Numerical Identification of a *Streptomyces* Strain Producing Spores in Submerged Culture

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Chemotaxonomic and numerical identification were carried out for a isolate of *Streptomyces* strain SMF301 producing spores in submerged culture. Fifty taxonomic unit characters were tested and the data were analyzed numerically using the TAXON program. The isolate SMF301 was identified to cluster 1A of *Streptomyces* and best matched to *Streptomyces limosus* which is a synonym of *Streptomyces albidoflavus*. Therefore, it was concluded that the isolate was identified to be a member of *Streptomyces albidoflavus*.

KEY WORDS □ ***Streptomyces albidoflavus*, Submerged spores, Numerical identification.**

The actinomycetes have been well recognized as producers of antibiotics and other useful bioactive metabolites. They are of interest also due to the unique morphological characters and spore formation. They grow as vegetative mycelia to form branched filaments in submerged culture and produce substrate mycelia and aerial mycelia on solid media(2). The aerial mycelia are differentiated into arthrospores aligned to mycelia when the culture conditions are shifted down (3, 20, 21). It has been thought that cell differentiation is closely related to the biosynthesis of secondary metabolites (21, 25). Studies on the elucidation of the mechanisms involved in cell differentiation have been carried out mostly on solid cultures. However, spore formation in submerged cultures would give some advantages in the quantitative analysis of the processes, hence, few species of *Streptomyces* have been used for the purposes (5, 6, 11, 21).

In this context, we selected an actinomycete isolated from soil, which was found to produce extra-cellular alkaline protease and also abundant spores in submerged cultures (10, 22, 23). As a first step we tried to identify the isolate to species level using chemical analysis and a numerical identification procedure (31, 32).

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MATERIALS AND METHODS

Isolation and Selection of Actinomycetes Producing Submerged Spores

Soil samples collected at Pyungtaek, Korea were dried at an oven controlled to 80°C for 4 h in order to give a selective advantage for heat resistant spores. The dried soil samples were suspended in sterilized distilled water and then inoculated onto on plates of actinomycete isolation medium supplemented with nystatin (50 μg/ml), cycloheximide (50 μg/ml), and penicillin (8 μg/ml) to prevent the growth of fungi and unwanted bacteria (8). Tough and leathery colonies having spores developed after 2 weeks cultivation were selected and purified on starch casein-nitrate-agar (13). Spores of the isolates were inoculated into starch-casein-nitrate liquid medium prepared in baffled flasks and cultured at 28°C for 5 d. Morphological characters and spore formation in submerged culture were observed using a phase-contrast microscope (X 600, Nikon Labophot, Japan). Strain SMF301 was selected as it produced extensively branched mycelia and spores but no fragments in submerged culture.

Culture Conditions and Diagnosis of Taxonomic Unit Characters

The isolate was kept on the slope of starch-

casein-nitrate agar medium and spores separated from mycelia by passing through glass wool (7). The spores were suspended in glycerol-nutrient broth and kept in a deep freezer at -70°C (30). The frozen spore suspensions were thawed at ambient temperature and used as inocula for the following experiments. For the purpose of observing cultural and morphological characters, the media used followed the recommendations of the International Streptomyces Project (ISP) (26). The solid cultures were carried out on agar plates and submerged cultures were studied in baffled flasks using a rotary shaking incubator at 28°C.

Cells grown in submerged culture using starch-casein-nitrate medium at 28°C were harvested and washed three times with physiological saline solution. The washed cells were disrupted with an ultrasonicator (Sonic Dismembrator Model 300, Fisher, USA. 100W for 30 min) in an ice bath and the cell walls separated from the lysates by ultracentrifugation (×11,000 g, for 15 min). Analysis of diaminopimelic acid (33) and whole-cell sugars (27) were carried out using thin-layer chromatography procedures. After cell fatty acids were esterified with anhydrous 5% methanol-HCl, the fatty acid methyl esters (FAME) were analyzed by gas chromatography (4). Menaquinones (MK) were extracted from cells with chloroform-methanol (2 : 1) in the dark, purified on TLC and analyzed by HPLC (28). The fifty unit characters used for the numerical identification of *Streptomyces* major clusters were tested following Williams *et al.* (31, 32) as shown in Table 1. The operational taxonomic units of the isolate were analyzed using the TAXON program (A.C. Ward, unpublished).

