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New Antibiotics Produced by *Streptomyces melanosporofaciens*
I. Taxonomy of the producing microorganism

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*Streptomyces melanosporofaciens*가 생산하는 새로운 항생물질
Ⅰ. 생산균의 분류·동정

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Strain 88-GT-161 producing new phthalic acid derivative and basic macrolide antibiotics was identified as being *S. melanosporofaciens* based on numerical taxonomic data. However, 4 unit characters among 139 units were clearly different from the common properties of 6 strains belonging to cluster No. 32 represented by the name of *S. violaceoniger* or *S. violaceusniger*, leading us to designate as a variety of *S. melanosporofaciens*. This paper describes the taxonomic characteristics of the strain. Isolation and chemical structures, including biological activities of the active compounds produced by this strain will be presented elsewhere.

In the course of antibiotics screening, strain 88-GT-161 was found to produce antibiotic complex active against various microbes, such as Gram positive bacteria, *Candida albicans, Chlorella,* lepidopterous insects, and phytopathogenic fungi, especially against *Pyricularia oryzae* and *Rhizoctonia solani.*

Since the discovery of streptomycin from the culture filtrate of *S. griseus* by Waksman (1) in 1944 a great number of antibiotics have been isolated from Actinomycetes origins and they are still thought to be the richest source of microorganism for antimicrobial agents. However, continued development of new antibiotics made taxonomists assign new species name in order to prove the novelity of an antibiotic, resulting overclassification and proliferation of species or genus.

Key words: *S. melanosporofaciens, S. violaceusniger,* numerical taxonomy, new antibiotics
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One of the most remarkable development during the past two decades was the application of numerical taxonomic method to Actinomycetes genera classification (2-5). Until the latest revision of "Bergey's Manual of Systematic Bacteriology (6)" in 1989, the identification of Actinomycetes was mostly done by the methods described in "International Streptomyces Project (ISP, 7)". Still now the data based on ISP methods provide important characteristics to identify several strains of the genus *Streptomyces* and are applicable to compare each characters based on numerical taxonomic data base (4) which can clarify the relationships between each cluster groups or numbers in the genus. In addition, the main chemotaxonomical marker for instance, diaminopimelic acid isomer, gives definitive information for identification of new strians.

In that viewpoint, attempts were being made to identify strain 88-GT-161 via numerical taxonomy and

ISP methods, and chemotaxonomic approach to detect DAP isomers as well.

Materials and Methods

Culture

Strain 88-GT-161 was isolated from a soil collected in Mt. Paekam, Kyungpook Province. Test microorganisms employed for antimicrobial spectrum were obtained from the Institute of Applied Microbiology, Tokyo University (IAM), the Japan Collection of Microorganisms, RIKEN (JCM), the Institute for Fermentation, Osaka (IFO), and the American Type Culture Collection (ATCC). And *Escherichia coli* AB 1157 was supplied by the antibiotic laboratory in RIKEN, Japan.

Taxonomy

Identification of strain 88-GT-161 was carried out principally according to the methods described in "Bergey's Manual of Systematic Bacteriology (6)" based on the numerical classification and identification studies of Williams *et al.* (4), and "International Streptomyces Projects (ISP, 7)". Washed inoculum recommended by Shirling and Gottlieb (7) was used throughout the experiment and inoculum size for the test was 0.05 m*l* of the washed inoculum with the exception of spot-inoculation for antimicrobial spectrum and β-lactamase and β-lactamase inhibitor production tests. Observation of the culture was, unless otherwise mentioned, made after incubation at 27°C for 14 days. The taxonomic keys of Bergey's Manual, numerical classification of Williams *et al.* (4), and ISP (8-11) were used to compare characteristics of this strain with those of known species or strains of *Streptomyces* and related genera.

Spore chain morphology was observed by phase-contrast ($\times$400) and scanning electron (Hitachi S-570, $\times$20,000) microscopies. Spore surface ornamentation was determined according to the category of Dietz and Mathews (12).

