韓 國 菌 學 會 誌 第 12 卷 第 3 號
Kor. J. Mycol. Vol. 12, No. 3, 85~92, 1984

韓國 土壤菌중 抗生物質 生成菌에 관한 硏究(第 2 報)

스트렙토마이세스屬 菌株 DMC-64 號의 分離 및 抗菌作用

金 禎 禹·崔 應 七·金 炳 珏
서울大學校 藥學大學 微生物藥品化學敎室

Studies on Antibiotic Producers of Korean Soil Microbes(II)

Isolation and Antibiotic Activity of *Streptomyces* Strain DMC-64

Jung Woo Kim, Eung Chil Choi and Byong Kak Kim
Department of Microbial Chemistry, College of Pharmacy, Seoul National University, Seoul 151, Korea

Abstract: To isolate antibiotic-producing microorganisms from Korean soils, microbes were isolated from the soil samples and screened for antibacterial activity. A strain which was isolated from the soil sample collected in Choong Chung Book Do had a high antibacterial activity against gram-positive bacteria. The examination of morphological and physiological characteristics of that strain according to the International Streptomyces Project methods showed that it was one of *Streptomyces* species. After the antibacterial constituent of the strain was produced in submerged culture method, it was isolated and purified by XAD-2 and CM-Sephadex column chromatography. And it was found to be one of quinone type antibiotics.

Keywords: *Streptomyces* strain DMC-64, Antibiotic activity, Microbial metabolite, Quinone.

Studies to find novel antibiotics as chemotherapeutics have been conducted by many investigators. These studies include isolation of antibiotic substances, determination of their structures and taxonomy of microbes.

In Korea, a number of reports on antibiotics obtained from the genus *Streptomyces* were published. Researchers reported the results of screening for antibiotic producing *Streptomyces* strains from Korean soil samples (Lee *et al.*, 1981) and reported the studies on two strains, *S. globus* and *S. albus* which produced antibiotics (Seo *et al.*, 1977). Also there were several papers on the taxonomy of *Streptomyces* species (Lee *et al.*, 1976; Cho *et al.*, 1977). Besides those studies, reports on antibiotics of Korean *Streptomyces* species were scarce.

To find antibiotic producing strains, screening of Korean soil microorganisms was attempted. During the course of the screening, a strain was isolated from the soil sample collected in Choong Chung Book Do, and designated as strain DMC-64. This strain had a high antimicrobial activity against gram positive bacteria and was identified as a *Streptomyces* species.

This paper deals with taxonomical studies on the strain DMC-64 and fermentation, purification and physicochemical properties of the antibiotic of DMC-64.

Materials and Methods

Screening Method

1) Soil Samples

Soil samples were collected in various locations in

Korea.

2) Media

a) Oat meal medium (oatmeal 20g, distilled water 1,000ml, agar 20g, chloramphenicol 4mg) was used for isolation of colonies.

b) Nutrient Agar medium (Nutrient broth 8g, agar 20g, distilled water 1,000ml) was used for the determination of in vitro antimicrobial activity.

3) Test Organisms

Escherichia coli, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Mycobacterium smegmatis* were obtained from College of Medicine, Seoul National University.

4) Isolation Method

One gram of soil sample was suspended with 10ml of sterilized water in sterilized test tubes. After the suspension was mixed, one ml of this suspension was diluted (1 : 10,000) and 0.1ml of this diluted solution was smeared on the oatmeal medium. After three day incubation at 27±1°C, the colonies of *Actinomycetes* were selected and tested for antimicrobial activity by paper disk agar diffusion method.

5) Determination of Antimicrobial Activity

Fifteen-hour culture broths of the five test organisms were diluted with the warm Nutrient Agar medium (1 : 200) and poured into Petri dishes. The culture filtrates and acetone extracts of four-day old mycelia of the *Actinomycetes* were absorbed into paper disks. These paper disks were placed on the agar plate and the antimicrobial activity was determined by measuring the diameters of the inhibition zone after 15-hour incubation.

Identification of Strain DMC-64

1) Isolation of Strain DMC-64

During the screening procedure, a strain which had a very strong antimicrobial activity against gram positive bacteria as well as *Mycobacterium smegmatis*, *Bacillus subtilis* and *Staphylococcus aureus* was isolated from the soil sample collected in Koe San County, Choong Chung Book Do. This strain was used in this experiment and named DMC-64.

