelium.

Strain No. 8006 produces a vegetative mycelium, which does not fragment into spores, and an aerial mycelium, which later forms spore chains. Sporophores are compact spirals and hooks being classified in the Retinaculiaperti Section. (Fig. 3). Spores are ellipsoidal to cylindrical, averaging 0.5~1.2 by 1.4~2.0 μm in size. Spore surface of strain No. 8006 is smooth to slightly rough (Fig. 4).

Cultural and Physiological Characteristics

The cultural characteristics and summarized physiological properties are shown in Tables 2 and 3, respectively. On the most media, strains No. 5818 and No. 24 develop pale yellow vegetative growth moderately and the aerial mycelia are cottony and pinkish gray. No soluble pigment is formed on the media. However, the reverse color of colonies of strain No. 24 changes from pale yellow to indigo color on media containing yeast extract, but it is not pH indicator.

Aerial mass color of strain No. 8006 is pinkish gray and that of vegetative growth is pale yellow or colorless. This strain belongs to the so-called chromogenic group. Pigment other than melanoid is not formed. The three strains hydrolyzed starch well, and the hydrolytic activities on gelatin or milk are weak.

Fig. 1. Sporophore morphology of strain No. 5818 grown on ISP No. 4: optical micrograph (×200).

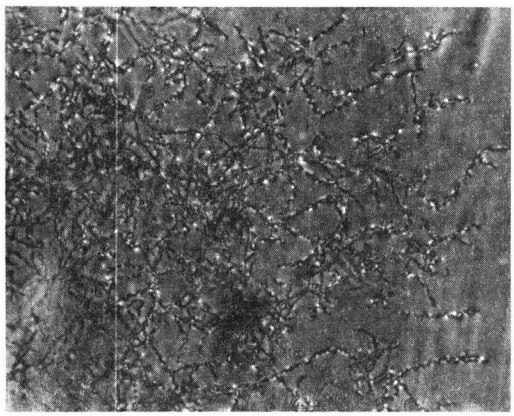


Fig. 2. Spores of strain No. 5818 grown on starch-inorganic salts agar, electron micrograph (×7,000).

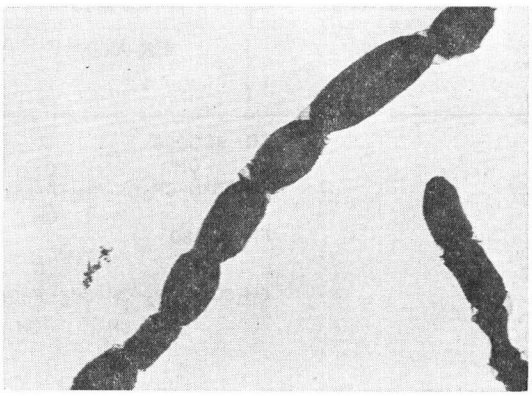


Fig. 3. Sporophore morphology of strain No. 8006 grown on sucrose-nitrate agar: optical micrograph (×400).

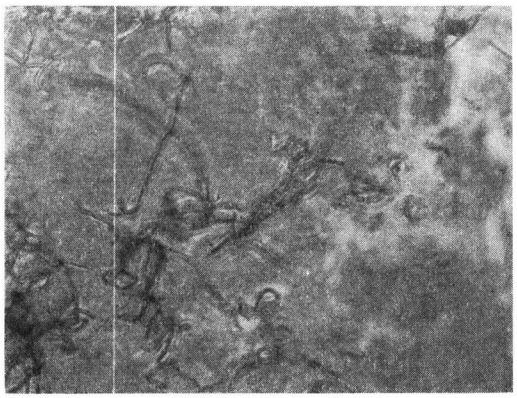
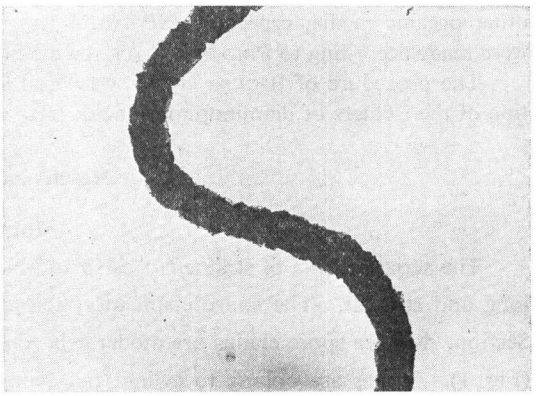


Fig. 4. Spores of strain No. 8006 grown on starch-inorganic salts agar, electron micrograph (×7,000).