Cell wall hydrolysate was prepared by Kawamoto's method (13) with the exception of enzyme treatment. Strain 88-GT-161 was cultured in ISP No. 1 submerged medium until the logarithmic growth phase (48 hr). Crude cell wall was hydrolysed by autoclaving (121°C/15 min.) in a sealed Pyrex tube containing 6 N HCl. Two-dimensional TLC (Sigmacell Type 100 cellulose) described by Harper (14) was employed for the detection of DAP isomers.

Cultural characteristics were observed on 15 different kinds of agar media recommended by Shirling and Gottlieb (7) and Hamada *et al.* (13). And color designation was made by matching the culture to "ISCC-NBS Centroid Color Charts (15)".

Antimicrobial activity was investigated by spot-inoculation followed by overlaying test microorganisms according to the method of Williams *et al.* (4).

Egg yolk media for enzyme activity assay was prepared by the method described by Gerhardt *et al.* (16). Hippurate hydrolysis was examined by the "method 2" described also by Gerhardt *et al.* β-Lactamase and β-lactamase inhibitor production was determined by O'Callaghan's method (17) using Cefinase (BBL) disc.

Degradation activity was investigated in modified Bennett's agar (MBA) medium (18) with glucose replaced by glycerol. DNase test agar and tryptone agar for RNase test were prepared according to the method described by Gerhardt *et al.* (16).

Christensen urea agar (16) slant was used for urea degradation test and a change in indicator from orange-yellow to red-purple was regarded as positive. Degradation test for allantoin was made by the method of Williams *et al.* (3). Sierra's medium (19) containing polyoxyethylene sorbitan mono-oleate (1%) was used for tween-80 degradation and formation of opaque crystal around colony was determined as positive. All the degradation tests except allantoin, urea, aesculin, and arbutin were examined on agar plate.

Resistance of strain 88-GT-161 to 11 different kinds of antibiotics at the defined concentrations were examined on MBA. The antibiotics were purchased from Sigma Chemical Company and each antibiotic solution was sterilized by millipore (0.25 μm) filtration.

Growth at different temperatures was determined also on MBA slant and incubation at 37°C and 45°C was done by enclosing the inoculated culture slants in plastic bags containing tap water in order to prevent the slants from excessive desiccation. Growth at pH 4.3 was observed on MBA whose pH was adjusted with sterilized 1 N HCl after autoclaving.

Growth in the presence of chemical inhibitors at the diagnostic concentrations described in the Bergey's Manual was made on MBA and results were read after 3 weeks of incubation.

Utilization of 11 nitrogen and 25 carbon sources for growth was tested by the methods described in the Bergey's Manual (6) and ISP (7), respectively.

Table 1. Morphological characteristics of strain 88-GT-161

Characteristics	*S. violaceusniger*	Strain 88-GT-161
Morphology and pigmentation:		
presence of spore (aerial mycelium)	100	+
Spore chain morphology:		
Rectiflexibiles	0	−
Retinaculiaperti	0	−
Spirales	100	+
Verticillati	0	−
Spore chain ornamentation:		
Smooth	0	−
Warty	0	−
Spiny	0	−
Hairy	0	−
Rugose	100	+
Production of aerial spore mass	100	+

*Values in the *S. violaceusniger* column are the percentage of strains with positive character states.

Results

Morphological characteristics

Morphological characteristics of strain 88-GT-161 are shown in Table 1. Spore and aerial mycelium were observed (Fig. 1 and 2). Spore chain showed compact *spirales* with cylindrical cut at the pointed head. Spore surface ornamentation was typical rugose type (Fig. 2).

Cultural and physiological characteristics

As shown in Table 2, spore mass color was gray series and no characteristic color was observed in substrate mycelium and diffusible pigment. Melanin was produced neither on peptone-yeast extract-iron agar nor on tyrosine agar. Mycelium fragmentation, sclerotia formation and sporulation on substrate mycelium were not observed.

