*Journal of Microbiology and Biotechnology*
Vol. 5. No. 6, 324-334, 1995

# Numerical Identification of *Streptomyces flaveus* Producing Antibiotic Substances Inhibitory to Plant Pathogenic Fungi

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The actinomycete strain A 11 was antagonistic to plant pathogenic fungi *Phytophthora capsici* and *Magnaporthe grisea*. Based on the diaminopimelic acid (DAP) type and morphological characteristics examined by scanning electron microscopy, the strain A 11 was confirmed to belong to the genus *Streptomyces*. Based on Willcox probability and similarity level, the strain A 11 was numerically identified as *Streptomyces flaveus* using TAXON program of Ward and Goodfellow. Antibiotic production of *S. flaveus* strain A 11 was most favorable when cultured on glycerol yeast extract peptone (GYP) agar for 20 days at 28°C. The crude antibiotics from solid GYP agar cultures of the strain A 11 were most effective against *Phytophthora capsici* and *Sclerotinia sclerotiorum* among the fungi tested. Antifungal activity of the antibiotics against *Alternaria solani, Botryosphaeria dothidea, Cercospora capsici, Magnaporthe grisea,* and *Rhizoctonia solani* was somewhat high, whereas *Colletotrichum gloeosporioides* and *Fusarium oxysporum* f. sp. *cucumerinum* were rarely inhibited even at high concentrations.

Screening for new antibiotics has been actively accelerated since Fleming discovered penicillin from *Penicillium natatum* in 1928. Actinomycin was isolated from actinomycetes by Waksman in 1941. The discovery of streptothricin and streptomycin provided the possibility for the use of antibiotics in medicine. Chloramphenicol, tetracycline, and macrolide antibiotics were further detected. To date, more than 10, 000 antibiotics have been isolated from various microbial sources, such as actinomycetes, bacteria, fungi, mushrooms, etc. About 74% of these antibiotics were produced by actinomycetes. The 75% of antibiotics produced from actinomycetes were also derived from the genus *Streptomyces* (20).

Antibiotics mostly used in medical fields began to be used in agriculture, since streptomycin was found to be active against some plant pathogenic bacteria. The intensive use of synthetic fungicides for control of plant diseases has caused problems of fungicide resistance and many side effects unfavorable against the environment (6, 9). However, antibiotics of microbial origin have few side effects to the environment and show little toxicity to host plants. They also have selective activity against plant pathogens, can be decomposed quickly after use, and do not bring about residual toxicity in soils (19). Agricultural antibiotics such as blasticidins (36), kasugamycin (37), polyoxin (12), and validamycin (13) are being used practically for the control of economically important plant diseases.

In general, microorganisms, especially actinomycetes, produce antibiotics that have various biological activities (25). In particular, antifungal active compounds are reported increasingly year by year (20). A variety of antifungal antibiotics were produced from various actinomycetes. Deisovalerylblastmycin (11) and gopalamicin (22) from *Streptomyces*, anthracycline antibiotics spartanamicins A and B (21) from *Micromonospora*, mildiomycin (10) from *Streptoverticillium*, and setamycin (27) and cystargin (39) from *Kitasatosporia* have been well demonstrated for antifungal activity.

It has long been known that some actinomycete strains of the same species could produce different antibiotics, whereas some strains belonging to different species produced the same antibiotics (15, 17). Antibiotic production by actinomycetes, therefore, may not be species-specific but strain-specific. Antibiotics of actinomycete origin show wide varieties of chemical structures encompassing aminoglycosides, anthracyclines, glycopeptides, β-lactams, macrolides, nucleosides, peptides, polyenes, polyethers, and tetracyclines (25).

Antibiotics are sometimes produced in nutritionally limited media or under limiting cultural conditions, indicating that antibiotic biosynthesis is subjected to various regulatory mechanisms (25). Antibiotics have

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Key words: *Streptomyces flaveus*, numerical identification, antifungal antibiotics, plant-pathogenic fungi

been regarded as typical secondary metabolites (5). Biosynthesis of secondary metabolites may be affected by final product repression, carbon catabolite repression, nitrogen repression (35), inorganic phosphate regulation (26), or ammonium regulation (18, 26). Thus, productivity of targeted antibiotics in microorganisms can be greatly enhanced by controlling the cultural conditions.

In the present studies, we numerically identified *Streptomyces flaveus* strain A 11 active against plant pathogenic fungi using TAXON program. The strain A 11 produced some antibiotic substances inhibitory to some plant pathogenic fungi.

## MATERIALS AND METHODS

### Antibiotic-producing Actinomycete Strain A 11

Actinomycete strain A 11 antagonistic to various plant pathogenic fungi (1) was provided by Molecular Plant Patholgy Laboratory, Department of Agricultural Biology, Korea University, Korea. Actinomyete strain A 11 was isolated from a soil sample collected in pepper-growng fields in Suweon, Korea (1). The organism was usually grown at 28°C on glycerol asparagine agar containing 10 g glycerol, 1 g L-asparagine, 1 g $K_2HPO_4$, 1 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 1 mg $MnCl_2 \cdot 4H_2O$, 20 g agar, and 1 liter $H_2O$. For long-term maintenance, the strain A 11 was preserved in glycerol asparagine broth containing 15% glycerol at -70°C.

