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Studies on Streptomycetes

Part I. *Streptomyces atratus* nov. sp.,* Producing New Antituberculous Antibiotics Rufomycin A and B

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Investigation was made on the mycological and antibacterial properties of a strain No. 46408 isolated from a soil sample collected in Shimoueda, Wakayama Prefecture. The strain No. 46408 was compared with similar strains, *St. hygroscopicus* and *St. halstedii*, and it was judged to belong to a new species and therefore named *Streptomyces atratus* nov. sp. Also a strain A-165-Z1, which produces ilamycin, was referred to on its mycological properties and assumed to resemble *St. atratus*. *St. atratus* was found to produce new antibiotics, rufomycin A and B, specially active against acid-fast bacteria.

INTRODUCTION

Among antibiotics produced by *streptomycetes*, a group consisting of anti-smegmatis factor[1], anti-phlei factor[2], actithiazic acid (acidomycin)[3], angustmycin[4], tubercidin[5], tubermycin[6], and bovinocidin[7] etc. have been known to be specially active against acid-fast bacteria.

But today streptomycin, dihydrostreptomycin, cycloserine, viomycin and kanamycin, etc. are used as antituberculotic and other antibiotics active against mycobacterium are not used practically because of their weak activity or uneffectiveness in vivo. On the other hand, strains resistant to isonicotinic acid hydrazide, streptomycin and kanamycin have come into existence, and so discovery of a new antituberculous agent active against the resistant strains has been hoped.

In the course of screening the antibiotics produced by *streptomycetes*, the authors isolated a *Streptomyces* strain No. 46408 (referred to as No. 46408 hereinafter) from a soil sample collected in 1957 in Shimoueda, Wakayama Prefecture.

The strain produces new antibiotics, rufomycin A and B, which are specially active against acid-fast bacteria and also against strains resistant to isonicotinic acid hydrozide, streptomycin and kanamycin. In the present paper are reported the mycological and antibacterial properties of No. 46408 and results of comparison of the strain with related strains. Recently, Umezawa et al. reported a strain A-165-Z1[8], which produces ilamycin active against acid-fast bacteria.

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* K. Nakazawa, et al., *Japanese patent* 1961-17398 (1961. 9. 26).

1) A. Kelner and H.E. Morton, *Proc. Soc. Exp. Biol. Med.*, **63**, 227 (1946).

2) N. Ouchi, *J. Antibiotics (Japan)*, **3**, 517 (1949~1950).

3) J.R. Schenck and A.F. DeRose, *Arch. Biochem. Biophys.*, **40**, 263 (1952). K. Ogata, et al., *Japanese patent*, 5799 (1953).

4) H. Yünsten, H. Yonehara and H. Ui, *J. Antibiotics (Japan)*, Ser. A., **7**, 113 (1954).

5) K. Anzai, G. Nakamura and S. Suzuki, *ibid.*, Ser. A., **10**, 201 (1954).

6) K. Isono, K. Anzai and S. Suzuki, *ibid.*, Ser. A., **13**, 133 (1960).

7) K. Anzai and S. Suzuki, *ibid.*, Ser. A., **13**, 133 (1960).

8) T. Takita, K. Ohi, Y. Okami and H. Umezawa, The 34th Annual Meeting of Japan Bacteriological Society, Yokohama, April 6, 1961.

EXPERIMENTAL AND RESULTS

A) Morphological and Cultural Characteristics.

The aerial mycelium of No. 46408 develops well and is powdery on glucose asparagine agar. Its color is at first white and changes to pale mouse gray or deep mouse gray and later to slate color. The sporophore forms mainly open loops or loops and rarely spirals. The spore is sphericall or ellipsoidal, $0.8\sim1.3\times1.2\sim2.0\ \mu$ and smooth on the surface (Fig. 1).

The cultural characteristics of the original strain are shown in Table I.

