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Numerical Identification of a *Streptomyces* Strain Producing β-Lactamase Inhibitor

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Numerical identification was carried out for an isolate of *Streptomyces* strain producing the extracellular β-lactamase inhibitor. Fifty taxonomic unit characters were tested and the data were analyzed numerically using the TAXON program. The isolate was identified to the major cluster 5 of *Streptomyces* and it was best matched to *Streptomyces omiyaensis* which is a synonym of *Streptomyces exfoliatus*. Therefore, it was concluded that the isolate was identified to be a strain (SMF19) of *Streptomyces exfoliatus*.

KEY WORDS □ **Numerical identification, β-lactamase inhibitor, *Streptomyces exfoliatus*, *Streptomyces omiyaensis***

Streptomycetes are well-known as a rich source of antibiotics and proof of the novelty of an antibiotic has frequently rested on the discription of the producer as a new species. This practice, coupled with the failure of taxonomists to find reliable tests for the identification of streptomycetes, has resulted in the proliferation of species, many of which were proposed on trivial differences in morphological and cultural properties (9). The definition and recognition of *Streptomyces* species have provided taxonomists with a major problem for many years. The first attempt to construct a numerical classification of streptomycetes using a wide range of characters was made by Silverstri *et al.* (8). Thereafter a number of attempts to construct both numerical classification and identification systems were followed but they were based on a relatively small number of characters. A more comprehensive numerical classification of streptomycetes and related genera was constructed by Williams *et al.* (12). The data from this provided a basis for the construction of probabilistic identification matrix for streptomycetes (13) using various new computer programs.

We isolated a strain of streptomycete producing extracellular β-lactamase inhibitor (2). We attemped to identify the strain by numerical analysis using the TAXON program developed by Dr. Ward (unpublished).

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MATERIALS AND METHODS

Microorganism and Culture Conditions

The microorganism used in this study was *Streptomyces* strain SMF19 producing β-lactamase inhibitor (2). The isolate was kept on the slope of starch-casein-nitrate agar medium (3). Spores developed on the slopes were separated from mycelia by passing through glass wool and the separated spores were suspended in glycerol-nutrient broth (1) and kept in a deep freezer at −70°C (10). The frozen spore suspensions were thawed at ambient temperature and used as inocula for the following experiments. Cultural and morphological characters were observed with the media of the International *Streptomyces* Project (ISP) (7). 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.

Chemotaxonomical Characters

Cells grown in submerged culture 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 3 min) in ice bath and the cell walls separated from the lysates by ultracentrifugation (×11,000 g, for 15 min). After acid hydrolysis, analysis of diaminopimelic acid and whole cell sugars were carried out using thin layer chromatography procedures (5).

Table 1. *Taxonomic unit characters used in the identification of an isolate of using the probability matrix of the Streptomyces major cluster and TAXON program**

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

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

Taxonomic Unit Characters and Determination of Identification Scores

The taxonomic unit characters used for the numerical identification of *Streptomyces* major cluster were tested following Williams *et al.* (12, 13) as shown in Table 1. The identification scores of the isolate were determined using the TAXON program.

Willcox probability (11): This is the likelihood of an unknown isolate (u) against taxon J divided by the sum of the likelihoods of u against all taxa. The nearer the score approaches 1.0, the better is the fit of an unknown isolate with a group in the matrix.

Taxonomic distance: This expresses the distance of an unknown isolate from the centroid of the group with which it is being compared. Lower score indicates closer relatedness to the group. The score can be calculated the following relations: $[\Sigma(U_i - P_{ij})^2/m]^{1/2}$. Where m is the number of characters, U_i is the score of u on character i (either 1 for positive or 0 for negative), and P_{ij} is the propotion of positives given by strains of taxon J on character i.

95% Taxon radius: This expresses the taxon radius within which 95% of members of taxon J are included.