### Identification of Actinomycete Strain A 11

To determine the genus of actinomycete strain A 11, the type of 2,6-diaminopimelic acid (DAP), known as one of components of cell wall of actinomycete mycelia, was analyzed using the methods of ISP (International *Streptomyces* Project) suggested by Shirling and Gottlieb (32) and Bergey's Mannual of Systematic Bacteriology (48).

Actinomycete strain A 11 was cultured on yeast extract-dextose broth (7.5 g yeast extract , 20 g dextrose, 1 liter $H_2O$, adjusted to pH 7.4 before autoclaving) for 5 days at 28°C using a rotary shaking incubator. Cultured broth was filtered with Whatman No. 1 paper, washed with distilled water and then ethanol, and freeze-dried. Dried cells (12 mg) were placed into a cap tube (13×100 mm) containing 5 ml 6 N HCl, sealed compactly, and hydrolyzed by heating the tube for 18 h in a boiling water bath. The hydrolysate was filtered with Whatman No. 1 paper and evaporated to dryness to remove residual HCl. This residue was dissolved in 1 ml distilled water and loaded on TLC plate (Cellulose NM 300, 0.5 mm layer, Macherey Nagel). Five μl of 0.01 M DL-DAP (Sigma) containing *meso*- and LL-DAP isomers were also loaded on TLC plates as standards. The separation of DAP was conducted by thin-layer chromaography developed with methanol-water-10 N HCl-pyridine (80 : 26 : 2.5 : 20) (3, 4, 34). After air-drying of the TLC plates, developed spots were visualized by spraying them with 0.2% ninhydrin in acetone and heating for 25 min at 100°C.

To examine spore chain morphology, actinomycete strain A11 was incubated for 10 days on inorganic salts starch agar (ISP medium 4) (10 g soluble starch, 2 g $CaCO_3$, 2 g $(NH_4)_2SO_4$, 1 g $K_2HPO_4$, 1 g $MgSO_4 \cdot 7H_2O$, 1 g NaCl, 1 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 1 mg $MnCl_2 \cdot 7H_2O$, 20 g agar, 1 liter $H_2O$, adjusted to pH 7.2). Spore chain morphology of the strain A 11 was examined using light and scanning electron microscopy (SEM). Spore chain morphology was examined by light microscopy at ×400 magnificaion. Specimen for SEM was prepared by the method of Williams and Davies (43). Among morphological categories suggested by Pridham *et al.* (30), the two categories, *Rectiflexibles* and *Spirales* were employed for evaluation of spore chain morphology.

### Numerical Identification of Actinomycete Strain A 11 using TAXON Program

Numerical identification of actinomycete strain A 11 was conducted using TAXON program (41) to determine the species of the strain A 11. TAXON is a computer program that identifies unknown strains by testing 50 unit characters and analyzing the results numerically based on data collected by Williams *et al.* (44). Tests of 50 unit characters for numerical identification were done following the method of Williams *et al.* (44, 45) and Langham *et al.* (16). Taxonomic 50 unit characters and their code name for computer are presented in Table 1.

Identification scores of actinomycete strain A 11 were determined using the TAXON program. Percentages in the matrix are converted by the program to proportions, $P_{ij}$ for the *i*th character of taxon *J*. The character state values of an unknown (*u*) are input into the TAXON program. Compared with each taxon in turn, identification coefficients are calculated and the best identifications printed. Only the *m* characters scored as + or - are considered, not those for which the value of *u* is unrecorded. Five of the identification coefficients provided by the program are described below.

i) Willcox probability ($L_{UJ}$) (42). This is the likelihood of unknown strain (*u*) against taxon *J* divided by the sum of the likelihoods of *u* against all *q* taxa, i.e., $L_{UJ}/\Sigma^{q} L_{UJ}$. The nearer the score approaches 1.0, the better is the fitness of an unknown strain with a group