In the course of screening the strain with high

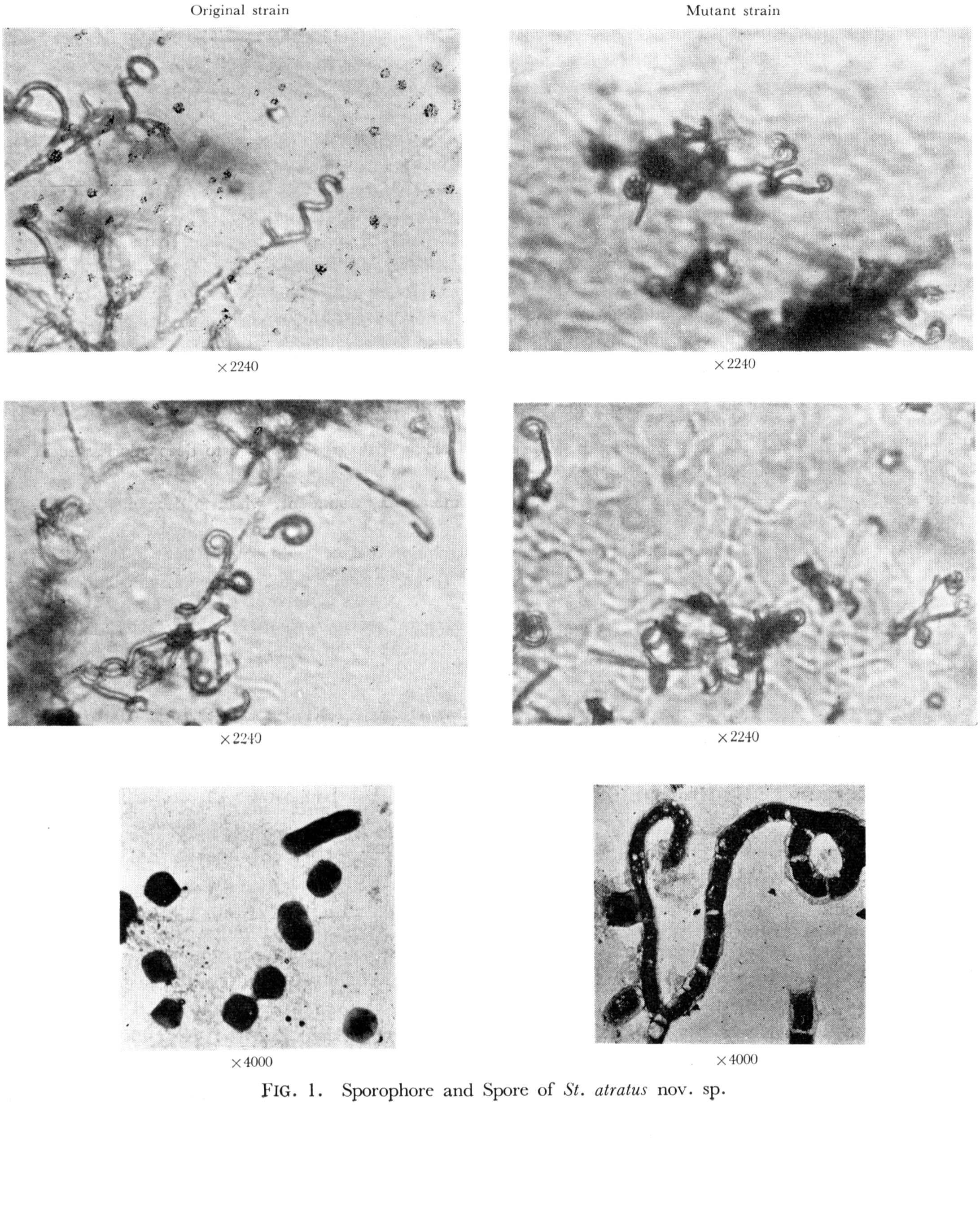

FIG. 1. Sporophore and Spore of *St. atratus* nov. sp.

TABLE I. CULTURAL CHARACTERISTICS OF *Streptomyces atratus* NOV. SP.