RESULTS AND DISCUSSION

Morphological and Chemotaxonomical Characters

The isolate, SMF19, produced typical streptomycete mycelia and also spores on solid media. Colonies developing on the media tested were tough and leathery. The substrate mycelia were yellow-brown on glycerol-asparagine agar medium (ISP5). Diffusible pigments were not detected on ISP5 medium. Melanin production was not

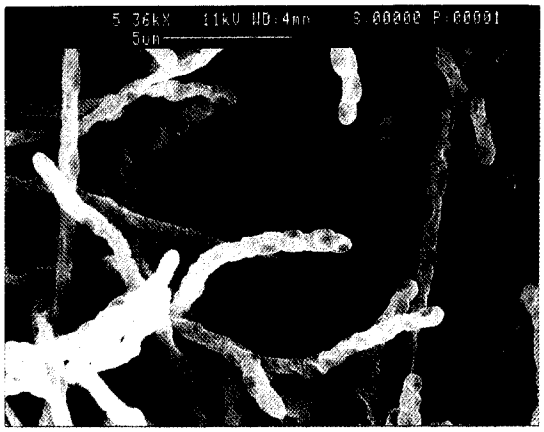

Fig. 1. *Spore chain of the isolate cultured on inorganic salts starch agar medium (ISP4) (by scanning electron microscope).*

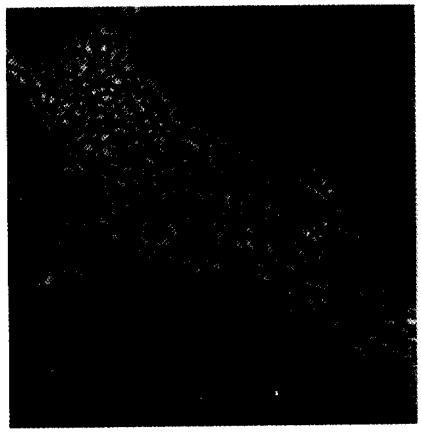

Fig. 2. *Vegetative hyphae of the isolate SMF19 cultured in Bennet liquid medium.*
Phase contrast microphotograph (×800)

Fig. 3. *Cellulose thin layer chromatogram of cell wall diaminopimelic acid(DAP) isomer of the isolate SMF19.*
1. cell wall hydrolysate 2. DAP isomers

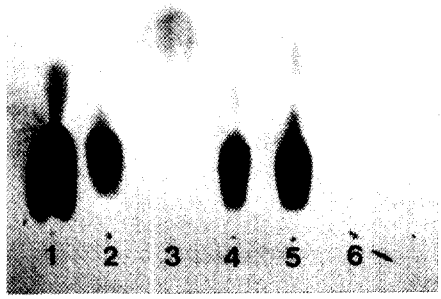

Fig. 4. *Cellulose thin layer chromatogram of whole cell sugar extract of the isolate SMF19.*
1. glucose 2. arabinose 3. rhamnose 4. galactose 5. mannose 6. whole cell extract

detected on peptone yeast iron agar medium (ISP 6) but detected on tyrosine agar medium (ISP7). The color of the aerial spore mass grown on inorganic salts starch agar medium (ISP4) was grey. The isolate SMF19 developed long rectiflexible spores in chains and spore surface ornament was smooth (Fig. 1). Vegetative hyphae grown in submerged culture using Bennet liquid medium were extensively branched but not fragmented and verticils were not observed (Fig. 2). The diaminopimelic acid (DAP) in the cell wall of the isolate was identified to be a LL-DAP isomer (Fig. 3) and the diagnostic sugars in the whole cell hydrolysate were not detected (Fig. 4). These results indicated that the isolate had chemotype I cell wall and type C whole cell sugar pattern. From the morphological and chemotaxonomical characterization, it was concluded that the isolate SMF19 belonged to the genus *Streptomyces* (6).