RESULTS AND DISCUSSION

Strain Selection and its Morphological Characters

Strain SMF301, isolated from soil, was selected since it produced typical streptomycete mycelia

Table 1. *Taxonomic Unit Characters used in the Identification of the unknown Streptomyces*

1. *Morphology and pigmentation*
   - Spore chain morphology : rectiflexibilis(RFS), spirales(SPI)
   - Color of spore mass : red(RED), gray(GRY)
   - Mycelial pigment : red/orange(ROS)
   - Diffusible pigment : production(PIG), yellow/brown(YBP)
   - Melanin production on : PYI medium(MPI), tyrosine medium(MTY)
2. *Antimicrobial activity*
   - *Bacillus subtilis*(SUB)
   - *Candida albicans*(ALB)
   - *Streptomyces murinus*(MUR)
   - *Micrococcus luteus*(LUT)
   - *Saccharomyces cerevisiae*(CER)
   - *Aspergillus niger*(NIG)
3. *Biochemical tests*
   - Lecithinase(LEC)
   - Pectin hydrolysis(PEC)
   - H_2S production(H2S)
   - Lipolysis(LIP)
   - Nitrate reduction(NO3)
   - Hippurate hydrolysis(HIP)
4. *Degradative tests*
   - Elastin(ELA)
   - Arbutin(ARB)
   - Xanthine(XAN)
5. *Antibiotic resistance*
   - Neomycin(NEO)
   - Oleandomycin(OLE)
   - Rifampicin(RIF)
   - Penicillin G(PEN)
6. *Growth test*
   - 45°C(45C)
   - Sodium azide(01Z)
   - Potassium tellurite(01T)
   - NaCl(7NA)
   - Phenol(PHN)
   - Thallous acetate(T01)
7. *Compounds as sole source of nitrogen*
   - DL-α-amino-n-butyric acid(BUT)
   - L-Valine(VAL)
   - L-Histidine(HIS)
   - L-Cysteine(CYS)
   - L-Phenylalanine(PHE)
   - L-Hydroxyproline(HYD)
8. *Organic compounds as sole source of carbon*
   - Sucrose(SUC)
   - Mannitol(MAN)
   - Raffinose(RAF)
   - Adonitol(ADO)
   - D-Melibiose(MEB)
   - meso-Inositol(INO)
   - L-Rhamnose(RHA)
   - D-Melezitose(MEZ)
   - Dextran(DEX)
   - Xylitol(XYT)

*The three letters in parenthesis are the code names for computer analysis.

but also produced abundant spores in both submerged culture and on solid media. Colony developing on the media tested were tough and leathery and the substrate mycelia were brown on all the media used (ISP media 1-7 and Bennett's media) except on inorganic salts-starch agar(ISP4) where the mycelia were yellow. Soluble pigments, including melanin, were not detected on glycerol-asparagine agar (ISP5). The color of the aerial spore mass developing on the surface of colonies was white (Fig. 1A and 1B). Formation of aerial mycelia on ISP4 agar medium and the formation of rectiflexibilis chains of spores on the aerial mycelia were clearly observed, spore surface ornamentation was smooth (Fig. 2). Vegetative hyphae grown in submerged culture using ISP 1 liquid medium were extensively branched but not fragmented and verticils were not observed. More interestingly it was observed that vegetative mycelia in submerged culture also developed rectiflexibile chain of spores (Fig. 3 and 4).