2) Biochemical Studies

(1) Determination of Isomers of Diaminopimellic Acid (DAP)

Dried cells of strain DMC-64 (1mg) were mixed with one ml of 6N HCl in the sealed Pyrex tube and heated at 100°C for 18 hours. After cooling, it was filtered and washed with one ml of water. The filtrate was evaporated in a rotary evaporator at 40°C to eliminate HCl. The residue was dissolved with 0.3ml of water and five micrometer of this solution was spotted on a TLC plate coated with cellulose ("microcrystalline") (Staneck *et al.*, 1974). Commercial DAP mixture was used as a standard compound. The solvent system was MeOH : water : 10N HCl : pyridine (80 : 17.5 : 2.5 : 10) and the spray reagent was acetonic ninhydrine (0.1% wt/vol). The observation was carried out after two-minute heating at 100°C.

(2) Determination of Monosaccharides in Whole Cell Hydrolyzates

Dried cells of strain DMC-64 (50mg) were mixed with one ml of 2N H_2SO_4 in the sealed Pyrex tube and heated at 100°C for two hours. This hydrolyzate was neutralized (pH 5.0~5.5) with saturated Ba $(OH)_2$ solution and precipitated $BaSO_4$ was eliminated by centrifugation at 6,000rpm. The supernatant was evaporated in a rotary evaporator at 40°C. The residue was dissolved in 0.4ml of water and five microliters of this solution were spotted on the TLC plate coated with cellulose ("microcrystalline"). Galactose, arabinose and xylose were used as standard compounds. The solvent system was ethylacetate : pyridine : water (100 : 35 : 25) mixture and the spray reagent was acid aniline phthalate (two ml of aniline, 3.3g of phthalic acid, 100ml of n-butanol saturated with water). The observation was carried out after two-minute heating at 100°C.

3) Macroscopic Features

The color of aerial mycelium, substrate mycelium, pigment diffusing into the medium and the growth of strain DMC-64 were observed after three week incubation at 28±1°C on the ISP media and some glucose containing media. The compositions of the used media were as follows.

(a) Yeast extract-malt extract agar (ISP No. 2 medium): Yeast extract 4.0g, malt extract 10.0g, glucose 4.0g, agar 20.0g, distilled water one liter.

(b) Oat meal agar (ISP No. 3 medium): Oat meal

20g, agar 18g, distilled water one liter, trace salts solution 1.0ml($FeSO_4 \cdot 7H_2O$ 0.1g, $MnCl_2 \cdot 4H_2O$ 0.1g, $ZnSO_4 \cdot 7H_2O$ 0.1g, distilled water one liter)

(c) Inorganic salts-starch agar(ISP No. 4 medium): Soluble starch 10.0g, $(NH_4)_2SO_4$ 2.0g, K_2HPO_4 (anhydrous basis) 1.0g, $MgSO_4 \cdot 7H_2O$ 1.0g, $CaCO_3$ 2.0g, agar 12.0g, trace salts solution 1.0ml, distilled water one liter.

(d) Glycerol-asparagine agar (ISP No. 5 medium): Glycerol. 10.0g, L-asparagine(anhydrous basis) 1.0g, K_2HPO_4 1.0g, agar 12.0g, trace salts solution 1.0 ml, distilled water one liter.

(e) Glucose-asparagine agar (Krainsky's medium): Glucose 10g, asparagine 0.5g, K_2HPO_4 0.5g, agar 15g, distilled water one liter.

(f) Glucose-nitrate agar: Glucose 5.4g, $NaNO_3$ 1.5g, KH_2PO_4 1.0g, $MgSO_4 \cdot 7H_2O$ 0.5g, thiamine HCl 2ml of a 1,000ppm stock solution, distilled water one liter.

(g) Glucose-peptone agar: Glucose 10g, peptone 2g, $MgSO_4 \cdot 7H_2O$ 0.5g, KH_2PO_4 0.5, agar 15g, distilled water one liter.

4) Microscopic Features

Strain DMC-64 was inoculated on a yeast extract-malt extract agar medium layer on a slide glass which was suitable for microscopy. The aerial mycelium and aerial spore chains were observed continuously for three weeks by the light microscopy.