**Table 1.** Fifty unit characters for numerically identifying *Streptomyces* sp. strain A 11 using TAXON program.

| Unit character | Code name | Strain A 11 |
|---|---|---|
| 1. Spore chain morphology *Rectiflexibiles* | RFS | -[a] |
| 2. Sporo chain morphology *Spirales* | SPI | + |
| 3. Color of spore mass red | RED | - |
| 4. Color of spore mass grey | GRY | + |
| 5. Mycelial pigment red-orange | ROS | - |
| 6. Diffusible pigment produced | PIG | - |
| 7. Diffusible pigment yellow-brown | YBP | - |
| 8. Melanin production on pepetone yeast extract iron agar medium | MPI | - |
| 9. Melanin production on tyrosine agar medium | MTY | - |
| 10-15. Antimicrobial activity against: *Bacillus subtilis* NCIMB 3610 | SUB | - |
| *Candida albicans* CBS 562 | ALB | - |
| *Streptomyces murinus* ISP 5091 | MUR | - |
| *Micrococcus luteus* NCIMB 196 | LUT | - |
| *Saccharomyces cerevisiae* CBS 1171 | CER | - |
| *Aspergilus niger* LIV 131 | NIG | - |
| 16. Lecithinase activity | LEC | - |
| 17. Lipolysis | LIP | - |
| 18. Pectin degradation | PEC | - |
| 19. Nitrate reduction | NO3 | + |
| 20. Hydrogen sulfide production | H2S | + |
| 21. Hippurate hydrolysis | HIP | - |
| 22. Elastin degradation | ELA | + |
| 23. Xanthine degradation | XAN | - |
| 24. Arbutin degradation | ARB | + |
| 25-28. Antibiotic resistance to: Neomycin (500 μg/ml) | NEO | + |
| Rifampicin (50 μg/ml) | RIF | + |
| Oleandomycin (100 μg/ml) | OLE | + |
| Penicillin G (10 i.u.) | PEN | - |
| 29. Growth at 45°C | 45C | - |
| 30-34. Growth with (%, w/v): NaCl (7.0) | 7NA | - |
| Sodium azide (0.01) | 01Z | + |
| Phenol (0.1) | PHN | - |
| Potassium tellurite (0.001) | 01T | + |
| Thallous acetate (0.001) | T01 | - |
| 35-50. Utilization of: DL-α-Amino-n-butyric acid | BUT | - |
| L-Cysteine | CYS | - |
| L-Valine | VAL | + |
| L-Phenylalanine | PHE | + |
| L-Histidine | HIS | - |
| L-Hydroxyproline | HYD | - |
| Sucrose | SUC | + |
| *meso*-Inositol | INO | + |
| Mannitol | MAN | + |
| L-Rhamnose | RHA | + |
| Raffinose | RAF | + |
| D-Melezitose | MEZ | - |
| Adonitol | ADO | - |
| D-Melibiose | MEB | + |
| Dextran | DEX | - |
| Xylitol | XYT | - |

[a]Symbols + and - represnet positive and negative results, respectively.

in the matrix.

ii) Taxonomic distance (d). This is given by $\sqrt{[\Sigma (u_i-P_{ij})^2/m^1]}$. This expresses the distance of an unknown strain from the centroid of the group with which it is being compared; hence low scores indicate relatedness.

iii) 95% taxon radius. This represents the radius of taxonomic groups including 95% groups of taxon *J.*

iv) % probability of strain further away. This indicates what % of all the cluster groups is represented by strains in cluster groups outside the identified strain; high scores show relatedness.

v) Simple matching coefficient ($S_{SM}$). This is for use on binary characters only. It is simply the proportion of characters that have the same state (both negative or both positive). This coefficient has the great virtue of intuitive simplicity.

**Medium for Antibiotic Production**

Antifungal activity was tested with five media to select the medium favorable for the antibiotic production from actinomycete strain A 11 identified as *Streptomyces flaveus*. The five media used were glycerol asparagine agar (10 g glycerol, 1 g L-asparagine, 1 g $K_2HPO_4$, 1 mg $ZnSO_4 \cdot 7H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 1 mg $MnCl_2 \cdot 4H_2O$, 20 g agar, and 1 liter $H_2O$), glucose starch peptone agar (5 g glucose, 5 g solunble starch, 10 g peptone, 5 g meat extract, 3 g NaCl, 20 g agar, and 1 liter $H_2O$, pH 7.0-7.2) (2), glycerol yeast extract peptone agar (25 g glycerol, 10 g yeast extract, 5 g peptone, 3 g $CaCO_3$, 2 g NaCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g $K_2HPO_4$, 20 g agar, and 1 liter $H_2O$, pH 7.0) (8), V8-juice agar (20% v8-juice, 20 g agar, and 1 liter $H_2O$), and yeast extract agar ( 4 g yeast extract, 10 g malt extract, 4 g glucose, 20 g agar, and 1 liter $H_2O$, pH 7.4) (24).

*Streptomyces flaveus* strain A 11 was cultured for 14 days on each of the above five media. Solid culture agars were harvested from plates and extracted with methanol. Methanol-extracts (1 liter per culture medium) were concentrated *in vacuo* using a rotary evaporator. Bioassay of concentrated culture filtrates was conducted against *Phytophthora capsici, Magnaporthe grisea,* and *Colletotrichum gloeosporioides* using the paper disk method.

**Culture Time for Antibiotic Production**

*Streptomyces flaveus* strain A 11 was cultured on glycerol yeast extract peptone agar at 28°C to determine the culture time optimal for antibiotic production. *S. flaveus* strain A 11- grown solid agars were collected from plates every three days after incubation, extracted with methanol, and concentrated *in vacuo* using a rotary evaporator. The 500 ml culture filtrates were yielded at different time intervals and extracted with methanol. Antifungal activity of concentrated culture filtrates was bioassayed using *P. capsici, M. grisea,* and *C. gloeosporioides.*

**Large-scale Production of Antibiotics**

*S. flaveus* strain A 11 was incubated on the glycerol yeast extract peptone agar for 20 days at 28°C. Mycelia of the strain A 11 were inoculated by streaking to the edges of petri dishes to grow compactly on glycerol yeast extract peptone agar. Solid agars (24 kg) with the actinomycete cultures were harvested from 1500 plates and extracted with methanol (30 liter). Methanol extracts of culture filtrates were concentrated *in vacuo* using a rotary evaporator.

