Journal of Microbiology and Biotechnology
Vol. 2, No. 3, 220-225, 1992

Numerical Identification of a *Streptomyces* Strain Producing Thiol Protease Inhibitor

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Chemotaxonomic and numerical identification were carried out for an isolate of *Streptomyces* strain SMF13 producing thiol protease inhibitor. Fifty taxonomic unit characters were tested and the data were analyzed numerically using the TAXON program. The isolate SMF13 was identified to be a member of the cluster 5 of *Streptomyces* and 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 of *Streptomyces exfoliatus*.

The genus *Streptomyces* accomodates aerobic Gram-positive bacteria that form extensive branching mycelia and spores (2, 5, 14). LL-diaminopimelic acid and glycine in the cell wall components are thought to be the most distinguished characters of the genus (2, 7, 8, 12) and high content of G+C (69~73 mol%) is one of their remarkable characters (11). *Streptomyces* species are well-known as good producers of antibiotics, enzymes, and biologically active metabolites. Many attempts have been made to meet new species to produce novel metabolites, hence, several hundred species of *Streptomyces* have been described based upon the subjectively chosen criteria of morphological and physiological characters (15). However, such approaches are subjected to provide identifications of little general predictive values. Strong demands to develop more elaborated systematics for the taxonomical classification in the genus have been arisen. In this context, various methods were developed and provided diagnotic tables for the identification of unkown *Streptomyces* isolates (1, 17).

In order to elucidate the biological roles of protease inhibitors in *Streptomyces* spp, we isolated a strain (SMF 13) of *Streptomyces* producing protease inhibitor extracellularly (3, 4, 10). we tried to identify the isolate to species level using numerical identification procedure (18, 19).

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Key words: *Streptomyces exfoliatus*, protease inhibitor, numerical identification.

MATERIALS AND METHODS

Microorganism and Culture Conditions

The microorganism used in this study was an isolate, SMF13, of actinomycetes isolated from soil in Korea. The characters of the isolate were reported in elsewhere (3, 4, 10). The isolate was transferred on the slope of starch-casein-nitrate agar medium each month and kept in a refrigerator. Spores formed on the medium were separated from mycelia by passing through glass wool and the spores were suspended in glycerol-nutrient broth then kept in a deep freezer at −70℃ (16). 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) (13).

Chemotaxonomical Characters

Cells grown in submerged culture using Bennet medium at 28℃ were harvested and washed three times with physiological saline solution. The washed cells were disrupted with an ultrasonicator (Sonic Dismembrator Model 300, Fisher, USA. 100 W for 3 min) in an 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 (8).

Diagnosis of Taxonomic Unit Characters and Numerical Identification

The fifty unit characters used for the numerical identification of *Streptomyces* major clusters were tested following Williams *et al.* (18, 19) as shown in Table 1. The characters were analyzed numerically using the TAXON program and the taxonomical relations of the isolate to the identified species of *Streptomyces* were evaluated with the following indices (17, 18): (1) Willcox probability which is the likelihood of unknown isolate (u) against taxon J divided by the sum of the likelihoods of against all taxa, i.e. $L_{uJ}/\Sigma L_{uJ}$. The nearer the score approaches 1.0, the better is the fit of an unknown with a group in the matrix. (2) Taxonomic distance which is given by $[\Sigma(U_i-P_{ij})^2/m]^{1/2}$ 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. This expresses the distance of unknown from the centroid of the group with which it is being compared; hence low scores indicate relatedness. (3) 95% Taxon radius that expresses the taxon radius within which 95% of members of taxon J are included.

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), 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(H2S)	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℃ (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.

RESULTS AND DISCUSSION

Morphological and Chemotaxonomical Characters

Colonies of the isolate (SMF13) developed on the solid media were tough and leathery. And substrate mycelia on the glycerol-asparagine agar solid media (ISP 5) were yellowish-brown whereas diffusible pigments were not detected. Melanin production was detected on the peptone yeast iron agar medium (ISP6) but not on the tyrosine agar (ISP7). The color of the aerial spore mass developing on the surface of colonies on inorganic salts starch agar (ISP4) was grey. The aerial mycelia turned to rectiflexcible chain of spores and spore surface ornamentation 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 type of diaminopimellic acid in the cell wall of the isolate was identified from the cell wall hydrolysate as LL- DAP; glycine was also detected (Fig. 3). This indicates that the isolate has a wall chemotype I and peptidoglycan type A3. However no diagnostic sugars were detected in the whole-cell hydrolysate (Fig. 4), which suggested that the whole-cell sugar pattern was type C. From the morphological and chemotaxonomical characterization, it was concluded that the isolate (SMF13) clearly belonged to the genus *Streptomyces* (9).

