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On the Isolation of Antibiotic Producing *Streptomyces* spp. from Soil

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抗生物質 生產 *Streptomyces*의 分離및 同定에 관하여

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ABSTRACT

Among the Streptomyces isolated from soil, two strains which have high antibacterial activity against various pathogenic bacteria are identified as *Streptomyces globosus* and *Streptomyces albus* subsp. according to I. S. P. Methods and Bergy's Manual of Determinative Bacteriology. Morphological and physiological characteristics of them on several media were observed.

Antibiotics from *S. albus* subsp. or *S. globosus* was identified as tetracycline or streptomycin-like substances respectively by the paper chromatographic behavior in eight solvent systems of V. Betina. And it was revealed that these antibiotic substances are stable to temperature and weak acid.

INTRODUCTION

Over 1,000 kinds of Streptomyces spp. have been discovered, which have been formed 50-60% of antibiotic producing organisms (Korzybski *et. al.*, 1967; Hans Zahner, 1972) and over the years as more and more antibiotics have been discovered, it has been found that most of them, especially these of medical importances, fall into the general category of secondary metabolites (W. Mass, 1972). On the other hand, Streptomyces are well known and widely studied fungilike bacteria playing very important role in clarifying ecological properties in soil, producing antiseptics, and exterminating plant germs.

For the studies on Streptomyces in Korea, a report on screening of them which produce antibiotics in Jeonrado soil has been described partially and Lee *et al.* (1976) has reported antibacterial activity of some microorganisms at the level of genus. And also identified 24 Streptomyces strains in 1974.

In this paper, the selection and classification of antibiotics producing *Streptomyces* spp. were carried in according to antimicrobial spectrum and physiological and morphological characteristics. And also the properties of antibiotics from these strains was determined using the paper chromatography.

MATERIALS AND METHODS

1. Organisms

Among the isolated 90 *Streptomyces* spp. from soil, two strains that display the

best antimicrobial activity were used in all these experiments.

2. Isolation of *Streptomyces*

Soils were collected from May, 1975 to November at the region of Gyeong'gi-do, Jeonra-do and Gyeongsang-do. Collected soils were diluted with dist. water into 10^3-10^4 times (Johnson *et al.*, 1960). A loopful inoculum was inoculated in Emerson's agar, glucose-asparagine agar and Lawrence (1950) selective medium, and these inoculated plates were incubated at 28-30°C for 5-7 days.

Isolated 90 strains stored in the 4°C room, using agar slant medium.

3. Identification of isolated *Streptomyces*

For the identification of two strains of *Streptomyces*, morphological and physiological characteristics of isolated *Streptomyces* were observed by the I.S.P. Methods (Methods for Characterization of *Streptomyces* species, 1966) and Bergy's Manual of Determinative Bateriology (1974). For studies of morphological characteristics, color of mature sporulated aerial mycelium (spores *en masse*), morphology of spore chain and reverse color of colony were determined on yeast extract-malt extract agar (Pridham *et al.*, 1956, 1957), inorganic salts-starch agar(Künster,1959), glycerol-asparagine agar (Pridham *et al.*, 1961), Czapek's sucrose agar after 14 and 21 day-incubation. And spore wall ornamentation was observed with Electron Microscope (JEM-T_7).

For physiologial tests, melanin pigment formation was studied on culture media, trypton-yeast extract broth and tyrosine agar slant (Shinobu's modification of Masumto's), after 3-4 day-incubation. Carbon utilization was investigated on Pridham and Gottlieb carbon utilization media with the comparison of the growth of strains (T.G. Pridham & D. Gottlieb, 1948). Likewise nitrate reduction on nitrate broth, soluble pigment production other than melanin pigment, Streptomycin-sensitivity and comparative growth between 28°C and 37°C-culture were tested for precised taxonomic studies.

4. Fermentation and extraction of antibiotics from two *Streptomyces* spp.

One loopful of spore suspension was transfered to 100ml portions of fermenting medium in 500ml flasks. Fermentation was achieved at 28°C for 150hrs on rotary shaker at 200rpm. Fermeting medium having the following composition; starch (2.5 %), soybean meal (1.5 %), $CaCO_3$ (0.3 %), K_2HPO_4 (0.2 %), $MgSO_4$ $7H_2O$ (0.1%), $CoCl_2 \cdot 6H_2O$(2.5×10^{-5}mg/ml), *p*H 7.0±0.2 was used for all experiments.