**Bioassay of Culture Filtrates for Antifungal Activity**

Methanol extracts of culture filtrates of *S. flaveus* strain A 11 were concentrated *in vacuo* and freeze-dried. Some quantity of yellow-brownish oily powders (120 g) obtained was dissolved in a small volume of distilled water and added to potato dextrose agar to give serial concentrations of 1, 10, 100, and 1,000 μg/ml. Mycelial disks (8 mm in diameter) of 16 plant pathogenic fungi including *P. capsici* and *M. grisea* were placed on potato dextrose agar containing crude antibiotic powders. Diameter of fungal mycelia was measured when control plates (8 cm in diameter) were covered completely with fungal mycelia, and the inhibitoin rate of mycelial growth at each concentration was determined.

## RESULTS

**Analysis of Diaminopimelic Acid (DAP) Type and Morphological Chracteristics**

Cell wall hydrolysates of actinomycete strain A 11 were developed on a cellulose TLC plate. Diaminopimelic acid present in the cell wall turned out to be LL-DAP (Fig. 1). Spore chains of strain A 11 formed *Spirales* type, as observed by light microscopy (data not shown). Under the scanning electron microscope, actinomycete strain A 11 was observed to have long

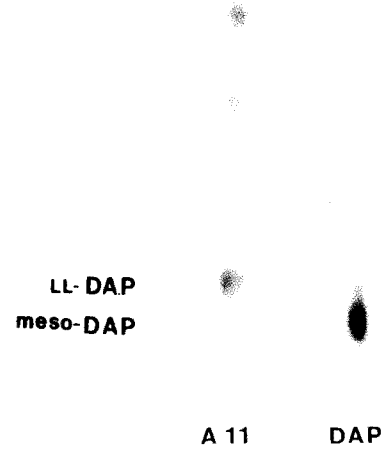


**Fig. 1.** Thin layer chromatogram of cell wall hydrolysates of actinomycete strain A 11.

spores of cylindrical forms on aerial mycelia, and spore surface ornamentation was smooth among the five groups classified by Dietz and Mathews (7) (Fig. 2). Special structures, such as zoospore and sporangium, were not found in strain A 11. Based on the DAP type of cell wall and the morphological characteristics, strain A 11 belonged to the genus *Streptomyces*.

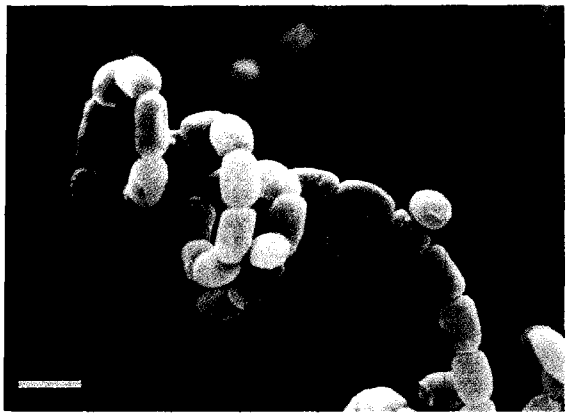

**Fig. 2.** Scanning electron micrograph of spore chains of actinomycete strain A 11 grown on inorganic salts-starch agar for 10 days.
Bar represents 1 μm.

**Numerical Identification of *Streptomyces* sp. Strain A 11 using TAXON Program**

Numerical identification of *Streptomyces* sp. strain A 11 was conducted using the TAXON program based on the data of characters of *Streptomyces* spp. classified by Williams *et al.* (44) (Table 1). Results of *Streptomyces* sp. strain A 11 obtained from the tests of 50 unit characters were input into the TAXON program to compare with the data of the other strains. Generally, correct identification of an unknown strain requires that the Willcox probability approaches 1.0, that taxonomic distance is included within 95% taxon radius and has a low score, and that % probability of strain further away has a high score.

Numerical identification was conducted using the TAXON program to determine which major clusters *Streptomyces* sp. strain A 11 belongs to (Table 2). Willcox probability which strain A 11 might fall into the taxon major clusters was 0.993309, which was

**Table 2.** Numerical identification of taxon major clusters possibly including *Streptomyces* sp. strain A 11 using TAXON program.