Vegetative growth:	Well-developed. Hyphae 0.5 to 0.8 micron in diameter. Colorless to deep mouse gray (Rdg*. LI, 15''''-i) and chamois (Rdg. XXX, 19''-b).
Aerial mycelium:	Powdery, white, later deep mouse gray to slate color (Rdg. LIII, carbon gray-k). Hyphae short tangled, branched, 1.5 to 1.8 micron in diameter. Sporophores produce many open loops and loops and rarely spirals with two to three turns.
Czapek's agar:	Growth (G) thin, spreading. Colorless to deep mouse gray. Aerial mycelium (AM) fairly abundant, powdery, deep mouse gray. Soluble pigment (SP) none.
Glucose Czapek's agar:	(G) abundant, colorless, later deep colonial buff (Rdg. XXX, 21''-b) to dark mouse gray. (AM) fairly abundant, powdery, white to deep mouse gray. (SP) none.
Glycerine Czapek's agar:	(G) abundant, colorless. (AM) Fairly abundant, white to mouse gray (Rdg. LI, 15''''). (SP) none.
Glucose asparagine agar:	(G) abundant, spreading, colorless, later Saccardo's olive (Rdg. XVI, 19'-m) to black. (AM) powdery, white, later deep mouse gray to slate color. (SP) none.
Yeast extract agar:	(G) abundant, wrinkled, colorless. (AM) very scant, white. (SP) none.
Nutrient agar:	(G) fairly abundant, wrinkled, colorless. (AM) none. (SP) none.
Glucose nutrient agar:	(G) abundant, wrinkled, colorless. (AM) abundant, white, later dark mouse gray to blackish mouse gray (Rdg. LI, 15''''-m) (SP) none.
Glycerine nutrient agar:	(G) abundant, folded, colorless to yellowish glaucous (Rdg. XLI, 25'''-f). (AM) scant, white to mouse gray. (SP) none to yellowish glaucous.
Nutrient broth:	(G) colorless, in the form of sediment. (AM) none. (SP) none.
Glucose nutrient broth:	Same as on nutrient broth.
Glycerine nutrient broth:	Same as on nutrient broth.
Cellulose:	No growth.
Egg medium:	(G) fairly abundant, wrinkled, colorless with greenish tinge to deep colorless buff (Rdg. XXX, 21''-b). (AM) scant, white. (SP) none.
Starch agar:	(G) abundant, colorless. (AM) moderately abundant, white to deep mouse gray. (SP) none.
Nitrate:	Reduction to nitrite on Czapek's solution and peptone solution with 0.2% $NaNO_3$.
Milk:	(G) colorless on the surface. (AM) none. (SP) none. Peptonization without coagulation.
Nutrient gelatin:	(G) colorless. (SP) none. Liquefaction.
Carrot plug:	(G) colorless to clay color (Rdg. XXIX, 17''). (AM) white to deep mouse gray. (SP) none.
Potato plug:	(G) abundant, folded, honey yellow (Rdg. XXX, 19'') to Orange Cinnamon (Rdg. XXIX, 13''). (AM) scant, white. (SP) none.
Starch hydrolysis:	Hydrolyzed zones per growth diameter are four to six.
Calcium malate agar:	(G) abundant, restricted, colorless to colonial buff (Rdg. LI, 15''''). (AM) scant, white to mouse gray. (SP) none.
Tyrosinate agar:	(G) thin, colorless. (AM) none. (SP) none.

* Rdg.; R. Ridgway, Color Standard and Color Nomenclature.

potency, a mutant was obtained by mono-spore culture. Cultural characteristics of the mutant are shown in Table II. Differing from the original strain, the aerial mycelium of the mutant is white to yellow or pale mouse gray with yellowish tinge and the reverse of its growth is yellow on most agar media, but its morphological and antibacterial properties are the same as those of the original strain (Fig. 1).

Carbon source utilizabilities of No. 46408 and the mutant were investigated by the method of Pridham et al.[9] The results are shown in Table III.

B) Comparison Between No. 46408 and Related Species. Among the known species of *Streptomyces,*

9) T.G. Pridham and D. Gottlieb, *J. Bacteriol.*, **56**, 107 (1948).