5) Physiological Tests

(1) Formation of Melanin Pigment

The formation of melanin pigment was observed two days after the inoculation of strain DMC-64 on the following medium: Glycerol 15.0g, L-tyrosine 0.5g, L-asparagine 1.0g, K_2HPO_4 0.5g, $MgSO_4 \cdot 7H_2O$ 0.5g, NaCl 0.5g, $FeSO_4 \cdot 7H_2O$ 0.01g, trace salts solution 1.0ml, agar 20.0g, distilled water one liter.

(2) Utilization of Carbon Sources

This was performed according to the procedure recommended by Shirling and Gottlieb (Shirling *et al.*, 1966; Pridham *et al.*, 1948). Sterilized ten carbon sources were added to already autoclaved carbon utilization medium and the strain DMC-64 was inoculated on the medium. After two-week incubation at 27±1°C, they were observed for the degree of the growth compared with the glucose-containing medium (positive standard) and carbon-free medium (negative standard).

a) Carbon Utilization Medium: $(NH_4)_2SO_4$ 2.64g, KH_2PO_4 2.38g, $K_2HPO_4 \cdot 3H_2O$ 5.56g, $MgSO_4 \cdot 7H_2O$ 1.00g, $CuSO_4 \cdot 5H_2O$ 6.40mg, $FeSO_4 \cdot 7H_2O$ 1.10mg, $MnCl_2 \cdot 4H_2O$ 7.90mg, $ZnSO_4 \cdot 7H_2O$ 1.50mg, agar 15g, distilled water one liter.

b) Used Carbon Sources: D-Glucose, D-xylose, L-arabinose, L-rhamnose, D-fructose, raffinose, D-mannitol, salicin, sucrose, i-inositol.

(3) Gelatin Hydrolysis

Strain DMC-64 was inoculated on the N.B. agar medium supplemented with 0.4% gelatin and incubated at 27±1°C for four days. The plate was flooded with gelatin-precipitating reagent (15% $HgCl_2$ in 20% concentrated HCl) and observed for existence of clear zones around colonies.

(4) Starch Hydrolysis

Strain DMC-64 was inoculated on the N.B. agar medium containing 0.2% soluble starch and incubated at 27±1°C for four days. Then the color changes were observed around the colonies after adding iodine solution.

Fermentation

1) Fermentation Procedure

A stock culture of strain DMC-64 was used to inoculate 100ml of the seed culture medium in a 500ml flask, and incubation was carried out at 27±1°C on a rotary shaker. A three-day culture (50ml) was transferred into 500ml of the production culture medium in a two liter flask and shake culture was carried for five days at 27±1°C. The composition of the seed and production medium was 2% oat meal medium.

2) Time Course of the Antibiotic Production by DMC-64

A stock culture of strain DMC-64 was inoculated into 100ml of the oat meal medium in a 500ml flask and incubated at 27±1°C on a rotary shaker. A three-day culture (10ml) was transferred into 100ml of oat meal medium in a 500ml flask and incubated. To determine antimicrobial activity, cell growth and pH change of the culture broth, culture filtrate was obtained from one flask per a day for seven days.

Antimicrobial activity was determined by disk method on Nutrient agar, using *Bacillus subtilis* ATCC 6633 as test organism and was expressed by the diameter of inhibition zones. Packed cell volume were determined by centrifugation at 400×g for 10 minutes.

Antimicrobial Activity

The determination of antimicrobial activity was performed according to the method already described in the screening method. Five-day culture filtrate was used to determine antimicrobial activity against ten test organisms and 15-hour culture broths of test organisms were diluted (1 : 200) with N.B. agar medium. Fifty microliters of culture filtrate and standard antibiotic solutions (chloramphenicol 8mg/ml, ampicillin 2mg/ml, gentamicin 0.2mg/ml) per a disk were used. The test organisms were as follows: *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 6538, *Streptococcus fragilis*, *Bacillus subtilis* ATCC 6633, *B. megaterium*, *B. licheniformis*, *Sarcina lutea*, *Mycobacterium smegmatis*, *Escherichia coli*, *Pseudomonas aeruginosa*.