Numerical Identification using the TAXON Program

In order to identify the isolate to species level, the fifty taxonomical unit characters of the isolate SMF19 was numerically analyzed using the TAXON program based on 26 major clusters which have been previously defined as a result

Table 2. *Identification scores of the isolate SMF19 to the clusters of Streptomyces by TAXON program*

Major cluster	TAXON distance	95% TAXON radius	Probability of SMF19 further away (%)	Identification Score*
5	0.4267	0.4455	12.4069	0.997914
1C	0.4739	0.3883	0.0013	0.000871
1B	0.4749	0.4404	0.5311	0.000612
19	0.5141	0.4508	0.0532	0.000472
3	0.4611	0.3631	0.0001	0.000131

*Willcox probability

Table 3. *Comparison of taxonomic scores between hypothetical median organism (HMO), centrotype, outer-most member strain (OMS), and the isolate SMF19 in cluster 5*

Member strain in cluster 5	TAXON distance	95% TAXON radius	Probability of members further away (%)	Identification Score*
H M O	0.2800	0.4455	99.6117	>0.999999
Centrotype (*S. roseolus*)	0.3242	0.4455	93.4908	0.999529
O M S (*S. umbrinus*)	0.4837	0.4455	0.4159	0.986515
Isolate, SMF19	0.4267	0.4455	12.4069	0.997914

*Willcox Probability

of classification of the streptomycetes (4). In the TAXON program, the criteria adopted for a successful identification were: (a) a willcox probability greater than 0.850 with low scores for taxonomic distance; (b) first group scores substantially better than those against the next best two alternatives; (c) characters against should be zero or few. The willcox probability of the isolate SMF19 to the major cluster 5 was 0.997914 and much higher than those of the next nearest cluster, cluster 1C (Table 2). It was indicated that the isolate could belong to the cluster 5.

The identification scores of the hypothetical median organism (HMO), centrotype (*S. roseolus*), the outer-most member strain (*S. umbrinus*), and the isolate SMF19 in the cluster 5 were compared (Table 3). The taxon distance of the Hypothetical Median Organism (HMO) was small and the willcox probability was 0.999999. The taxon radius within which 95% of members was relatively higher than the taxon distance of HMO. The willcox probabilities of centrotype (*S. roseolus*) and outer-most member (*S. umbrinus*) of cluster 5 were 0.999529 and 0.986515 respectively. In addition the probabilities of the HMO and centrotype to be included in the cluster (probability of strain further away) were very high. From these results, it was thought that cluster 5 was a very compact group.

The taxon distance of the isolate was further than that of the centrotype but nearer than that of the outer-most member strain. And the taxon distance value of the isolate SMF19 was smaller than 95% taxon radius of cluster 5. The probability of the strain to be included in the cluster was higher than that of the outer-most member. The willcox probability of the isolate was between that of the centrotype (*S. roseolus*) and that of the outer-most member (*S. umbrinus*) of cluster 5. These results cleary indicated that the isolate located inner part of cluster 5, which was more closer to the hypothetical median organism

Table 4. *Simple matching coefficient (S_{SM}) of the isolate SMF19 to member organisms in Streptomyces cluster 5*

ISP No.	Strain	ATCC No.	S_{SM} (%)
5552	*Streptomyces omiyaensis*	27454	82
5558	*Streptomyces hydrogenans*	19631	78
5329	*Streptomyces termitum*	25499	76
5174	*Streptomyces roseolus*	23210	76
5064	*Streptomyces gardneri*	23911	70
5314	*Streptomyces nashvillensis*	25476	70
5541	*Streptomyces flavochromogenes*	14841	70
5122	*Streptomyces roseosporus*	23958	68
TI	*Streptomyces* sp.		68
5164	*Streptomyces litmocidini*	19914	66
5279	*Streptomyces violaceorectus*	25514	66
5022	*Streptomyces filamentosus*	19753	66
5016	*Streptomyces narbonensis*	19790	64
5175	*Streptomyces roseoviridis*	23959	60
5060	*Streptomyces exfoliatus*	12627	58
5196	*Streptomyces zaomyceticus*	27482	58
5012	*Streptomyces cineroruber*	19740	56
5278	*Streptomyces umbrinus*	19929	56

*Cited from Williams *et al.* (12).