| Taxon Major Cluster | Taxonomic distance[a] | 95% Taxon radius[b] | % Prob of strain further away[c] | Willcox Probability[d] |
|---|---|---|---|---|
| 19 (*Streptomyces diastaticus*)[e] | 0.4362 | 0.4508 | 10.2084 | 0.993309 |
| 20 (*S. olivaceoviridis*) | 0.4829 | 0.3720 | 0.0000 | 0.000001 |
| 1C (*S. albidoflavus*) | 0.4907 | 0.3883 | 0.0001 | 0.004685 |
| 15 (*S. chromofuscus*) | 0.4914 | 0.4271 | 0.0351 | 0.001637 |
| 3 (*S. atroolivaceus*) | 0.4933 | 0.3631 | 0.0000 | 0.000000 |
| 37 (*S. griseoflavus*) | 0.5022 | 0.3658 | 0.0000 | 0.000000 |
| 12 (*S. rochei*) | 0.5040 | 0.4173 | 0.0025 | 0.000343 |
| 32 (*S. violaceoniger*) | 0.5175 | 0.3854 | 0.0000 | 0.000000 |
| 1B (*S. albidoflavus*) | 0.5228 | 0.4404 | 0.0068 | 0.000005 |
| 23 (*S. microflavus*) | 0.5243 | 0.3931 | 0.0000 | 0.000000 |
| 5 (*S. exfoliatus*) | 0.5294 | 0.4455 | 0.0065 | 0.000013 |
| 18 (*S. cyaneus*) | 0.5354 | 0.4497 | 0.0058 | 0.000006 |
| 1A (*S. albidoflavus*) | 0.5354 | 0.3782 | 0.0000 | 0.000000 |
| 6 (*S. violaceus*) | 0.5366 | 0.4126 | 0.0000 | 0.000000 |
| 65 (*Kitasatoa spp.*) | 0.5407 | 0.3374 | 0.0000 | 0.000000 |
| 21 (*S. griseoruber*) | 0.5432 | 0.3709 | 0.0000 | 0.000000 |
| 40 (*S. phaeochromogenes*) | 0.5539 | 0.3805 | 0.0000 | 0.000000 |
| 10 (*S. fulvissimus*) | 0.5669 | 0.4036 | 0.0000 | 0.000000 |
| 31 (*S. antibioticus*) | 0.5745 | 0.4131 | 0.0000 | 0.000000 |
| 29 (*S. lydicus*) | 0.5823 | 0.3831 | 0.0000 | 0.000000 |
| 33 (*S. chromogenus*) | 0.5862 | 0.3955 | 0.0000 | 0.000000 |
| 16 (*S. albus*) | 0.5870 | 0.3347 | 0.0000 | 0.000000 |
| 61 (*S. lavendulae*) | 0.6135 | 0.4118 | 0.0000 | 0.000000 |
| 42 (*S. rimosus*) | 0.6146 | 0.3507 | 0.0000 | 0.000000 |
| 17 (*S. griseoviridis*) | 0.6489 | 0.3943 | 0.0000 | 0.000000 |
| 30 (*S. filipinensis*) | 0.6547 | 0.3845 | 0.0000 | 0.000000 |

[a]This expresses the distance of an unknown strain from the centroid of the group with which it is being compared.
[b]This represents the radius of taxonomic groups including 95% groups of taxon *J*.
[c]This indicates what percent of all the cluster groups is represented by strains in cluster groups outside the identified strain.
[d]This is the likelihood of unknown strain (*u*) against taxon *J* divided by the sum of the likelihood of *u* against all *q* taxa.
[e]*Streptomyces* spp. in parenthesis are representative species belonging to the taxon major clusters of *Streptomyces*.

much higher than the 0.004685 of Willcox probability to the taxon major cluster 1 C. Taxonomic distance value (0.4362) of strain A 11 existed within its 95% taxon radius (0.4508). Percent probability of strain further away of strain A 11 (10.2084) to the taxon major cluster 19 was distinctly higher than that of the other major clusters. Therefore, it was likely that the strain best matched to *Streptomyces* sp. strain A 11 might fall into the taxon major cluster 19.

The numerical identification scores of *Streptomyces* sp. strain A 11 were compared with those of a hypothetical median organism (HMO), centrotype (*Streptomyces phaeoviridis*), the outer-most member (*Streptomyces coralus*), and the best match strain (*Streptomyces flaveus*) among the taxon major cluster 19 members (Table 3). Taxonomic distance (0.4362) of strain A 11 was within 95% taxon radius (0.4508). This score was smaller than that of the outer-most member *S. coralus* (0.4435). However, % probability of strain further away meant by the probability of being placed in the center of cluster was 10.2084, which was lower than that of either centrotype (92.9354) or best match strain (69.5300). Thus, it was supposed that the strain A 11 was placed in regions further away from the center of major cluster 19.

Simple matching coefficients ($S_{SM}$) of *Streptomyces* sp. strain A 11 to 20 strains in the taxon major cluster 19 were calculated based on the data of fifty unit characters (Table 4). The greater this value is, the higher the similarity level is. $S_{SM}$ of the best match strain *S. flaveus* (76.00%) was highest among those of the 20 strains.

In conclusion, the results of numerical identification using the TAXON program and the similarity level indicated that *Streptomyces* sp. strain A 11 was identical to a strain of *Streptomyces flaveus* included in taxon major cluster 19 (*Streptomyces diastaticus*).

**Medium for Antibiotic Production**

To select the medium favorable for antibiotic pro-

**Table 3.** Identification of hypothetical median organism (HMO), centrotype, outer-most member, best match strain and *Streptomyces* sp. strain A 11 based on the major cluster 19 of *Streptomyces* using TAXON program.

| Cluster 19 member | Taxonomic distance | 95% Taxon radius | % Prob. of strain further away | Willcox probability |
|---|---|---|---|---|
| HMO | 0.2658 | 0.4508 | 99.9072 | 0.997032 |
| Centrotype: *Streptomyces phaeoviridis* | 0.3296 | 0.4508 | 92.9354 | 0.997509 |
| Outer-most member: *Streptomyces coralus* | 0.4435 | 0.4508 | 7.2571 | 0.447286 |
| Best match strain: *Streptomyces flaveus* (*Microellobosporia flavea*) | 0.3670 | 0.4508 | 69.5300 | 0.999555 |
| Unidentified strain: *Streptomyces* sp. strain A 11 | 0.4362 | 0.4508 | 10.2084 | 0.993309 |