Purification of the Antibiotic of Strain DMC-64

A five-day culture broth of strain DMC-64 was filtered through filter paper (Toyo Roshi Co., Ltd.). The culture filtrate was passed through a column of Amberite XAD-2(Rohm and Haas Co.), and the eluate was discarded. The column was successively washed with water (×10) and 10% methanol (×3) Then the absorbed antibiotic was eluted with 50% methanol. The active eluate was evaporated and 0.075 M acetate buffer solution (pH 4.0) was added to it. This was chromatographed on CM-Sephadex C-25 (Pharmacia Fine Chemicals) column(3.2×40cm) and the eluted 0.075M acetate buffer solution was gradiently changed to pH 6.0. The active eluate was collected and passed through the XAD-2 column. After elution by 50% methanol, the eluate was evaporated *in vacuo*, and chromatographed on the CM-Sephadex C-25(NH_4^+) column(3.2×40cm). The column was developed with 0.01N NH_4OH solution, and then the normality was increased gradiently to 0.1N. The active fraction was passed through XAD-2 column as the same procedure previously described. The evaporated residue was dissolved in a small amount of methanol and then precipitated with ether. The precipitate was collected and dried. The color of this compound was dark brown. The procedure is outlined in Figure 1.

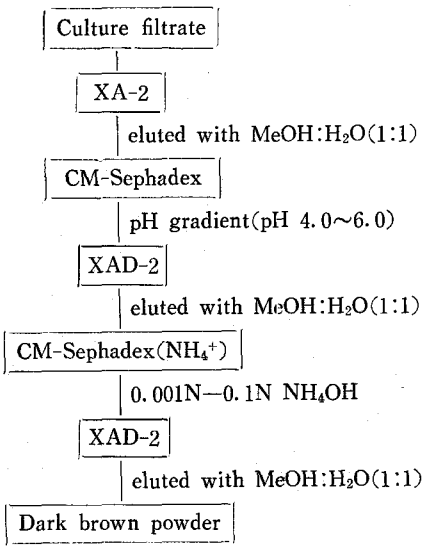


Fig. 1. Purification procedure.

Spectral Analysis

Physico-chemical properties of the purified antibiotic of DMC-64 were examined by spectroscopic methods. The conditions used for spectroscopy were as follows:

1) UV spectrum: Hitachi Model ESP-3T
   Reading Spectrometer
   (Solvent: distilled water)
2) IR spectrum: Beckman IR-20A
   (KBr disk)
3) NMR spectrum: Varian FT-80A
   (Solvent: DMSO, D_2O)

Results

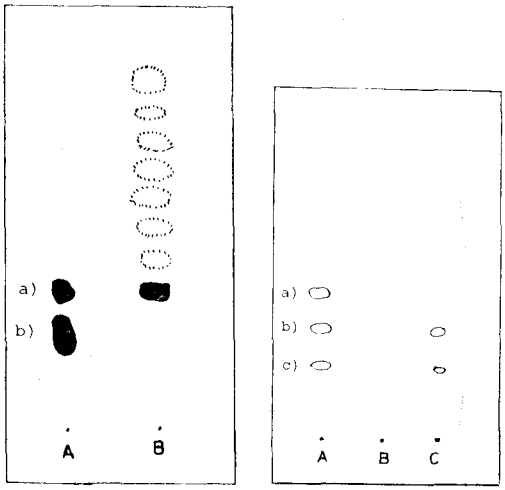
Identification of Strain DMC-64

1) Biochemical Studies

Fig. 2 and 3 showed that the whole cell hydrolyzates of strain DMC-64 contained L-DAP and no arabinose, galactose and xylose. This fact indicated that this strain was cell wall type I described in Bergey's Manual of Determinative Bacteriology.

2) Macroscopic Features

As shown in Table I, this strain grew very well



2 3

Fig. 2. Separation of DAP isomers by TLC. (A) standard DAP mixture, (B) hydrolyzates of strain DMC-64.
a) L-DAP b) meso-DAP.

Fig. 3. Separation of monosaccharides in whole cell hydrolyzates. (A) standard mixture, (B) strain DMC-64, (C) *Mycobacterium semegmatis.*
a) Xylose b) Arabinose c) Galactose.

on the glucose containing medium and ISP medium, and produced abundant aerial mycelia. The color of spore was gray, and the color of substrate mycelium and soluble pigment was yellow or brownish yellow.

3) Microscopic Features

Fig. 4 showed that the aerial mycelium including

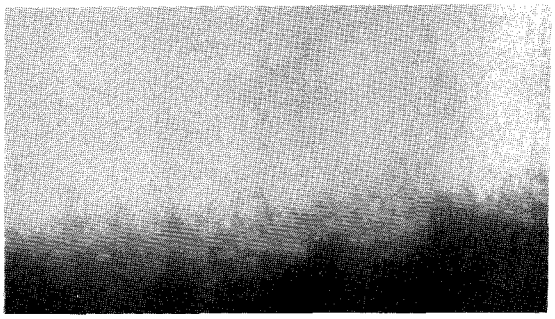


Fig. 4. Aerial mycelium of strain DMC-64 (×150).