TABLE II. CULTURAL CHARACTERISTICS OF A MUTANT OF *Streptomyces atratus*

Vegetative growth:	Well developed. Hyphae 0.5 to 0.8 micron in diameter. Colorless to mustard yellow (Rdg*. XVI, 19′-b).
Aerial mycelium:	White, later cream color (Rdg. XVI, 19′-f) to pallid mouse gray (Rdg. LI, 15′′′′-f) with yellowish tinge. Sporophores produce many open loops and loops and rarely spirals.
Czapek's agar:	Growth (G) restricted, colorless to yellow. Aerial mycelium (AM) white, later cream color to pallid mouse gray. Soluble pigment (SP) none.
Glucose Czapek's agar:	(G) abundant, raised up, mustard yellow to primuline yellow (Rdg. XVI, 19′). (AM) fairly abundant, white, later cream color to pale olive gray (Rdg. LI, 23′′′′-f). (SP) none to pale yellow.
Glycerine Czapek's agar:	(G) abundant, colorless to primuline yellow. (AM) very scant, white, later cream color to pale mouse gray. (SP) none.
Glucose asparagine agar:	(G) restricted, primuline yellow to old gold (Rdg. XVI, 19′-i). (AM) white, later pale yellow to pale pinkish buff (Rdg. XXIX, 17″-f). (SP) none.
Nutrient agar:	(G) fairly abundant, restricted, colorless. (AM) none. (SP) none.
Glucose nutrient agar:	(G) abundant, restricted, wrinkled, colorless. (AM) scant, white to pale yellow. (SP) none.
Glycerine nutrient agar:	Same as on Glucose nutrient agar.
Nutrient broth:	(G) colorless, in the form of sediment. (AM) none. (SP) none.
Glucose nutrient broth:	Same as on Nutrient broth.
Glycerine nutrient broth:	Same as on Nutrient broth.
Cellulose:	No growth.
Egg medium:	(G) abundant, wrinkled, colorless to naple yellow (Rdg. XVI, 23′-d). (AM) none. (SP) none.
Starch agar:	(G) abundant, spreading, colorless. (AM) very scant, white. (SP) none.
Milk:	(G) colorless on the surface. (AM) none. (SP) none. No peptonization.
Nutrient gelatin:	(G) Colorless. (AM) none. (SP) none. Liquefaction.
Nitrate:	Reduction to nitrate on Czapek's solution and peptone solution with 0.2% $NaNO_3$.
Carrot plug:	No growth.
Potato plug:	(G) abundant, folded, tawny-olive (Rdg. XXIX, 17″-i). (AM) scant, white to pale yellow. (SP) none.
Starch hydrolysis:	Hydrolyzed zones per growth diameter are four to six.
Calcium malate agar:	(G) abundant, colorless to naples yellow (Rdg. XVI, 23′-d). (AM) fairly abunant, white later massicot yellow to pale smoke gray (Rdg. XLVI, 21′′′′-f). (SP) none.
Tyrosinate agar:	(G) restricted, colorless to old gold. (AM) very scant, white. (SP) none.

* Rdg.: R. Ridgway, Color Standard and Color Nomenclature.

St. hygroscopicus and *St. halstedii* were found to behave like No. 46408 on glucose asparagine agar. So No. 46408 was compared with *St. hygroscopicus* IFO 3148 and *St. halstedii* IFO 3199 and some differences in their cultural characteristics were found. The results are shown in Table IV.

The cultural and physiological properties of No. 46408 differ from those of *St. hygroscopicus* IFO 3148 on the following media; glucose asparagine agar, potato plug, carrot plug, calcium malate agar, tyrosinate agar, gelatin and Czapek's solution containing nitrate. And the properties of No. 46408 differ from those of *St. halstedii* IFO 3199 on the following media; egg medium, yeast extract agar, calcium malate agar, milk, and gelatin.

Differences of the properties of No. 46408 from those of the related species in comparative carbon source utilization test are shown in Table V.

Judging from the above mentioned characteristics, No. 46408 was assumed to belong to a new species and therefore, named *Streptomyces atratus* nov. sp. The culture has been deposited at the Institute for Fermentation, Osaka, and also at the American Type Culture Collection, Washington, D.C., U.S.A. under accession numbers of IFO-3897 and ATCC-14046, respectively.

The previously described strain A-165-Z1, which produces ilamycin, seems to resemble *St. atratus* in the morphological and cultural characteristics, but the report of the cultural properties of the former is incomplete, and therefore, it is impossible to compare the strain with *St. atratus* in detail.

TABLE III. UTILIZATION OF CARBON SOURCES

Carbon compound	Original strain	Mutant strain	Carbon compound	Original strain	Mutant strain
Glycerine	⧻	⧻	Dulcitol	−	±
Xylose	±∼+	⧺	Maltose	⧻	⧻
Arabinose	−	±	Lactose	⧺	⧺
Ribose	⧺	⧺	Saccharose	−	±∼+
Glucose	⧻	⧻	Cellobiose	⧻	⧻
Mannose	⧻	⧻	Raffinose	⧻	⧺
Rhamnose	+∼⧺	±	Dextrin	⧻	⧻
Galactose	⧻	⧻	Starch	⧻	⧻
Sorbose	−	±∼+	Inuline	−	−
Fructose	+	+	Salicin	⧻	⧻
Erythritol	−	−∼±	Na-acetate	⧺	⧺
Sorbitol	−	−	Na-citrate	⧺	⧺
Mannitol	−	−	Na-succinate	⧺	⧺
Inositol	−	−	Control	±	−
Adonitol	⧺	⧺			

⧻ heavy growth, ⧺ good growth, + fair growth, ± faint growth, − no growth.