Chemotaxonomical and Physiological Characters

The type of diaminopimelic acid in the cell wall of the isolate (SMF 301) was identified from the cell wall hydrolysate as LL-DAP; alanine, glutamic acid, and glycine were also detected(Fig. 5). This indicates that the isolate has a wall chemotype I (15) and peptidoglycan type A3(24). However no diagnostic sugars were found in the whole-cell hydrolysate(Fig. 6), which suggested that the whole-cell sugar pattern was type C (16). Since iso-C_{14}, iso-C_{15}, anteiso-C_{15}, iso-C_{16}, n-C_{16}, iso-C_{17} were found in the mycelia of the isolate, the fatty acid profile was considered to be the type characteristic of *Streptomyces* (Fig. 7). MK-9 (H_6) and MK-9(H_8) were found to be the predominant menaquinones (Fig. 8). From the chemotaxonomical and morphological characterization, it was concluded that the strain SMF301 clearly belonged to the genus *Streptomyces*(17).

Numerical Identification using the TAXON Program

In order to identify the isolate to species level, the fifty taxonomical characters collected for isolate (SMF301) were numerically analyzed using the TAXON program and the *Streptomycetes* probability matrices based on 26 major clusters and 40 minor clusters respectively(14). The identification score (Willcox probability) of the isolate to subcluster 1A was much higher than

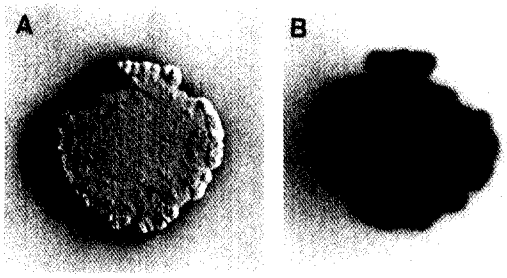

Fig. 1. *Color of spore mass(A) and pigmentation(B) of isolate SMF301 grown on Bennett's agar medium for 5 days.*

Fig. 3. *Mycelia, submerged spores and spore chains of isolate SMF301 cultured in Bennett's broth medium for 5 days. Phase contrast microphotograph, X1,500.*

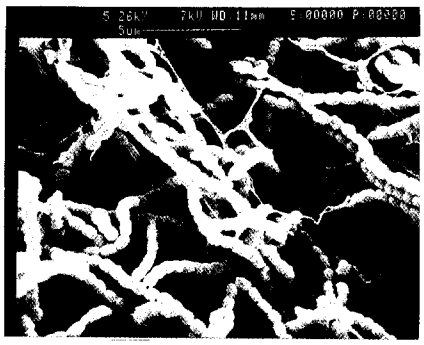

Fig. 2. *Scanning electron micrograph of isolate SMF 301 cultured on tryptone-yeast extract agar medium for 7 days.*

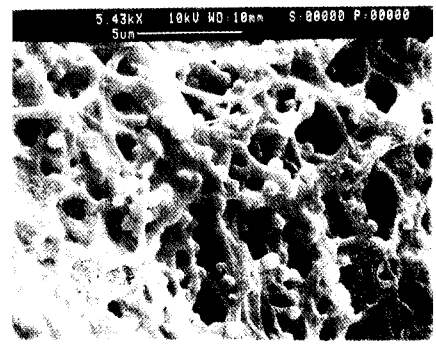

Fig. 4. *Scanning electron micrograph of isolate SMF 301 cultured on Bennett's broth medium for 5 days*

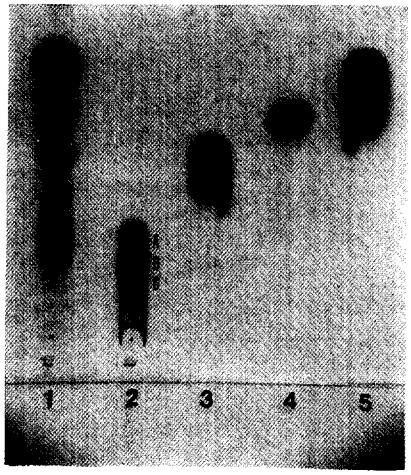

Fig. 5. *Cellulose thin layer chromatogram of cell wall diaminopimelic acid (DAP) isomers and amino acids of isolate SMF301.*
1. cell wall hydrolysate 2. DAP isomers 3. glycine 4. glutamic acid 5. alanine. A. LL-DAP, B. meso-DAP, C. 3-OH DAP.