Numerical Identification using the TAXON Program

In order to identify the isolate to species level, the fifty taxonomical characters collected for isolate (SMF13) were numerically analyzed using the TAXON program and the *Streptomycetes* probability matrices based on 26 major clusters and 40 minor clusters (6). In the numerical anaysis, 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

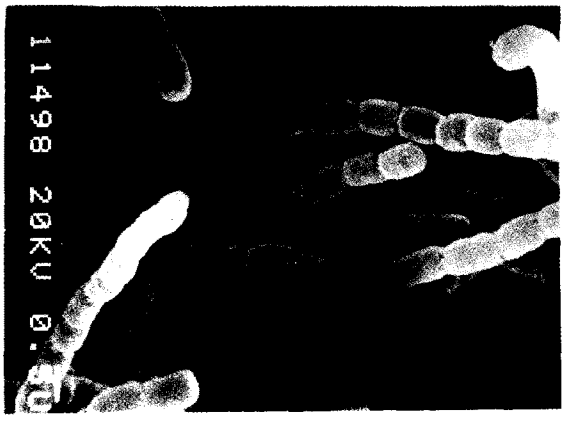

Fig. 1. Scanning electron micrograph of siolate SMF13 cultured on inorganic salt starch agar for 14 days.

Fig. 2. Mycelial forms of 3 day old submerged culture in Bennet medium by phase contrast microscopy.

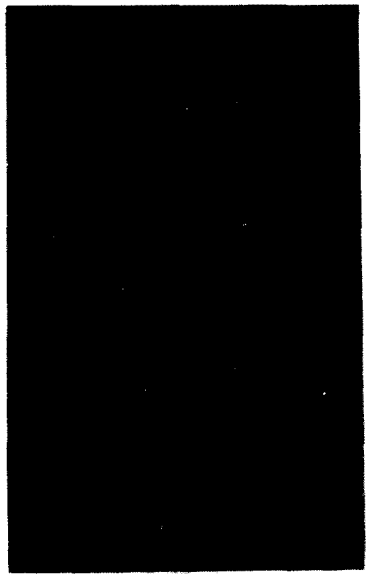

Fig. 3. Cellulose thin layer chromatogram of cell wall diaminopimelic acid (DAP) isomers and amino acids of isolate SMF13.
1. cell wall hydrolysate 2. DAP isomer (A: LL-DAP, B: meso-DAP, C: 3-OH DAP) 3. glycine

Fig. 4. Cellulose thin layer chromatogram of whole cell sugar extract of isolate SMF13.
1. whole cell extract 2. glucose 3. arabinose 4. rhamnose 5. galactose 6. mannose

be zero or few (17).

The identification score (Willcox probability) of the isolate to cluster 5 was 0.999120 and much higher than that of the next nearest cluster, cluster 1C, (Table 2). The Willcox probability of the Hypothetical Median Organism (HMO) in the cluster 5 was 0.999999 and that of the centrotype in the cluster 5 (*S. roseolus*) was 0. 999529 (Table 3). The Willcox probability of the isolate was much greater than that of the outer-most member of cluster 5 (0.986515). The TAXON distances of the HMO and the centrotype were 0.2800 and 0.3242 and those of the isolate and the outer-most member were 0.3690 and 0.4837, respectively (Table 3). The TAXON distance of the isolate was smaller than the 95% taxon radius in which 95% of members were included. In addition, the probabilities of the HMO and centrotype strain further away which imply the probability of the strain to be included in the cluster were very high (Table 3). From these results, it was thought that the cluster 5 was a compact group and that the isolate was identified as a member of the cluster 5, which located inner part of the cluster.

Table 2. Identification scores of the strain SMF13 to the clusters of *Streptomyces* by TAXON program.