Shown in Table 3 are the cultural characteristics of strain 88-GT-161 investigated by ISP (7) and Hamada's (13) methods. Growth on ISP No. 2, 3, 4, 5, glucose-asparagine agar, potato dextrose agar, and starch agar media was good, but poor on ISP No. 6, 7, and Czapek's agar media. This strain hardly grew on potato and carrot plug. Aerial mycelium on those media where growth was good showed white color at first and changed to gray nearing 2 weeks of incubation. It was then changed to black and slimy after maturation, and accumulation of moist exudate was observed on the sporulating surface after 3 weeks of incubation. Non-melanoid reddish brown pigment was produced on tyrosine agar medium after 4-day incubation but the color was not changed by flooding 0.05 N NaOH or HCl.

Fig. 1. Light micrograph of aerial mycelium and spore chain of strain 88-GT-161.

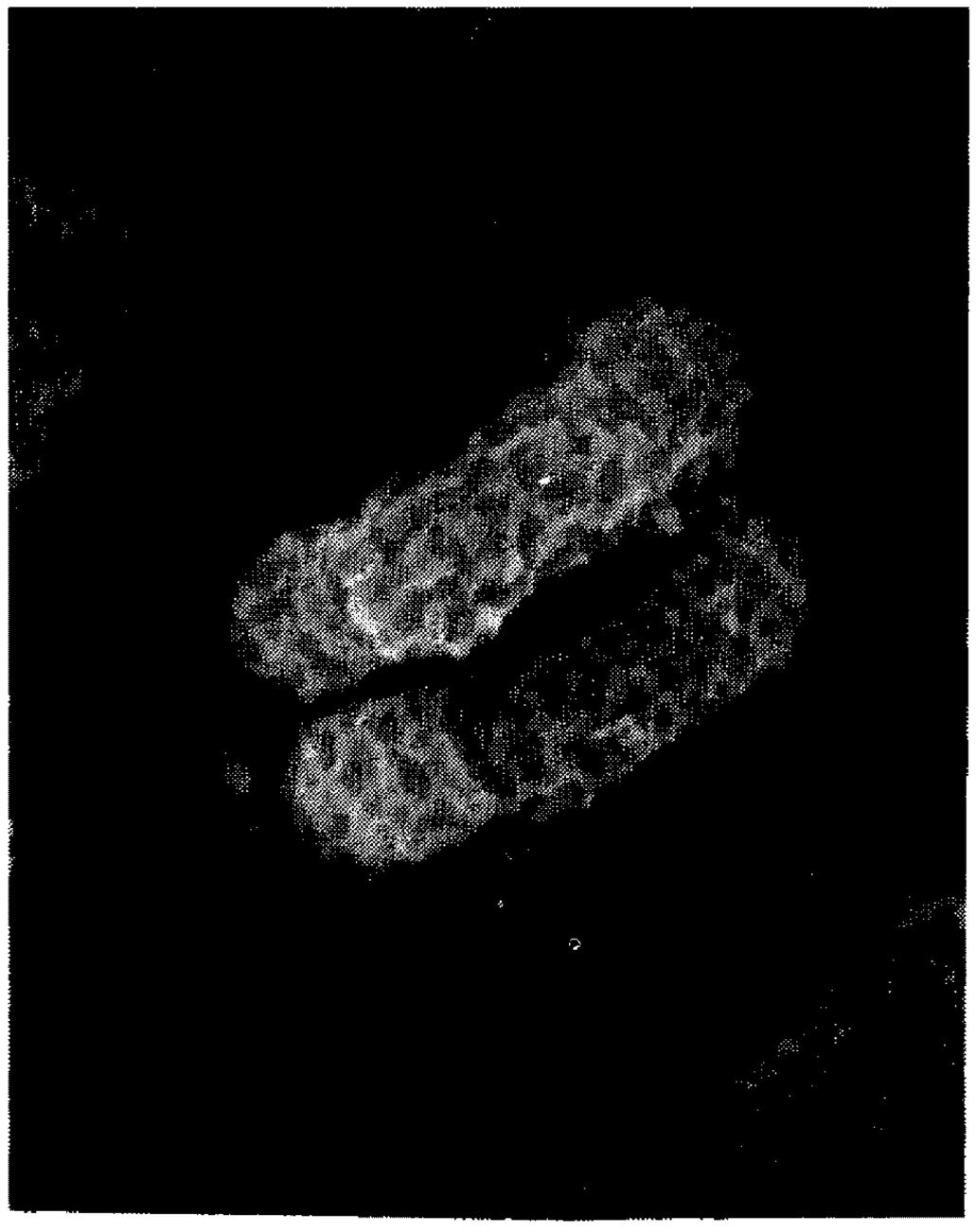

Fig. 2. Scanning electron micrograph of spore surface of strain 88-GT-161.

Other physiological characteristics

As shown in Table 4 cell wall chemotype of strain

Table 2. Cultural and physiological characteristics of strain 88-GT-161

Characteristics	*S. violaceusniger*	Strain 88-GT-161
Colour of spore mass:		
Red	0	–
Yellow	0	–
Gray	100	+
Green	0	–
Blue	0	–
Violet	0	–
White	0	–
No distinctive substrate mycelial pigments	100	+
Pigmentation of substrate mycelium		
Red/orange	0	–
Green	0	–
Blue	0	–
Violet	0	–
Production of diffusible pigments	0	–
Pigmentation of diffusible pigments:		
Red/orange	0	–
Yellow/brown	0	–
Green	0	–
Blue	0	–
Violet	0	–
Sensitivity of substrate pigment to pH	0	–
Sensitivity of diffusible pigment to pH	0	–
Melanin production on peptone/yeast/iron agar	0	–
Melanin production on tyrosine agar	0	–
Fragmentation of mycelium	0	–
Sclerotia formation	0	–
Sporulation of substrate mycelium	0	–

*Values in the *S. violaceusniger* column are the same as in Table 1.