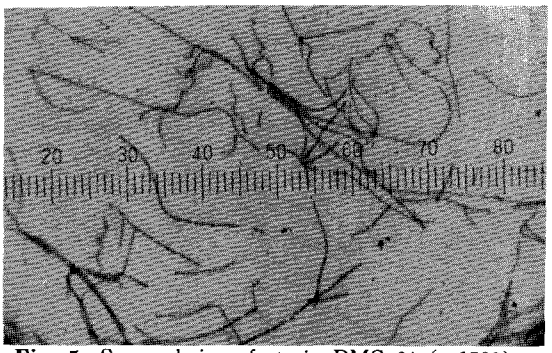


Fig. 5. Spore chains of strain DMC-64 (×1500).

the spore chain was rectus-flexibilis and did not contain spiral form. Fig. 5 showed that the diameters of spores were 0.5~1.0 micrometer because one small scale was 1.3 micrometer.

4) Physiological Tests

The results of starch hydrolysis and gelatin hydrolysis were both positive and the melanin pigment was not formed. The results of the utilization of

Table I. Cultural characteristics of Strain DMC-64.

Medium	Growth	Reverse phase	Aerial mycelium	Soluble pigment
Oatmeal agar(ISP No. 3 medium)	good	slightly gray	gray	none
Yeast extract malt extract agar (ISP No. 2 medium)	good	dark reddish brown	gray	reddish brown
Inorganic salts-starch agar (ISP No. 4 medium)	moderate	slightly yellow	gray	none
Glycerol-asparagine agar (ISP No. 5 medium)	moderate	reddish yellow	white	slightly yellow
Glucose-asparagine agar	good	reddish yellow	whitish gray	yellow
Glucose-peptone agar	moderate	brownish yellow	whitish gray	brownish yellow
Glucose-nitrate agar	moderate	whitish gray	whitish gray	none

Table II. Utilization of carbon sources by Strain DMC-64.

Carbon Sources	Use
D-Glucose	+
D-Xylose	+
L-Arabinose	−
L-Rhamnose	−
D-Fructose	±
D-Galactose	−
Raffinose	−
D-Mannitol	−
Inositol	−
Salicin	+
Sucrose	−

(+ : utilized, ± : weakly utilized, − : not utilized)

carbon sources were shown in Table II.

Fermentation

The results were outlined in Figure 6. The variation of pH was not significant, the full growth appeared after three days, and the antimicrobial activity against *B. subtilis* ATCC 6633 reached maximum at five days after the inoculation.

Biological Activity

Strain DMC-64 had an antimicrobial activity against gram-positive bacteria and no activity against gram-negative bacteria. The results were shown in

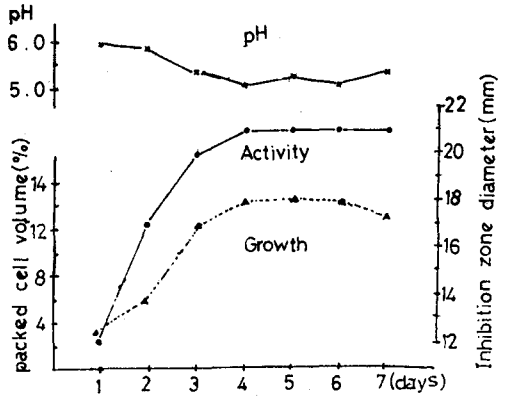


Fig. 6. Fermentation of the antibiotic of DMC-64.

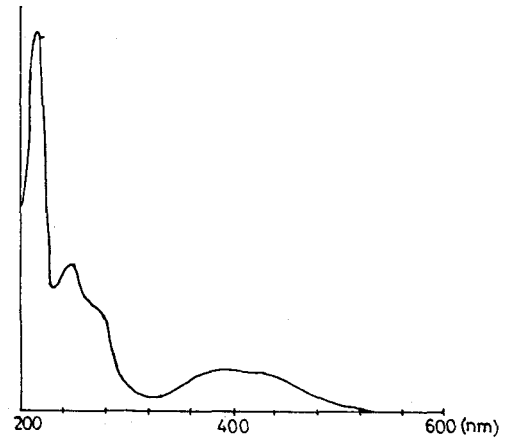


Fig. 7. UV absorption spectrum of the antibiotic of DMC-64 in H_2O.