The harvested broth was filtered at *p*H 5.0 with filter aid, thereafter the filtrates was extracted as following; 50ml of acetone is added to 50 ml of the filtrate to precipitate the proteins and other compounds that might interfere in antibiotic activities in the filtrate. The mixture is heated to 50°C for 10 min. and cooled at room temperature. After removal of precipitation, the aqueous concentrate was extracted with ethyl acetate at *p*H 6.0. The solvent extract was washed with distilled water. After filtration, it was centrifuged at 3,000 rpm.

The extracted solution of harvested broth was used for measurement of antibiotic activity and paper chromatography (Z. Barath, P. Betina, and P. Nemec, 1964).

5. Antibiotic assay

Antibiotic activity was determined by the paper-disc plate method (W.W. Davis and T.R. Stout, 1971), using *Staphylococcus aureus* ATCC 6538p as the test organism. Varing the quantities of ethyl acetate solution of extracted antibiotics were applied to 7.0mm diameter discs of Whatman No. 1 filter paper and allowed to dry at room temperature for at least 1 hr. The discs were placed on nutrient agar plates which had been overlayed with soft agar (1.0%) containing nutrient broth and test organisms. The diameter of the zone of inhibition was recorded after 24hrs at 37°C.

6. Paper Chromatography

The antibiotics produced by two *Streptomyces* strains were first analyzed in the following four solvent systems;

I. Distilled water
II. n-Butanol saturated with water
III. Ethyl acetate saturated with water
IV. Benzene saturated with water

According to their R_f values in the above solvent system, antibiotics were divided into five classes with fourteen subclasses (V. Betina, 1963). And the following additional solvent systems were used for individual classes of antimicrobial compounds.

A. Methanol-water (40 : 60)
B. n-Propanol-water (40 : 60)
C. Methanol-3% ammonium chloride in water (70 : 30)
D. Methyl ethylketone- n-butanol-water (30 : 5 : 65).

Strips of the Whatman No. 1 paper (1×18cm) were used, the origin being 2.5cm from the lower end of the strips. For the development of the chromatograms 1 inch glass cylinders were used with 5 ml of the solvents. After applying the antibiotic substance to the origin and drying in air, the chromatographic strips were immersed to a depth of 1.5 cm in the solvent. Ascending development at 20±1°C, without preliminary saturation of chromatograms with vapours of the solvent, was used. The development was stopped when the solvent front reached a distance of 10cm from the origin. After drying the chromatograms in air, detection was carried out either bioautographically, using *Staphylococcus aureus* ATCC 6538p. 1M phosphate buffer (*p*H 7.9±0.1) was used as disc-dipping solution.

RESULTS AND DISCUSSION

1. Antimicrobial spectrum

Antimicroial spectrum of the two isolates of *Streptomyces* spp. against a variety of test microorganisms were observed by the cross-sreak test using nutrient agar and BBL agar mostly, blood agar for *Pneumoniae* and *Neisseria* sp. and 2% glucose-Sabouraud agar for fungi. Test organisms were cross streaked against each Streptomyces of 3-day incubation at 28°C on the media which were above, and these were incubated again at 37°C for 24 hrs. As shown Table 1, *S. globosus* and *S. albus* subsp. which were identified by the method of I.S.P. inhibit the growth of Gram positive, negative bacteria, *Mycobacterium* species, and fungi.

2. Identification of 2 strains of antibiotic producing *Streptomyces*

The microorganisms used in this experiment were identified as *Streptomyces albus* subsp. and *S. globosus* on the classification criteria. These were incubated on the various media at 28°C for 2 weeks

to investigate its growth characteristics.

On microscopic examination, the aerial hyphae are short branched in case of *S. globosus*, and long and wavy in *S. albus* subsp. The sporophores produce flexous and the structure of the spore surface was cylindrical smooth in *S. albus* subsp. by an electron micrscope. These are illustrated in plates 1 and 2.

In *S. globosus*, the aerial mycelium is ordinarily greenish gray and velvety or powdery, and soluble pigment was not produced, also didn't reduce nitrate into nitrite, but melanin pigment was not produced. In S. *albus* subsp. the aerial mycelium was ordinarily white or in some media it was grayish white and velvety. And in this strain, faint yellowish gray soluble pigment was produced on some media and could reduce nitrate into nitrite. But little melanin pigment was formed. Table 2 shows the cultural and physiological characteristics of *S. globosus* and *S. albus* subsp. on several media. The utilization of carbon source was tested according to the method of Pridham *et al.*, 1948. As shown in Table 3, the result shows that 2 strains of Streptomyces grew well in Mannitol and Dextrin while Raffinose and Rhamnose were not used

3. determination of produced antibiotics from *S. albus* subsp. and *S. globosus*.