88-GT-161 showed LL-DAP type. Gelatin liquefaction was observed not only in glucose peptone gelatin but also in simple gelatin media. Coagulation of skim milk showed negative reaction but peptonization was very weakly positive when incubated at 37°C. Optimum temperature and pH range for growth were 28-34°C and pH 6.5-7.5, respectively.

Antimicrobial activity

This strain showed antimicrobial activity against *B. subtilis, M. luteus, C. albicans, S. murinus,* and *A. niger* but did not show the activity against 3 other kinds of test microorganisms used in this assay (Table 5).

Enzyme activity

As the Table 6 shows proteolysis and lipolysis activities were observed but the lecithinase activity. Neither pectin nor chitin were hydrolyzed. H_2S production and hippurate hydrolysis were positive but nitrate reduction was negative. β-Lactamase was produced both on yeast-peptone plus glycerol (YPG) agar and Beecham's FS agar media but β-lactamase inhibitor was not formed on those media.

Degradation of organic compounds

Among the 18 different kinds of organic compounds used in this test, strain 88-GT-161 decomposed every compounds with the exception of guanine, L-tyrosine, xanthine, and allantoin. In the case of xanthine degradation, this strain showed negative reaction until the 3rd week of incubation but degradation occurred gradually after 4th week. What is more, it was notable that L-tyrosine was not degraded until the 4th week of incubation, suggesting difference between strain 88-GT-161 and 6 strains of *Streptomyces* spp. belonging to cluster No. 32, all of which showed positive reaciton (Table 7).

Resistance to antibiotics

As shown in Table 8, strain 88-GT-161 demonstrated resistance to rifampicin, oleandomycin, and penicillin G among the 11 different kinds of antibiotics used in this examination. This strain was most suceptible to streptomycin at the given concentration. But suceptibility was variable depending upon inoculum size in the case of lincomycin. It was known that all the 6 strains of cluster No. 32 showed resistance to cephaloridin and vancomycin but strain 88-GT-161 showed suceptibility to these 2 antibiotics.