**Table 4.** Simple matching coefficients ($S_{SM}$)[a] of *Streptomyces* sp. strain A 11 to the member organisms in *Streptomyces* major cluster 19 based on fifty unit characters.

| Cluster 19 member | ISP No. | ATCC No. | $S_{SM}$ (%) |
|---|---|---|---|
| *Streptomyces diastatochromogenes* | 5449 | 12309 | 68 |
| *S. vastus* | 5309 | 25506 | 68 |
| *S. coralus* | 5256 | 23901 | 62 |
| *S. phaeoviridis* | 5285 | 23947 | 66 |
| *S. flaveus (Microellobosporia flavea)* | M315 | 15332 | 76 |
| *S. bottropensis* | 5262 | 25435 | 68 |
| *S. minoenisis* | 5031 | 19787 | 64 |
| *S. tauricus* | 5560 | 27470 | 64 |
| *M. cinerea* | M301 | 15740 | 74 |
| *S. mirabilis* | 5553 | 27447 | 66 |
| *S. nigellus* | 5490 | 27450 | 74 |
| *S. humidis* | 5263 | 12760 | 68 |
| *S. rishiriensis* | 5489 | 14812 | 64 |
| *S. diastaticus* | 5496 | 3315 | 62 |
| *S. lincolnesis* | 5335 | 25466 | 66 |
| *S. glomeraurantiacus* | 5429 | 19839 | 60 |
| *S. galilaeus* | 5481 | 14969 | 58 |
| *Streptomyces* sp. | F1 | | 66 |
| *S. achromogenes* | 5028 | 12767 | 60 |
| *S. olivochromogenes* | 5451 | 3336 | 64 |

[a]This is the proportion of characters that have the same state (both positive or both negative).

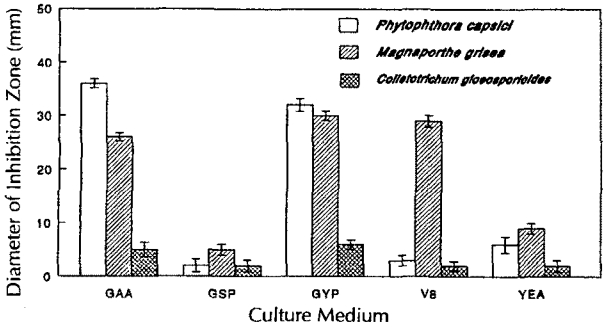


**Fig. 3.** Inhibitory effects of culture filtrates of *Streptomyces flaveus* strain A 11 grown on different agar media against *Phytophthora capsici, Magnaporthe grisea,* and *Colletotrichum gloeosporioides* on potato dextrose agar.
Inhibition of mycelial growth of the test plant pathogenic fungi was measured using paper disk method. Vertical bars represent standard deviations. GAA, GSP, GYP, V8, and YEA represent glycerol asparagine agar, glucose starch peptone agar, glycerol yeast extract peptone agar, V8 juice agar, and yeast extract agar, respectively.

duction by *Streptomyces flaveus* strain A 11, solid cultures grown on each of the five agar media were extracted with methanol and then concentrated. Bioassay was conducted with the culture filtrates against *Phytophthora capsici, Magnaporthe grisea,* and *Colletotrichum gloeosporioides* using the paper disk method (Fig. 3). Antifungal activity against *P. capsici* was most effective in the culture filtrates from glycerol asparagine agar (GAA) and glycerol yeast extract peptone (GYP) agar among the five media. Cultures on GYP agar, V8-juice agar, and GAA were also effective in inhibiting mycelial growth of *M. grisea.* In contrast, antibiotics active against *C. gloeosporioides* were not produced on any of the agar media tested. Thus, GYP agar capable of producing a large amount of antibiotics effective against both *P. capsici* and *M. grisea* was used for large-scale production of antibiotics from *S. flaveus* strain A 11.

**Culture Time for Antibiotic Production**

To determine the cultute time optimal for antibiotic production, *S. flaveus* strain A 11 was cultured on GYP agar for 25 days at 28°C. Solid agar cultures were harvested from plates every three days after incubation, followed by extraction with methanol. Antifungal activity of culture filtrates was tested against *P. capsici, M. grisea,* and *C. gloeosporioides* (Fig. 4). Antibiotics active against *P. capsici* began to form 3 days after incubation of *S. flaveus* strain A 11. Production of antibiotics inhibitory against both plant pathogenic fungi *M. grisea* and *P. capsici* distinctly increased after 6 days of incubation, hence reaching a maximum level at 20 day-cultures. However, all of the culture filtrates obtained throughout the culturing of *S. flaveus* strain A 11 did not effectively inhibit mycelial growth of *C. gloeosporioides.*