Strains No. 5818 and No. 24 utilize L-arabinose and D-glucose easily, and usually utilize D-xylose, D-fructose, galactose, maltose and glycerin. D-Glucose, mannose, salicin, maltose and glycerin are utilized for growth by strain No. 8006.

Whole cell hydrolysates of the three strains contain LL-diaminopimelic acid.

Identification of the Three Strains

Microscopic studies and cell wall pattern indicated that strains No. 5818, No. 24 and No. 8006 belong to the genus *Streptomyces*. Accordingly, comparisons of these strains were made with the published descriptions[6~10] of *Streptomyces* species. From the above-mentioned information, strains No. 5818 and No. 24 are considered to be closely related to *Streptomyces sindenensis*, *Streptomyces xanthocidicus* and *Streptomyces exfoliatus*. However, they can be differentiated as described as follows:

Streptomyces sindenensis: Mature spore chains are generally short. On ISP No. 4 agar, sporulating aerial mycelium is poorly developed. D-Mannitol is utilized for growth.

Streptomyces xanthocidicus: On ISP No. 5 and ISP No. 2 agar, sporulating aerial mycelium is abundant. Sucrose and raffinose are utilized for growth. Melanin production is variable.

Streptomyces exfoliatus: On ISP No. 5 agar, aerial mycelium is formed and sporulation is good on ISP No. 2 agar.

Table 2. Cultural characteristics of strain No. 5818, No. 24 and No. 8006.

Medium	Characteristics		
	No. 5818	No. 24	No. 8006
Sucrose-nitrate agar	AM: very thin, white VG: colorless, small colonies SP: none	AM: white to gray, very thin powdery VG: colorless, small colonies SP: none	AM: thin, white, short cottony VG: colorless, small colonies SP: none
Glucose-asparagine agar	AM: pinkish gray, short cottony VG: pale yellow, small colonies SP: none	AM: pinkish gray, short cottony VG: pale yellow, small colonies SP: none or trace of yellow	AM: white, short cottony VG: colorless to pale yellow, small colonies SP: none
Glycerin-asparagine agar (ISP No. 5)	AM: none VG: scant growth SP: none	AM: none VG: scant growth SP: none	AM: pinkish gray, short cottony VG: colorless to cream colored, small colonies SP: none
Starch-inorganic salts agar (ISP No. 4)	AM: gray to pinkish gray short cottony VG: pale yellow, abundant SP: none	AM: mouse gray to pinkish gray, short cottony VG: pale yellow, abundant SP: none or trace of yellow	AM: pinkish gray, short cottony VG: pale yellow, small colonies SP: none
Tyrosine agar	AM: none VG: scant growth SP: none	AM: none VG: scant growth SP: none	AM: thin, white powdery VG: colorless, small colonies SP: none
Nutrient agar	AM: none VG: scant growth SP: none	AM: none VG: scant growth SP: none	AM: none VG: cream colored, wrinkled colonies SP: faint brown
Yeast-malt extract agar (ISP No. 2)	AM: white to pink, short cottony VG: pale yellow, small colonies SP: none	AM: white, thin powdery VG: pale yellow to pale yellowish brown, wrinkled margin, indigo color SP: none	AM: pinkish gray, short cottony VG: yellowish brown, wrinkled colonies SP: trace
Oatmeal agar (ISP No. 3)	AM: pinkish gray, short cottony VG: pale yellow, small colonies SP: none	AM: pinkish gray, short cottony VG: pale yellow, small colonies SP: none	AM: pinkish gray, short cottony VG: pale yellow, small colonies SP: none
Glucose-peptone gelatin stab	AM: white to pink, short cottony VG: colorless SP: none	AM: white to pink, short cottony VG: colorless, faint growth SP: none	AM: white, powdery VG: yellowish brown, growth on surface SP: brown
Milk	AM: faint growth on surface VG: pale yellow SP: none	AM: white, very thin powdery VG: pale yellow, growth on surface ring SP: none	AM: none AM: cream colored growth on surface ring SP: none

(to be continued)

Table 2. (Continued)

Medium	Characteristics		
	No. 5818	No. 24	No. 8006
Peptone-yeast iron agar	AM: none VG: scant growth SP: none	AM: none VG: colorless to slightly indigo color, faint growth SP: none	AM: none VG: colorless, wrinkled colonies SP: brownish black

Symbols: AM, aerial mycelium; VG, vegetative growth; SP, soluble pigment.