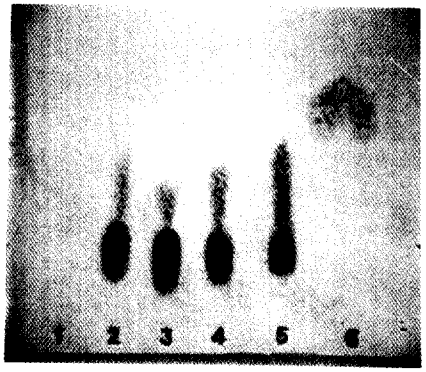

Fig. 6. *Cellulose thin layer chromatogram of whole cell sugar extract of isolate SMF301.*
(1. whole cell extract 2. arabinose 3. galactose 4. glucose 5. mannose 6. rhamnose)

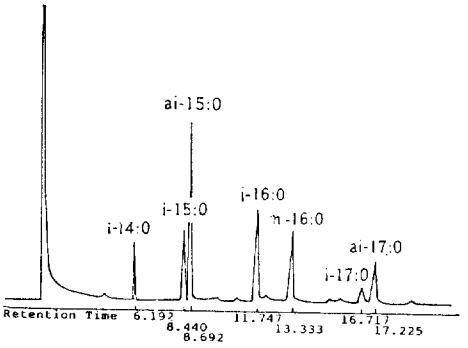

Fig. 7. *Gas chromatogram of cellular fatty acids of the isolate strain SMF301.*
Column: capillary column, detector: Flame ionization detector, Injection temperature: 250 °C, detector temperature: 250°C, column temperature: 180°C, carrier gas: N_2 gas(30 ml/min).

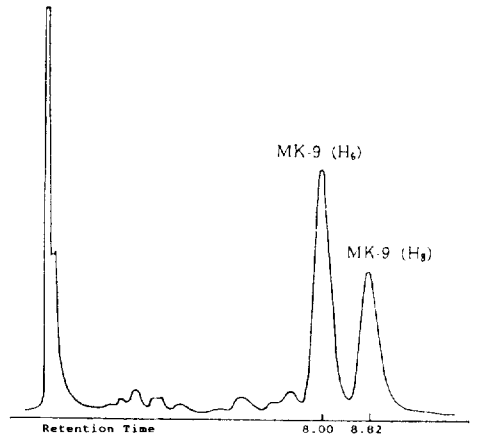

Fig. 8. *High performance liquid chromatogram of menaquinones (MK) prepared from isolate strain SMF301.*
Column: RP-C18, mobile phase: Methanol: isopropanol(2 : 1), detector: UV 270 nm, flow rate: 1 ml/min.

those of the next nearest cluster (cluster 42), hence, the isolate can be identified as a member of subcluster 1A (Table 2A). The taxonomic unit characters of the isolate were compared again with those of 20 member organisms in subcluster 1A of which *S. albidoflavus* is the type strain. It was found that the isolate was best matched to *S. limosus* in terms of positive responses: forty-one of the 50 taxonomic unit characters were matched to those of *S. limosus* (Table 3). The simple matching coefficient (S_{SM}) of the isolate to *S. limosus* was 82% and that to *S. albidoflavus* was 78%, these values were higher than those of the outer most member (60 %) (*Streptomyces* sp) (Table 4) of the group. The numerical analyses of the taxonomic unit characters of representative members in subcluster 1A are shown in Table 2B, C, and D.

The taxon distances between the Hypothetical Median Organism (HMO) and *S. albidoflavus* were smaller and relatively near to the centroid of subcluster 1A. The taxon radius within which 95% of members were included was relatively low. In addition the identification score (Willcox probability) and the probability of the strain further way were very high (Table 2B and 2C). From these results, it was thought that subcluster 1A was a very compact group and that subcluster

Table 2. *Numerical identification of Streptomyces strain SMF301 using the TAXON program.*

A: Identification of strain SMF301 to *Streptomyces* clusters.