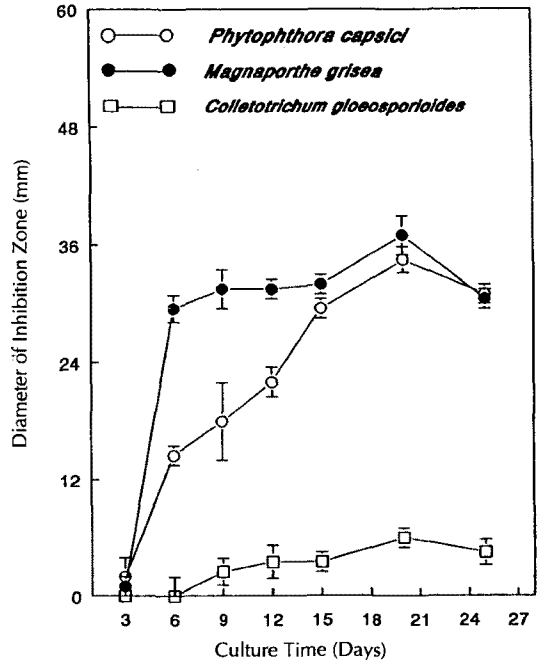


**Fig. 4.** Time course of inhibitory effects of culture filtrates of *Streptomyces flaveus* strain A 11 against *Phytophthora capsici, Magnaporthe grisea,* and *Colletotrichum gloeosporioides.*
Culture filtrates of the strain A 11 was harvested at various culture times after incubation on the glycerol yeast extract peptone agar. Vertical bars represent standard deviations.

**Large-scale Production of Antibiotics**

Solid GYP agar cultures (24 kg) of *S. flaveus* strain A 11 were extracted with methnol (30 liter). The culture filtrates were freeze-dried to yield yellow-brownish oily powders (120 g). Antifungal activity of the crude antibiotic powders against 16 plant pathogenic fungi was evaluated using the serial agar dilution method (Table 5). The crude antibiotic powders were most effective against *Phytophthora capsici* and *Sclerotinia sclerotiorum* among the fungi tested. In particular, mycelial growth of *S. sclerotiorum* was inhibited more than 50% at 100 μg/ml. Antifungal activity against *Alternaria solani, Botryosphaeria dothidea, Cercospora capsici, Magnaporthe grisea,* and *Rhizoctonia solani* was somewhat high, whereas *Colletotrichum gloeosporioides* and *Fusarium oxysporum* f. sp. *cucumerinum* was rarely inhibited even at 1,000 μg/ml.

## DISCUSSION

Based on the diaminopimelic acid (DAP) type of cell wall components, and morphological characteristics examined by scanning electrom microscopy

**Table 5.** Antifungal activity of culture filtrates[a] of *Streptomyces flaveus* strain A 11 to various plant pathogenic fungi.

| Test fungus | % Inhibition of mycelial growth[b] by concentration of | | | |
|---|---|---|---|---|
| | 1 | 10 | 100 | 1000 μg/ml |
| *Alternaria mali* | 2.5 | 4.1 | 6.5 | 23.8 |
| *Alternaria solani* | 20.7 | 29.1 | 31.8 | 59.0 |
| *Botryosphaeria dothidea* | 4.3 | 5.5 | 30.0 | 52.9 |
| *Cercospora capsici* | 22.0 | 22.3 | 43.5 | 57.0 |
| *Cercospora kikuchi* | 7.3 | 9.0 | 26.0 | 42.2 |
| *Cladosporium cucumerinum* | 11.2 | 23.1 | 38.0 | 49.2 |
| *Colletotrichum gloeosporioides* | 1.5 | 3.1 | 4.4 | 4.9 |
| *Cylindrocarpon destructans* | 2.5 | 3.4 | 10.1 | 45.8 |
| *Fusarium oxysporum f. sp. cucumerinum* | 2.0 | 4.0 | 7.2 | 15.3 |
| *Magnaporthe grisea* | 1.7 | 2.1 | 7.4 | 51.2 |
| *Mycosphaerella fragariae* | 5.9 | 9.5 | 13.2 | 22.7 |
| *Mycosphaerella melonis* | 0.9 | 14.8 | 20.3 | 24.4 |
| *Phytophthora capsici* | 9.4 | 11.9 | 19.4 | 67.9 |
| *Pythium ultimum* | 0 | 3.4 | 10.1 | 45.8 |
| *Rhizoctonia solani* | 6.7 | 16.4 | 17.3 | 57.5 |
| *Sclerotinia sclerotiorum* | 3.8 | 28.7 | 62.0 | 70.9 |

[a]Fungal mycelial growth was measured on potato dextrose agar containing different amounts of freeze-dried culture filtrates of *Streptomyces flaveus* strain A 11 when control plates ( 8 cm in diameter) were covered completely with fungal mycelia.

[b]% Inhibition of mycelial growth = $[1 - \frac{\text{Diameter of mycelial growth on antibiotic-treated plate}}{\text{Diameter of mycelial growth on untreated plate}}] \times 100$.

(Fig. 2), the strain A 11 isolated from a pepper growing field was confirmed to belong to the genus *Streptomyces*. To further determine the species of *Streptomyces* sp. strain A 11, numerical identification was undertaken using TAXON program based on the data of characters of *Streptomyces* species and probabilistic identification matrix proposed by Williams *et al.* (44, 45) and Langham *et al.* (16). TAXON is a computer program for identifying unknown bacteria and actinomycetes, which was developed by Ward and Goodfellow, University of Newcastle upon Tyne, UK (41). Numerical identification of *Streptomyces* sp. strain A 11 was done by comparison with the numerical classification data of the existing *Streptomyces* strains.