Growth at different temperatures, pH 4.3, and in the presence of chemical inhibitors

Table 3. Cultural characteristics of strain 88-GT-161

Medium	Growth	Spore mass color	R.S.*	S.P.**
Yeast ext. -malt ext. agar (ISP No. 2)	good	white	P.Y	none
Oatmeal agar (ISP No. 3)	good	I. gray	P.Y	none
Inorganic salts-starch agar (ISP No. 4)	good	Y. white	P.Y	none
Glycerol-asparagine agar (ISP No. 5)	good	grayish white	P.Y	none
Peptone-yeast ext. iron agar (ISP No. 6)	moderate	none	P.Y	none
Tyrosine agar (ISP No. 7)	poor	I. gray	m.o	S. Br
Sucrose-nitrate agar	moderate	I. gray	I. gray	none
Glucose-asparagine agar	good	I. gray	I. Y	P.Y
Nutrient agar	moderate	Y. white	P.Y	none
Bennett's agar	moderate	I. gray	P.Y	none
Potato dextrose agar	good	I. gray	I.Y	none
Czapek's agar	poor	I. gray	I. gray	none
Carrot plug	poor	none	none	none
Potato plug	poor	none	none	none
Starch agar	good	Y. white	Y. white	none

* Reverse side color, ** Soluble pigment. Color name was assigned according to ISCC-NBS Centroid Color Charats[15].

Table 4. Other physiological characteristics of strain 88-GT-161

Characteristics	Strain 88-GT-161
Cell wall composition	LL-DAP
Sporangium and motile spore	–
Melanoid pigment production in tryptone-yeast ext. broth (ISP No. 1)	–
Gelatin liquefaction :	
Glucose-peptone gelation (27°C)	+
Gelatin (27°C)	+
Skim milk (27°C & 37°C) :	
Coagulation	–
Peptonization	+
Cellulose decomposition	–
Optimum temperature for growth	28-34°C
Optimum pH for growth	6.5-7.5
Horse blood hemolysis	–

Table 5. Antimicrobial activity of stain 88-GT-161

Test microorganism	*S. violaceus-niger*	Strain 88-GT-161
Bacillus subtilis IAM 1069	67	+
Pseudomonas fluorescens IAM 1201	0	–
Escherichia coli AB 1157	0	–
Micrococcus luteus JCM 1464	100	+
Candida albicans IFO 6258	17	+
Saccharomyces cerevisiae IFO 1008	50	–
Streptomyces murinus JCM 4333	50	+
Aspergillus niger ATCC 9642	33	+

* Values in the *S. violaceusniger* column are the same as in Table 1.

Strain 88-GT-161 could not grow at the given temperatures and at pH 4.3 (Table 9). As Table 10 shows, strain 88-GT-161 did not grow at the NaCl concentration above 4%, but grew in the presence of sodium

Table 6. Enzyme activity of strain 88-GT-161

Enzyme	*S. violaceus-niger*	Strain 88-GT-161
Lecithinase	0	–
Proteolysis	83	+
Lipolysis	100	+
Pectin hydrolysis	50	–
Chitin hydrolysis	83	–
Nitrate reduction	83	–
H_2S production	100	+
Hippurate hydrolysis	67	+
β-Lactamase production on YPG agar	40	+
β-Lactamase production on Beecham's FS agar	60	+
Production of *Klebsiella* β-lactamase inhibitor	0	–

* Values in the *S. violaceusniger* column are the same as in Table 1.

Table 7. Degradation of organic compounds by strain 88-GT-161

Organic compound	*S. violaceusniger*	Strain 88-GT-161
Hypoxanthine	83	+
Guanine	17	–
Elastin	83	+
L-Tyrosine	100	–
Adenine	100	+
Xanthine	0	–
DNA	100	+
RNA	100	+
Tween-80	100	+
Starch	100	+
Xylan	33	+
Casein	100	+
Testosterone	100	+
Urea	100	+
Allantoin	50	–
Gelatin	100	+
Aesculin	100	+
Arbutin	100	+

* Values in the *S. violaceusniger* column are the same as in Table 1.