Cluster	TAXONOMIC distance	95% TAXONOMIC radius	Probability of SMF301 further away (%)	Identification Score*
1A(*S. albidoflavus*)	0.4339	0.3782	0.0400	0.998950
42(*S. rimosus*)	0.4441	0.3507	0.0001	0.000516
1B(*S. anulatus*)	0.5006	0.4404	0.0619	0.000534
1C(*S. halstedii*)	0.5259	0.3883	0.0000	0.000000
40(*S. phaeochromagenes*)	0.5482	0.3805	0.0000	0.000000

*Willcox Probability

B: Identification of the Hypothetical Median Organisms (HMO) of *Streptomyces albidoflavus* and related strains

Cluster	TAXONOMIC distance	95% TAXONOMIC radius	Probability of HMO further away (%)	Identification Score*
1A(*S. albidoflavus*)	0.2205	0.3782	99.9285	>0.999999
1B(*S. anulatus*)	0.4334	0.4404	7.2052	0.000000
1C(*S. halstedii*)	0.4337	0.3883	0.1348	0.000000
3(*S. atroolivaceus*)	0.4804	0.3631	0.0000	0.000000
15(*S. chromofuscus*)	0.4951	0.4271	0.0243	0.000000
SMF301 to HMO in Cluster 1A Matched=38 Mismatched=12				

*Willcox Probability

C: Identification of *S. albidoflavus* strain to the clusters of *Streptomyces*.

Cluster	TAXONOMIC distance	95% TAXONOMIC radius	Probability of centrotype further away (%)	Identification Score*
1A(*S. albidoflavus*)	0.2462	0.3782	99.1805	>0.999999
1B(*S. anulatus*)	0.4453	0.4404	3.7859	0.000000
1C(*S. halstedii*)	0.4455	0.3883	0.0400	0.000000
3(*S. atroolivaceus*)	0.4687	0.3631	0.0000	0.000000
15(*S. chromofuscus*)	0.4924	0.4271	0.0317	0.000000
Matched=47 Mismatched=3				

*Willcox Probability

D: Identification of *S. limosus* (the best matched organism to SMF 301) to *Streptomyces* clusters.

Cluster	TAXONOMIC distance	95% TAXONOMIC radius	Probability of HMO further away (%)	Identification Score*
1A(*S. albidoflavus*)	0.3204	0.3782	55.0628	0.999788
1B(*S. anulatus*)	0.4160	0.4404	15.7728	0.000212
1C(*S. halstedii*)	0.4524	0.3883	0.0187	0.000000
40(*S. phaeochromogenes*)	0.4638	0.3805	0.0014	0.000000
12(*S. rochei*)	0.4692	0.4173	0.1010	0.000001
Best matched to SMF301 Matched=41 Mismatched=9				

*Willcox Probability

Table 3. *Comparison of taxonomic unit characters between member organisms in Cluster 1A of Streptomyces and strain SMF301. And willcox probabilities calculated using the TAXON program.*

Taxonomic unit characters (TAXON code)	S_{sm}*value in cluster 1A	HMO** in cluster 1A	*Streptomyces albidoflavus* ISP	*Streptomyces limousus* ISP	SMF301
RFS	70	+	+	+	+
SPI	1	−	−	−	−
RED	1	−	−	−	−
GRY	1	−	−	−	−
ROS	5	−	−	−	−
PIG	20	−	−	−	−
YBP	15	−	−	−	−
MPI	1	−	−	−	−
MTY	5	−	−	−	−
SUB	1	−	−	−	−
LUT	1	−	−	−	−
ALB	85	+	+	+	+
CER	85	+	+	+	+
MUR	10	−	−	−	−
NIG	55	+	+	+	+
LEC	5	−	−	−	+
LIP	99	+	+	+	+
PEC	5	−	−	−	−
NO3	25	−	−	−	+
H2S	90	+	+	+	−
HIP	1	−	−	−	−
ELA	99	+	+	+	+
XAN	99	+	+	+	+
ARB	95	+	+	+	+
NEO	1	−	−	−	+
RIF	45	−	−	−	−
OLE	80	+	+	+	+
PEN	99	+	+	+	+
45C	1	−	−	−	−
7NA	95	+	+	+	+
01Z	85	+	+	+	+
PHN	95	+	+	+	+
01T	95	+	+	+	+
T01	25	−	+	−	+
BUT	70	+	+	+	+
CYS	70	+	+	+	+
VAL	35	−	−	+	+
PHE	75	+	+	+	+
HIS	45	−	+	+	+
HYD	1	−	−	−	−
SUC	45	−	−	+	−
INO	55	+	+	+	+
MAN	99	+	+	+	+
RHA	20	−	−	+	−
RAF	5	−	−	−	+
MEZ	50	+	−	+	+
ADO	45	−	−	+	+
DEX	20	−	−	+	+
MEB	30	−	−	−	+
XYT	5	−	−	+	+
Willcox Probability		>0.999999	0.999999	0.999788	0.998950