The authors wish to compare *St. atratus* with the strain A-165-Z1[5], after publication of a detailed report on the cultural characteristics of the latter.

C) Antibiotic Activity. The antibacterial spectrum of No. 46408 was investigated by the cross streak method using bouillon agar and glycerine bouillon agar. The strain No. 46408 was streaked on agar plates and incubated at 28°C for four days. The plates were then cross-streaked with test organisms shown in Table VI and were further incubated at 37°C for twenty hours for Gram positive and negative bacteria, and for fourty-four hours for acid-fast bacteria. Finally the inhibition length of each test organism was measured. The results are shown in Table VI. No. 46408 inhibits the growth of only *B. subtilis* on the glycerine bouillon agar plate, and does not inhibit the growth of other test organisms. Then fermentation was run with the liquid medium with the following composition at 28°C for four days on a rotary shaker at 220 r.p.m.; 2.0% glucose, 0.6% Ehrlich meat extract, 0.5% polypeton, 0.3% rice bran, 0.5% sodium chloride, and 0.3% calcium carbonate (adjusted to pH 7.0). The culture filtrate and the

TABLE IV. DIFFERENTIATION OF CULTURAL CHARACTERISTICS OF *St. atratus* FROM THOSE OF RESEMBLING SPECIES

Medium	*St. atratus*	*St. halstedii*	*St. hygroscopicus*
Glucose asparagine agar	Growth colorless later black	Growth colorless later black	Growth colorless
Bouillon agar	Growth colorless	Growth colorless later brownish black	Growth colorless
Egg medium	Growth deep colonial buff	Growth colorless	No growth
Yeast extract agar	Growth colorless	Growth colorless later brownish black	Growth colorless
Potato plug	Growth honey yellow	Growth colorless to cream color	Growth colorless to cream color
Carrot plug	Color of plug no change	No growth	Color of plug dark gray
Cellulose	No growth	No growth	No growth
Milk	Strong peptonization without coagulation	Weak peptonization with coagulation	Fairly strong peptonization without coagulation
Gelatin	Strong liquefaction	Weak liquefaction	Weak liquefaction
Nitrate reduction	Positive	Positive	Negative
Calcium malate agar	Growth colorless to colonial buff	Growth colorless	Growth colorless
Tyrosinate agar	Growth colorless	Growth vinaceous buff	Growth vinaceous buff to yellow

TABLE V. DIFFERENTIATION OF CARBON SOURCE UTILIZATION OF *St. atratus* FROM THOSE OF RESEMBLING SPECIES

Carbon compd. \ Species	*St. atratus*	*St. halstedii*	*St. hygroscopicus*
Xylose	$\pm \sim +$	+++	+
Sorbose	−	−	+
Sorbitol	−	−	+++
Mannitol	−	−	+++
Rhamnose	$+ \sim ++$	−	−

TABLE VI. ANTIBACTERIAL SPECTRUM BY CROSS STREAK METHOD

Test organisms	Inhibition length	
	Bouillon agar (mm)	Glycerin bouillon agar (mm)
Escherichia coli	0	0
Proteus vulgaris	0	0
Pseudomonas aeruginosa	0	
Serratia marcescens	0	
Bacillus subtilis	0	13
Bacillus cereus	0	0
Staphylococcus aureus	0	0
Mycobacterium 607		0
Mycobacterium avium		0
〃 (SM*-resistant)		0

SM*=streptomycin.

solution obtained by extraction of the mycelium with methanol were active against only acid-fast bacteria and not against Gram positive and negative bacteria. This active substance showed the same activity on acidic, neutral and basic assay media and therefore, seems to be a so-called physiologicaly neutral substance.

SUMMARY

Investigation was made on the morphological and cultural properties and carbon source utilization of the strain No. 46408 isolated from a soil sample collected in Shimoueda, Wakayama Prefecture.

The strain was compared with related species, *St. hygroscopicus* and *St. halstedii,* on their cultural properties and carbon source utilization.

As a result, the strain No. 46408 was found to be a new species and named *St. atratus* nov. sp. Also a strain A-165-Z1 was referred to on its mycological properties and supposed to resemble *St. atratus.*

St. atratus produces an antibiotic, which is specially active against acid-fast bacteria and physiologically neutral.

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