Table III. Antibacterial spectrum.

Test Organism	Culture Filtrate	Chloramphenicol (8mg/ml)	Ampicillin (2mg/ml)	Gentamicin (0.2mg/ml)
Staphylococcus aureus ATCC 25923	17*	24	21	12
Staphylococcus aureus ATCC 6538	19	23	24	13
Streptococcus fragilis	20	29	19	13
Bacillus subtilis ATCC 6633	21	26	17	12
Bacillus megaterium	—	21	13	12
Bacillus licheniformis	20	22	14	12
Sarcina lutea	27	44	46	17
Mycobacterium smegmatis	19	26	25	13
Escherichia coli	—	15	19	12
Pseudomonas aeruginosa	—	15	—	12

inhibition zone, mm, —: no inhibition

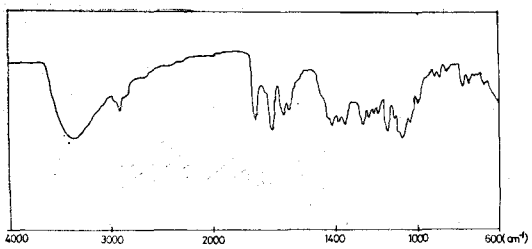


Fig. 8. IR absorption spectrum of the antibiotic of DMC-64 in KBr.

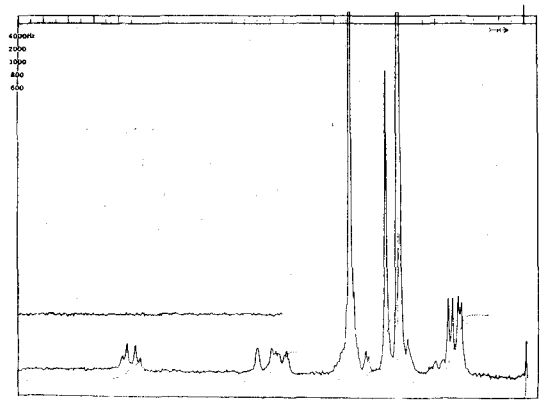


Fig. 9. NMR spectrum of the antibiotic of DMC-64 (80 MHz, D_2O+DMSO).

Table III.

Spectral Analysis

UV, IR and NMR spectra of the antibiotic of DMC-64 were shown in Fig. 7, 8 and 9. Its mass spectrum showed that this compound was very unstable for detecting the molecular ion peak.

Discussion

Strain DMC-64 was selected among 100 antibiotic producing strains which were isolated from various Korean soil samples, because it had a very effective antimicrobial activity against gram positive bacteria, especially *Mycobacterium smegmatis*. This strain had characteristics of *Streptomyces* species, and its representative characteristics were as follows: its whole cell hydrolyzate contained L-DAP and no galactose, xylose and arbinose; the diameter of its spore was about 0.5~1.0 micrometer; the gram staining was positive; aerial haphae and aerial spores existed; the diameters of the colonies were less than one cm; the results of starch hydrolysis an gelatin hydrolysis were both positive.

Rectus flexibilis of spore chain, the gray colony, no formation of melanin pigment and the utilization of carbon sources were used to determine the species of strain DMC-64. According to Bergey's Manual of Determinative Bacteriology, strain DMC-64 had properties similar to those of *S. aburaviensis*. But strain DMC-64 and *S. aburaviensis* were not the same species because of the different properties of utilization of salicin and the chromogenicity. Therefore it was concluded that strain DMC-64 was a different species compared with all the species listed in Bergey's Manual of Determinative Bacteriology.

It was found that the strongest activity of antibiotic of DMC-64 was obtained from the 5-day culture broth. This fact showed that five days were adequate for producing the antibiotic.

The aqueous solutions of this antibiotic with acidic to neutral pH range were orange-colored, but its alkaline solutions were dark violet. Therefore, the antibiotic of strain DMC-64 was regarded as an indicator type. From its UV and IR spectra, it was considered as quinone antibiotic (Hata *et al.*, 1971) because it had an absorption peak at 210nm, 255nm and 280nm in UV spectrum and had an absorption peak at 1,620cm^{-1} and 1,655cm^{-1} in IR spectrum. Also in the NMR spectrum, the aromatic hydrogen peaks were observed at 7.87 and 7.70 ppm.