Table 5. *Comparison of taxonomic unit characters between member organisms in Cluster 5 of Streptomyces*

Taxonomic unit characters (TAXON code)	Positive value (%)	HMO in cluster 5	*Streptomyces roseolus* ISP	*Streptomyces omiyaensis* ISP	SMF-19
RFS	99	+	+	+	+
SPI	1	−	−	−	−
RED	39	−	−	−	−
GRY	39	−	−	+	+
ROS	11	−	−	−	−
PIG	39	−	−	−	−
YBP	28	−	−	−	−
MPI	61	+	−	−	−
MTY	61	+	−	−	+
SUB	56	+	−	−	−
LUT	44	−	−	−	+
ALB	1	−	−	−	−
CER	1	−	−	−	+
MUR	39	−	−	−	−
NIG	6	−	−	−	−
LEC	50	+	−	−	−
LIP	94	+	+	+	−
PEC	61	+	+	+	+
NO3	83	+	+	+	+
H2S	89	+	+	+	+
HIP	44	−	−	+	−
ELA	89	+	+	+	+
XAN	94	+	+	+	+
ARB	99	+	+	+	−
NEO	11	−	−	+	−
RIF	11	−	−	−	−
OLE	44	−	+	+	+
PEN	44	−	−	+	+
45C	17	−	−	−	−
7NA	22	−	−	−	+
01Z	22	−	−	−	−
PHN	72	+	−	+	+
01T	83	+	+	+	+
T01	6	−	−	−	+
BUT	61	+	−	+	+
CYS	50	+	−	+	+
VAL	50	+	−	+	+
PHE	83	+	+	+	+
HIS	78	+	+	+	+
HYD	89	+	+	+	+
SUC	28	−	−	−	−
INO	6	−	−	−	−
MAN	1	−	−	−	−
RHA	61	−	+	+	+
RAF	33	−	−	−	−
MEZ	22	−	−	−	−
ADO	1	−	−	−	−
DEX	6	−	−	−	−
MEB	28	−	−	−	−
XYT	1	−	−	−	−
Matching to SMF19		38	38	41	50
Mismatching to SMF19		12	12	9	0
Willcox* probability		>0.999999	0.999529	0.929468	0.997914

*The willcox probability calculated by the TAXON program.

than the outer-most member strain.

Simple matching coefficients of the isolate to members in the cluster 5 were obtained. The simple matching coefficient (S_{SM}) of the isolate to *S. omiyaensis* was 82% and that to *S. roseolus* was 76% (Table 4). These values were higher than that of to the outer most member (*S. umbrinus*; 56%).

The taxonomic unit characters of the isolate SMF19 were compared with those of the hypothetical median organism (HMO), centrotype (*S. roseolus*) and the best-matched member (*S. omiyaensis*) in cluster 5 (Table 5). It was evident that the isolate was best matched to *S. omiyaensis* in terms of positive responses.

In recent reports, the member species in cluster 5 were reclassified as *S. exfoliatus* based upon the numerical analyses and comparisons of the similarity levels (6). Therefore we concluded that the isolate SMF19 was one strain (SMF19) of *S. exfoliatus*.

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초 록: β-Lactamase 저해 물질을 생산하는 *Streptomyces*속 분리균주의 수리동정
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세포외로 β-lactamase 저해물질을 생산하는 *Streptomyces*속 한 균주(SMF19)를 분리하여 형태적 관찰 및 수리동정을 실시하였다. 50개의 분류단위 형질을 분석하였고 이 실험결과를 TAXON program에 적용하여 종의 수리동정을 실시하였다. 그 결과 분리균 SMF19는 *Streptomyces*의 제 5 주군집에 속하며 이 군집에 속하는 *S. omiyaensis*와 유사성이 가장 높은 것으로 나타났다. *S. omiyaensis*는 주 군집 5의 대표균주인 *S. exfoliatus*의 synonym이므로 분리균은 *S. exfoliatus*의 한 균주로 동정되었다.