Table 4. *Simple matching coefficient(S_{SM}) of isolate SMF301 to member organisms in Streptomyces subcluster 1A*

ISP NO.	Stants	ATCC NO.	S_{SM} (%)
5131	*Streptomyces limosus*	19778	82
5296	*Streptomyces craterifer*	25445	80
5455	*Streptomyces albidoflavus*	25422	78
5130	*Streptomyces felleus*	19752	78
5585	*Streptomyces tetanusemus*	27471	78
135	Streptomyces sp	LIV 135	76
5394	*Streptomyces sampsonii*	25495	74
195	*Streptomyces* sp	LIV 195	74
5537	*Streptomyces saprophyticus*	3351	72
5077	*Streptomyces rutgersensis*	3350	72
5364	*Streptomyces citres*	25441	72
5485	*Streptomyces coriofaciens*	14155	70
5321	*Streptomyces krainskii*	25465	68
5001	*Streptomyces canescens*	19736	68
5347	*Streptomyces ordorifer*	6246	66
464	*Streptomyces* sp	LIV 464	66
5233	*Streptomyces coelicolor*	23899	64
5372	*Streptomyces intermedius*	3329	62
422	*Streptomyces* sp	LIV 422	60
5324	*Streptomyces gougerotii*	10975	56

*Cited from Williams *et al.* (1983a).

1A was clearly distinguished from the other cluster groups. However the taxon distance value of the isolate SMF301 was a little larger than 95% taxon radius and the probability of the strain to be included in the cluster (probability further away) was very low. These data indicated that the isolate could belong to the outer part of subcluster 1A.

Major Cluster 1 consists of 71 member species which are subdivided into subcluster 1A (type strain: *S. albidoflavus*), 1B (type strain: *S. anulatus*), and 1C (type strain: *S. halstedii*) at the 81% similarity level (32). This indicates that the 20 member species in cluster 1A are clearly defined at the 81% S_{SM} similarity level but are the related to cluster 1B and 1C strains at the 77.5% similarity level. *S. albidoflavus* together with *S. anulatus* and *S. halstedii* can be considered to be approximately equivalent to the *S. griseus* group (9, 18, 29). In reacent reports, member species in subcluster 1A were reclassified as *S. albidoflavus* (17). The species reclassified as *S. albidoflavus* were previously considered to be synonyms of *S. coelicolor* based on high DNA homologies (12, 19).

From the numerical analyses and comparisons of the similarity levels, it can be concluded that the isolate can be identified as a member of *S. albidoflavus*.

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초 록: 심층 배양에서 포자를 형성하는 *Streptomyces* sp. 분리균주의 수리동정

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액체배양시 沈層포자를 형성하는 *Streptomyces* 一株를 분리하여 화학적 분류 및 수리동정을 실시하였다. 50개 분류 단위 형질을 분석하였고, 이 실험 결과를 이용하여 종의 수리동정을 위해서는 TAXON program을 사용하였다. 수리동정 결과 분리주는 *Streptomyces* 주군집 1A에 속하며, 주균집 1A의 중심균주인 *Streptomyces albidoflavus*와 synonym으로 사용되는 *Streptomyces limousus*와 가장 형질의 유사성이 높았다. 따라서 분리주는 *Streptomyces albidoflavus*의 아균집에 속하는 한 균주로 판단된다.