Table 8. Resistance of strain 88-GT-161 to antibiotics

Antibiotic (μg/ml)	*S. violaceusniger*	Strain 88-GT-161
Gentamicin (100)	0	–
Neomycin (50)	0	–
Streptomycin (100)	33	–
Tobramycin (50)	0	–
Rifampicin (50)	83	+
Cephaloridin (100)	100	–
Vancomycin (50)	100	–
Demethylchlorote-tracycline (500)	17	–
Oleandomycin (100)	50	+
Lincomycin (100)	100	+
Penicillin G (10 i.u)	17	+

* Values in the *S. violaceusniger* column are the same as in Table 1.

Table 9. Growth of strain 88-GT-161 at different temperatures

Characteristics	*S. violaceusniger*	Strain 88-GT-161
4°C	0	–
10°C	0	–
37°C	67	–
45°C	50	–
pH 4.3	0	–

* Values in the *S. violaceusniger* column are the same as in Table 1.

azide (0.01, 0.02% w/v) and phenylethanol (0.1%). Growth inhibition was observed in the presence of phenylethanol (0.3%) and phenol (0.1%). On the other hand, growth of this strain was not inhibited by the presence of potassium tellurite (0.001%), thallous acetate (0.001%), and crystal violet (0.0001%).

Growth on sole nitrogen source

Table 11 shows utilization of nitrogen sources for growth by strain 88-GT-161. Potassium nitrate, L-threonine, L-serine, L-phenylalanine, L-histidine, and L-arginine were well utilized for growth, but DL-α-amino-n-butyric acid, and L-valine were hardly used by this strain for growth.

Table 10. Growth of strain 88-GT-161 in the presence of chemical inhibitors

Chemical inhibitor (% w/v)	*S. violaceus-niger*	Strain 88-GT-161
Sodium chloride (4)	67	–
Sodium chloride (7)	0	–
Sodium chloride (10)	0	–
Sodium chloride (13)	0	–
Sodium azide (0.01)	50	+
Sodium azide (0.02)	33	+
Phenylethanol (0.1)	83	+
Phenylethanol (0.3)	0	–
Phenol (0.1)	0	–
Potassium tellurite (0.001)	17	+
Potassium tellurite (0.01)	0	–
Thallous acetate (0.001)	0	+
Thallous acetate (0.01)	0	–
Crystal violet (0.0001)	100	+

* Values in the *S. violaceusniger* column are the same as in Table 1.

Table 11. Growth of strain 88-GT-161 on sole nitrogen source

Nitrogen source (0.1% w/v)	*S. violaceusniger*	Strain 88-GT-161
DL- α-Amino-n-butyric acid	100	+
Potassium nitrate	100	+
L-Cysteine	33	–
L-Valine	33	–
L-Threonine	83	+
L-Serine	100	+
L-Phenylalanine	67	+
L-Methionine	17	+
L-Histidine	100	+
L-Arginine	83	+
L-Hydroxyproline	83	+

* Values in the *S. violaceusniger* column are the same in Table 1.

Table 12. Growth of strain 88-GT-161 on sole carbon source

Carbon source (1.0% w/v)	*S. violace-usniger*	Strain 88-GT-161
L-Arabinose	100	+
Sucrose	33	–
D-Xylose	67	+
meso-Inositol	67	+
Mannitol	100	+
D-Fructose	83	+
L-Rhamnose	83	+
Raffinose	83	+
D-Melezitose	83	–
D-Mannose	100	+
D-Lactose	100	+
Inulin	33	–
Adonitol	67	–
Salicin	83	+
Trehalose	100	+
D-Melibiose	83	+
Dextran	17	–
D-Galactose	83	+
Cellobiose	100	+
Xylitol	17	–
Sodium acetate (0.1% w/v)	50	–
Sodium citrate (0.1% w/v)	67	+
Sodium malonate (0.1% w/v)	33	–
Sodium propionate (0.1% w/v)	50	+
Sodium pyruvate (0.1% w/v)	67	+

* Values in the *S. violaceusniger* column are the same as in Table 1.