In order to classify antibiotics from *S. albus* subsp. and *S. globosus*, systematic chromatographic analysis(V. Betina, 1963) were applied to our experiment although they were crude concentrates. An antibiotically active microorganisms, *S. globosus* and *S. albus* subsp. are cultivated in

Table 1. Antibacterial Spectrum of *S. globosus* and *S. albus* subsp.

Test organsms	Medium	*S. globosus*	*S. albus* subsp.
Escherichia coli ATCC 11105	N	- - +	± ± ±
Klebsiella pneumoniae ATCC 10031	B	+ + +	+ +
Salmonella typhi	B B L	+ + +	+ + +
Pseudomonas pyocyanea ATCC 10490	N	- - +	+
Staphylococcus aureus ATCC 6538p	N	- - +	+ + +
Staphylococcus epidermis ATCC 12223	N	+ + +	+ + +
Micrococcus flavus NCIB 8165	N	+	+ + +
Serratia marcescens	B B L	+ +	- - +
Neisseria gonorrhoeae	B	+	+
Bacillus subtilis	N	+	- +
Lactobacilllus leichmanni	B B L	+ +	+
Streptococcus faecalis CN 5869	N	+ + +	-
Streptococcus thermophillus	N	+	+ + +
Mycobacterium smegmatis ATCC 607	B	+ + +	+ +
Candida albicans YU 1200	G	+ +	+
Saccharomyces cerevisiae	G	+	+

*Media;
N: nutrient agar B: blood agar BBL: BBL agar G: 2% glucose Sabouraud agar
**Inhibition distance by the cross-streak test
+ + +: strong inhibition + +: moderate inhibition +: slightly inhibition

Table 2. Cultural Characteristics of *S. globosus* and *S. albus* subsp.
G; Growth A; Aerial mycelium S; Soluble pigment

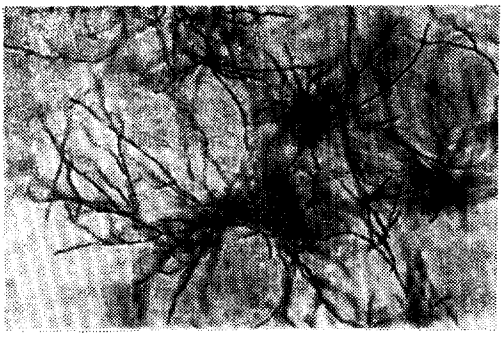
	Characteristics	
	S. globosus	*S. albus* subsp.
Emerson agar	A : olive gray	A : white
	G : moderate	G : moderate
	S : none	S : graish brown
Glucose asparagine agar	A : whitish gray	A : white
	G : moderate	G : good
	S : none	S : none
Yeast extract-Malt extract agar	A : greenish gray	A : greenish white
	G : good	G : excellent
	Melanin pigment: +	Melanin pigment: ±
Inorganic salts-starch agar	A : olive gray	A : white
	G : moderate	G : good
Glycerol arpargine agar	A : gray	A : greenish white
	G : poor	G : excellent
C'zapeck sucrose agar	A : yellowish gray	A : white
	G : excellent	G : poor
Nitrate solution	G : coloress	G : yellow pelletmass on surface
	S : none	S : brownish shadow
	Nitrite-production: −	Nitrite-production: +
Nutrient agar	A : greenish gray	A : yellowish white
	G : excellent	G : excellent
	Streptomycin sensitivity: +	Streptomycin sensitivity: +
Tyrosine agar	A : yellowish gray	A : white
	G : good	G : moderate
	Melanine pigment: +	Melanine pigment: −
28°C-culture	A : whitish gray	A : greenish white
	G : good	G : excellent
	S : none	S : graish shadow
37°C-culture	A : white	A : graish white
	G : poor	G : poor
	S : none	S : none

liquid medium at the stage of fermentation period including maximum antibiotic activity. Thereafter the mycelium is separated from the medium by filtration. The filtrates applied to paper chromatographic analysis and their solvent system and method were described in Materials and Methods previously. R_f values of them in systematic solvent system are indicated in Figure. It is speculated that antibiotics from *S. globosus* subsp. is tetracycline and *S. globosus* produces streptomycin-like substances. But antibiotics from *S. globosus* is particulary different from Streptomycin class antibiotics in solvent C. We can discuss that

Table 3. Utilization of carbon sources.