These facts suggested that this compound was a quinone indicator antibiotic and had properties similar to medermycin (Takano *et al.*, 1976) which also belongs to quinone type antibiotic.

Further studies would be needed to determine definitely the complete structure of the antibiotic of strain DMC-64.

Conclusion

The strain DMC-64 which was isolated from Korean soil, was identified as a species of the genus *Streptomyces* and was considered as a new strain.

The antibiotic of DMC-64 had an antimicrobial activity against gram-positive bacteria and a charac-

teristic color reaction of quinone structure. Its UV, IR and NMR spectra also showed characteristics of the quinone ring. Therefore this was regarded as a quinone type antibiotic.

적 요

한국의 토양에서 항생물질을 생성하는 균주를 분리하기 위하여, 토양으로 부터 분리한 균주의 항균력에 대한 Screening을 시행하였다. 그중 충청북도에서 채취한 토양에서 분리한 균주가 그람양성균에 매우 강한 항균력을 나타내어 이 균주에 대한 연구를 진행하였다. International Streptomyces Projeet methods 에 따라 형태학적 및 생리학적 성질을 관찰하여 *Streptomyces*에 속하는 종임을 확인하였다. 이 균주를 액내배양하여 얻은 항균성물질은 XAD-2와 CM-Sephadex를 사용하여 정제하여 quinone류에 속하는 항생물질이라는 것을 밝혔다.

Acknowledgments

This investigation was supported in part by a research grant from the Ministry of Education, Republic of Korea. The authors acknowledge with gratitude the support and wish to express gratitude to the late Professor Young Eun Kim for his advice and encouragement.

They also thank Dr. Kyoung Soo Chung, Mr. Ha Won Kim, Mr. Jin Woo Chung, Mr. Jin Hwan Kwak and other members of Department of Microbial Chemistry, College of Pharmacy, Seoul National University for their assistance and help during the experiment.

References

Buchanan, R.E., and Gibbons, N.E., eds (1974): Bergey's Manual of Determinative Bacteriology, 8th ed. Williams and Wilkins Company, Baltimore.

Cho S.H., Ahn C.S. and Kwon Y.H. (1977): The Isolation and Identification of Some *Streptomyces*. *Kor. J. Microbiol.* **15**:170-175.

Gehardt, Murray, Costilow, Nester, Wood, Krieg and Phillips. (1981): Manual of Methods for General Bacteriology. American Society for Microbiology, Washington, D.C.

Hata A.T., Omura, S., Nakagawa A. and Otani M. (1971): A New Antibiotic, Kinamycin. Isolation, Purification and Properties. *J. Antibiotics.* **24**: 353-359.

Lechevalier M.P. (1970): Chemical Composition as a Criterion in the Classification of Aerobic *Actinomycetes*. *Intern. J. Syst. Bac.* **20**: 435-443.

Lee D.Y., Kim S.W., and Ko K. (1981): Studies on the Development of Antibiotic-producing *Streptomyces* in the Western Area of Korea. *Kor. J. Mycol.* **9**: 103-108.

Lee, M.J., Hah Y.C., and Ahn C.S. (1976): Studies on the Isolation and Identification of Genus *Streptomyces*. *Kor. J. Microbiol* **14**: 25-35.

Pridham T.G. and Gottlieb D. (1948): The Utilization of Carbon Compounds by Some *Actinomycetales* as an Aid for Species Determination. *J. Bacteriol.* **56**:107-114.

Seo Y.M., and Hong S.W. (1977): On the Isolation of Antibioticproducing *Streptomyces* spp. from Soil. *Kor. J. Microbiol.* **15**:93-99.

Shirling E.B. and Gottlieb D. (1966): Methods for Characterization of *Streptomyces* species. *Intern. J. Syst. Bac.* **16**: 313-340.

Staneck J.H. and Roberts G.D. (1974): Simplified Approach to Identification of Aerobic *Actinomycetes* by Thin Layer Chromatography. *Appl. Microbiol.* **19**:226-231.

Takano, S., Hasuda, K., Ito A., Koide Y., Ishii, F., Haneda I. Chihara, S., Koyama Y. (1976): A New Antibiotic, Medermycin. *J. Antibiotics* **29**: 765-768.

〈Received February 21, 1984〉