Growth on sole carbon source

Utilization of 25 kinds of carbon sources is shown in Table 12. D-Xylose, *meso*-inositol, mannitol, D-fructose, L-rhamnose, D-mannose, D-lactose, trehalose, D-melibiose, D-galactose, and cellobiose were utilized very well for growth but sucrose, D-melezitose, inulin, adonitol, dextran, xylitol, sodium acetate, and sodium malonate were not used for growth by this strain. Other carbon sources were found to be used for growth but not much in the degree of utilization.

Discussion

Spore surface ornamentation of strain 88-GT-161 showed typical rugose type, which could serve as the reference point to exclude unprobable identity of genus or species. It could be found that among the 475

type strains of Actinomycetes so far reported in Bergey's Manual of Systematic Bacteriology (6), ISP (8-11), and numerical taxonomy of Williams *et al.* (4), one type strain of *Actinomadura* belonging to cluster group E and 7 strains of *Streptomyces* belonging to cluster group A show rugose type in cell wall ornamentation.

In the mean time, cell wall chemotype of *Actinomadura* is *meso*-DAP type, suggesting that strain 88-GT-161 showing LL-DAP type is clearly different from *Actinomadura* and belongs to cluster group A. Amongst cluster group A, all the 6 strains of *Streptomyces* in cluster No. 32 (*S. violaceusniger*) and 1 strain in cluster No. 39 (*S. longisporoflavus*) show rugose type in spore surface ornamentation. But 139 unit characters of *S. longisporoflavus* were significantly different from those of strain 88-GT-161. Especially, *S. longisporoflavus* shows no antimicrobial activity against 8 strains of test microorganisms. On the other hand, strain 88-GT-161 showed antimicrobial activity against all the test microorganisms exclusive of Gram negative bacteria and *Saccharomyces cerevisiae*. These findings led us to deduce that strain 88-GT-161 belonged to cluster No. 32 represented by the name of *S. violaceoniger* in William's report (4) or *S. violaceusniger* in the Bergey's Manual of Systematic Bacteriology (6).

It was then found that there are 6 strains in cluster No. 32; *S. endus* (ISP 5187), *S. hygroscopicus* (ISP 5578 and N 736), *S. melanosporofaciens* (ISP 538), *S. sparsogenus* (ISP 5356), and *S. violaceusniger* (ISP 5563). Characteristics of each of these 6 reference strains were compared with those of strain 88-GT-161, using ISP methods (8-11). It was found that morphological, physiological, cultural, and carbon sources utilization characteristics of strain 88-GT-161 were identical with those of *S. melanosporofaciens*. Especially, the facts that strain 88-GT-161 was unable to use sucrose for growth, produced non-melanoid reddish brown pigment on tyrosine agar medium (ISP No. 7), and accumulated moist black and slimy exudate on spore mass after maturation coincide with those properties of *S. melanosporofaciens* reported by Arcamone (20). On the other hand, properties of special interest were the differences in the following 4 unit characters. It has been known that all the 6 strains belonging to cluster No. 32 (*S. violaceusniger*) show positive reaction in L-tyrosine degradation and resistance to vancomycin and cephaloridin, and negative reaction of growth in the presence of thallous acetate at the concentration of 0.001%, contradictory to the properties of strain 88-GT-161.

From these findings it could be suggested that strain 88-GT-161 was a variety of *S. melanosporofaciens*.

요 약

새로운 phthalic acid 유도체 및 염기성 아르로라이드 항생물질을 생산하는 strain 88-GT-161 을 numerical taxonomic data base에 의거 *Streptomyces melanosporofaciens* 로 동정되었으나, 139 단위 특성 중 4 가지 단위에 있어 *S. violaceoniger* 혹은 *S. violaceusniger* 로 대표되는 cluster No.32에 속하는 6 균주의 공통된 특성과 분명히 다른 특성을 나타냈다. 따라서 본 논문에서는 strain 88-GT-161 의 분류·동정학적 특성에 대하여 보고하고자 한다.

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