Sources	Utilization	
	S.globosus	*S.albus* subsp.
D-Glucose	++	++
Xylose	±	+
L-Arabinose	++	—
L-Rhamnose	—	—
D-Fructose	++	+
D-Galactose	+	+
Raffinose	—	—
D-Mannitol	++	++
i-Inocitol	—	—
Sucrose	—	+
Cellulose	—	—
Sodium citrate	—	+
Inulin	—	±
Dextrin	++	++

++ : good growth ± : doubtful growth
+ : fair growth — : no growth



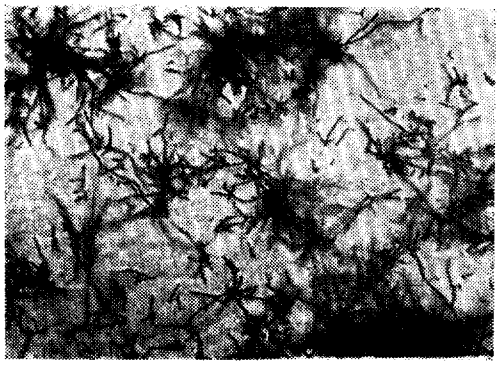


Plate 1. Sporophore chains(×600) of *S.globosus* on glycerol-aspragine agar, 21days. (up) Spore chains (×600) of *S. albus* subsp. on yeast extract-malt extract agar, 14 days.

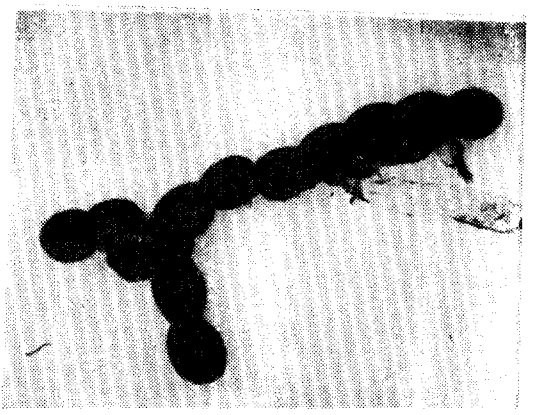


Plate 2. Spore surface ornamentation: electron micrographon on yeast extract—malt extract agar (×15,000). *S. globosus* (up), and *S. albus* subsp.

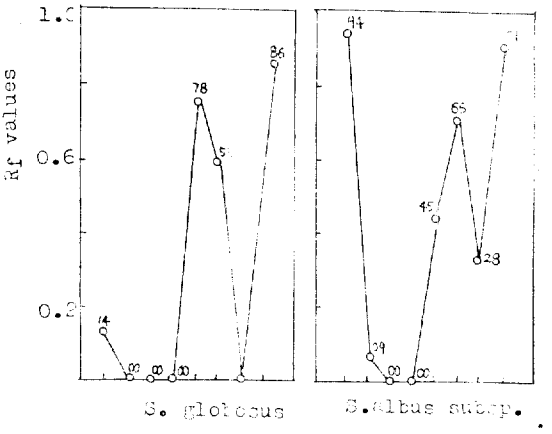


Figure Identification of antibiotics produced by *S. globosus* and *S. albus* subsp. using Paper-Chromatography.

antibiotics from *S. globosus* in unknow substance in Streptomycin class antibiotics, which is not mentioned by V. Betina (1963).

摘 要

우리나라 토양에서 분리한 90여 *Streptomyces* 균주 가운데 병원성균에 대해 역가가 높고 생리적 성질이 유사한 두 種을 I.S.P Methods에 의해서 *S. albus* subsp.와 *S. globosus*로 同定하고 여러가지 배지상에서 이들의 형태 생리적인 特性을 살펴보았다.

그리고 이들 두 *Streptomyces* 균주에서 나오는 항생물질을 동정하기 위하여 V. Betina가 고안한 paper chromatography pattern에 의거하여 실험하였던 바, *S. albus* subsp.는 tetracycline을 *S. globosus*는 streptomycin과 유사한 물질을 생성하는 것으로 나타났다. 또한 이들이 내는 항생물질은 약한 산성용액에서 안정을 유지하며 역가도 높게 나타났다.

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