Table 3. Physiological properties of strain No. 5818, No. 24 and No. 8006.

Property observed	Characteristics		
	No. 5818	No. 24	No. 8006
Action on milk	Coagulation followed peptonization	Coagulation followed weak peptonization	No coagulation, slow peptonization
Gelatin liquefaction	Not liquefied	Not liquefied	Not liquefied
Starch hydrolysis	Hydrolyzed	Hydrolyzed	Hydrolyzed
Melanin production	None	None	Produced on peptone-yeast-iron agar and tryptone-yeast extract broth, but not produced on tyrosine agar
Temperature requirement	Growth from 12° to 40°C Good sporulation at 27°C	Growth from 12° to 40°C Good sporulation at 27°C	Growth from 10° to 37°C Good sporulation at 26°C
Cell wall pattern	I (Containing LL-DAP)	I (Containing LL-DAP)	I (Containing LL-DAP)
Utilization of various carbon compounds			
L-Arabinose	++	++	−
D-Xylose	+	+	−
D-Glucose	++	++	+
D-Fructose	+	+	−
Sucrose	−	−	−
Inositol	−	−	−
L-Rhamnose	−	−	−
Raffinose	−	−	−
D-Mannitol	−	−	−
Mannose	−	−	+
Salicin	−	−	+
Galactose	+	+	±
Lactose	−	−	−
Maltose	+	+	+
Glycerin	+	+	+

Symbols: ++, good utilization; +, fair utilization; ±, doubtful utilization; −, no utilization.

As a result of the above comparisons, strains No. 5818 and No. 24 are considered new species of genus *Streptomyces*. The name *Streptomyces rubellomurinus* is proposed for strain No. 5818, referring to the distinctive pinkish gray aerial mass color. The type strain has been deposited in the American Type Culture Collection, Rockville, Md., as ATCC 31215.

Strain No. 24 closely resembles *Streptomyces rubellomurinus* No. 5818. However, this species was differentiated from No. 24 by the indigo color of the vegetative mycelium on media containing yeast extract. Strain No. 24 is considered a subspecies of *Streptomyces rubellomurinus*, and the name *Streptomyces rubellomurinus* subsp. *indigoferus* is proposed. This type strain is deposited as ATCC 31304.

The microbiological characteristics of strain No. 8006 are in good agreement with those of *Streptomyces lavendulae*, except for spore surface. Only difference observed between strain No. 8006 and *Streptomyces lavendulae* is in their spore morphology. This difference, however, does not seem to us sufficient to distinguish strain No. 8006 from *Streptomyces lavendulae*. And the strain is identified as *Streptomyces lavendulae*. *Streptomyces lavendulae* No. 8006 is deposited as ATCC 31279.

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References

1) OKUHARA, M.; Y. KURODA, T. GOTO, M. OKAMOTO, H. TERANO, M. KOHSAKA, H. AOKI & H. IMANAKA: Studies on new phosphonic acid antibiotics. I. FR-900098, isolation and characterization. J. Antibiotics 33: 13~17, 1980
2) OKUHARA, M.; Y. KURODA, T. GOTO, M. OKAMOTO, H. TERANO, M. KOHSAKA, H. AOKI & H. IMANAKA: Studies on new phosphonic acid antibiotics. III. Isolation and characterization of FR-31564, FR-32863 and FR-33289. J. Antibiotics 33: 24~28, 1980
3) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
4) WAKSMAN, S. A.: The Actinomycetes. Vol. 2. Classification, Identification and Descriptions of Genera and Species. The Williams and Wilkins Co., Baltimore, 1961
5) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
6) PRIDHAM, T. G. & H. D. TRESNER: BERGEY's Manual of Determinative Bacteriology, 8th ed., pp. 748~829, The Williams and Wilkins Co., Baltimore, 1974
7) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strains of *Streptomyces*. II. Species descriptions from first study. Int. J. Syst. Bacteriol. 18: 69~189, 1968
8) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type strains of *Streptomyces*. III. Additional species descriptions from first and second studies. Int. J. Syst. Bacteriol. 18: 279~392, 1969
9) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. IV. Species description from the second, third and fourth studies. Int. J. Syst. Bacteriol. 19: 391~512, 1969
10) SHIRLING, E. B. & D. GOTTLIEB: Cooperative description of type cultures of *Streptomyces*. V. Additional description. Int. J. Syst. Bacteriol. 22: 265~394, 1972