Cluster	TAXON distance	95% TAXON radius	Probability of SMF13 further away (%)	Identification score*
5	0.3690	0.4455	63.5068	0.999120
1C	0.4179	0.3883	0.5720	0.000813
3	0.4243	0.3631	0.0159	0.000011
19	0.4542	0.4508	4.1639	0.000055
1B	0.4782	0.4404	0.4119	0.000000

*Willcox probability

Table 3. Comparisons of taxonomic scores between hypothetical median organism(HMO), centrotype, outer-most member strain(OMS), best matched organism(BMO) and isolate SMF13 in cluster 5.

Member strain in cluster 5	TAXON distance	95% TAXON radius	Probability of further away(%)	Identification score*
HMO	0.2800	0.4455	99.6117	>0.999999
Centrotype (*S. roseolus*)	0.3242	0.4455	93.4908	0.999529
OMS (*S. umbrinus*)	0.4837	0.4455	0.4159	0.986515
BMO (*S. omiyaensis*)	0.3409	0.4455	85.9599	0.929468
Isolate SMF13	0.3690	0.4455	63.5068	0.999120

*Willcox probability

Table 4. Simple matching coefficient(S_{SM}) of the isolate SMF13 to member organisms in *Streptomyces* cluster 5.

ISP NO.	Strain	ATCC NO.	S_{SM}(%)
5552	*Streptomyces omiyaensis*	27454	92
5558	*Streptomyces hydrogenans*	19631	84
5329	*Streptomyces termitum*	25499	82
5174	*Streptomyces roseolus*	23210	82
5064	*Streptomyces gardneri*	23911	80
5314	*Streptomyces nashvillensis*	25476	76
5122	*Streptomyces roseosporus*	23958	74
5541	*Streptomyces flavochromogenes*	14841	72
5164	*Streptomyces litmocidini*	19914	72
TI	*Streptomyces sp.*		70
5279	*Streptomyces violaceorectus*	25514	68
5022	*Streptomyces filamentosus*	19753	68
5175	*Streptomyces roseoviridis*	23959	66
5016	*Streptomyces narbonensis*	19790	66
5060	*Streptomyces exfoliatus*	12627	64
5196	*Streptomyces zaomyceticus*	27482	64
5012	*Streptomyces cineroruber*	19740	58
5278	*Streptomyces umbrinus*	19929	54

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

The taxonomic unit characters of the isolate were compared again with those of 18 member organisms in the cluster 5. From the simple matching coeffcient (S_{SM}) analysis, it was found that the isolate was best matched to *S. omiyaensis* and *S. umbrinus* was found again to be the worst matched member (Table 4).

The cluster 5 consists of 18 member species which are clearly defined at the 77.5% S_{SM} similarity level. However, it could be divided at the 79% S_{SM} and 63% S_J similarity levels into strains not producing melanin or other diffusible pigments and those that all produced melanin and sometimes other pigments. The group I included the type strain such as *S. exfoliatus*, *S. fiamentosus*, *S. hydrogenans*, *S. omiyaensis*, *S. roseolus*, *S. roseosporus*, *S. termitum*, *S. cinereuber*, *S. gardneri*, *S. litmocidini*. The group II included the allied species such as *S. narbonensis*, *S. nashvillensis*, *S. roseoviridis*, *S. umbrinus*, *S. violaceorectus*, *S. zoomyceticus*. From the comparison of taxonomic unit characters among member organism in the cluster 5, it was found that the isolate was a member of the cluster 5 belonged to the group I (Table 5).

However in recent reports, all member species in the cluster 5 were reclassified as *S. exfoliatus* (9). Therefore, we concluded that the isolate was a member strain (SMF 13) of *S. exfoliatus*.

Table 5. Comparison of taxonomic unit characters among member organisms in cluster 5 of *Streptomyces* and the Willcox probability calculated by TAXON program.

Taxonomic unit characters (TAXON code)	Positive character states (%)	HMO in cluster 5	*Streptomyces roseolus* ISP	*Streptomyces omiyaensis* ISP	SMF13
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 SMF13		41	42	46	50
Mismatching to SMF13		9	8	4	0
Willcox probability		>0.999999	0.999529	0.929468	0.999120

Acknowledgement

This work was supported by the KOSEF research grant to the RCMM in Seoul National University. The authors are very grateful for the supports and Y. T. Rho for the cell wall analyses is also acknowledged.

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(Accepted 15 September 1992)