To date most attempts to classify streptomycetes have been based on a limited number of subjecting chosen features, with heavy empyhasis on morphological and cultural characteristics. Such classifications are artificial, have a narrow data base, and suffer from all of the disadvantages of monothetic groups. To overcome these disadvantages, numerical classification was suggested, in which streptomycete strains were classified systematically by testing many characteristics. A large-scale numerical phenetic survey of *Streptomyces* and related genera was undertaken by Williams *et al.* (44, 46, 47) to clarify the infrastructure of the genus and its relationships with other cell wall chemotype I genera. Information from the numerical taxonomic data base given by Williams *et al.* (44) was used to construct a probabilistic matrix for numerical identification of streptomycetes (16, 45). When the probabilistic identification matrix is stored in a computer, it can be used for the numerical identification of unknown strains, which provides a measure of the likelihood that the identification is correct (16, 45).

Unit characters for numerical identification of the strain A 11 were analyzed. Strain A 11 and its best match strain *Streptomyces flaveus* were identical in 38 of 50 characters, e.g., pigment production, antimicrobial activity, or utilization of carbon sources, etc. In contrast, they differed from each other in degradation tests, nitrate reduction, antibiotic resistance, or utilization of nitrogen sources.

The Willcox probability of the strain A 11 was 0.993309, which was distinctly higher than that of 0.004685 to the major cluster 1C (Table 2). This high Willcox probability implies that the strain A 11 is included in the major cluster 19. Major cluster 19, which is represented by *Streptomyces diastaticus*, contains twenty *Streptomyces* strains (44). Among them, the strain A 11 was best-matched to *Streptomyces flaveus* (Tables 3 and 4). By comparing with the Willcox probability of major clusters, it was possible to know in which major cluster the unidentified strain was included. Willcox probability of the strain A 11 to its best match strain *S. flaveus* was 0.999555, which was higher than those to HMO, centrotype organism, or outer-most member organism (Table 3). Comparison of the taxonomic distance and % probability of strain further away also revealed that the

strain A 11 was taxonomically placed further away from the centrotype organism. Simple matching coefficient of the strain A 11 to *S. flaveus* was 76%, which was higher among the cluster of 19 strains. Consequently, the $S_{SM}$ analysis also confirmed the strain A 11 to be *S. flaveus*. In conclusion, *Streptomyces* sp. strain A 11 was identified as *Streptomyces flaveus* using the TAXON program.

*Streptomyces flaveus* strain A 11 isolated from pepper-growing soils in Suweon, Korea (1), did not produce any antifungal substance sufficient to purify from its liquid cultures (data not presented). Both butanol and aqueous phases of culture filtrates obtained from various liquid cultures were not inhibitory to the fungi tested. However, solid cultures of *S. flaveus* strain A 11 grown on the agar media such as glycerol yeast extract peptone (GYP) agar and glycerol asparagine agar (GAA) showed a high level of inhibitory activity against plant pathogenic fungi *P. capsici* and *M. grisea* (Fig. 3). Some actinomycetes have been known to produce antibiotics when grown on agars, but not in submerged cultures (14, 23, 38).

A close relationship between antibiotic production and morphology of *Streptomyces* strains was proposed by some demonstrations that the mycelia of some *Streptomyces* strains were filamentous in antibiotic-producing agar colonies, when compared with the fragmented mycelia in the corresponding liquid cultures without antibiotic production (29, 31, 33). Antibiotic production of *Streptomyces* strains occurred in liquid cultures only by mutation of strains with the fragmented mycelia in the broth, or by changing the fragmented forms into filamentous forms using filtration methods (29, 33). When *S. flaveus* strain A 11 was cultured in liquid media, its mycelia were observed to be fragmented (no micrographs presented). This observation provides evidence to explain the finding that antibiotics were not produced in liquid culture of the strain A 11. However, the reasons why filamentous cells produce antibiotics but fragmented cells cannot in liqiud media should be clearly elucidated.

Among five culture media tested to determine the conditions for large-scale production of antibiotics, GYP agar was evaluated to be capable of producing a large amount of antibiotics highly inhibitory to plant pathogenic fungi (Fig. 3 and Table 5), indicating a possible role of glycerol in production of antifungal substances. Production of antibiotics by the strain A 11 on the GYP agar distinctly occurred after incubation for 6 days, reaching a maximum level at 20 days after incubation (Fig. 4), which indicated that *S. flaveus* strain A 11 needs a relatively long period to produce antibiotics in agar cultures, compared with other *Streptomyces* sp. Because in general the growth of *Streptomyces* sp. is somewhat slower in solid agar than in submerged cultures (40), the antibiotic production by *S. flaveus* strain A 11 may be delayed in agar cultures. Most of the antibiotics from microorganisms are secondary metabolites produced at the beginning of the idiophase when cell multiplication ceases (49). The kinds or quantities of targeted antibiotics may vary according to the components of media or cultural conditions. Although *S. flaveus* strain A 11 grew very slowly on the GYP agar relative to the several other agar media tested, the GYP agar was most favorable for production of antifungal antibiotics. Involvment of glycerol in the GYP agar may limit the vegetative growth of the strain A 11, but may also accelerate biosynthesis of secondary metabolites such as antifungal antibiotics.

## Acknowledgement

This research was supported by the Korea Industry-University Collaboration Foundation and Han Nong Corporation in 1994.

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**(Received September 15, 1995)**