APPLICATION OF THE RANDOM AMPLIFIED POLYMORPHIC DNA USING THE POLYMERASE CHAIN REACTION FOR EFFICIENT ELIMINATION OF DUPLICATE STRAINS IN MICROBIAL SCREENING

II. ACTINOMYCETES

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We evaluated the random amplified polymorphic DNA (RAPD) method using *Streptomyces lavendulae* and *Streptomyces virginiae* strains to eliminate duplicate actinomycete strains in our microbial screening program. The RAPD data were compared with phenotypic characteristics, DNA relatedness, HPLC analysis of metabolites and low-frequency restriction fragment analysis by pulsed-field gel electrophoresis. These results were consistent with each other. Therefore, we conclude that RAPD is a simple, efficient, and reliable method for the selection of actinomycete strains.

A large number of actinomycete strains isolated and selected empirically by taxonomists, have been subjected to microbial screenings. For improving the efficiency of screenings we need a method that could eliminate strains, particularly similar ones isolated from different samples.

Low-frequency restriction fragment analysis (LFRFA) of DNA by pulsed-field gel electrophoresis (PFGE) as a relatively new technique for analysing large DNA fragments has recently proved to be useful for identifying genetic variation of microorganisms[1,2]. It has been reported that mutants and variants of *Frankia* strains generally clustered closely with their parents[3].

The random amplified polymorphic DNA (RAPD) method is a DNA polymorphism analysis system based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. These primers detect polymorphisms in the absence of specific nucleotide sequence information[4,5].

We applied this method to evaluate actinomycete strains. In this study, we used *Streptomyces lavendulae* and *Streptomyces virginiae* strains which have been reported as producers of many kind of bioactive compounds and frequently isolated from soil samples. Moreover, *S. virginiae* has been given as a subjective synonym of *S. lavendulae* by S. T. WILLIAMS[6].

Materials and Methods

Microorganisms

Actinomycete strains used in this study are shown in Table 1. They were purchased from the Institute for Fermentation, Osaka, Japan, (IFO), American Type Culture Collection, Rockville, U.S.A., (ATCC), and Japan Collection of Microorganisms, Saitama, Japan, (JCM).

Cultural and Physiological Tests

Methods adopted by the International Streptomyces Project (ISP)[7] were used for our taxonomic studies. Activities of 19 enzymes were assayed by the API ZYM system (API System S. A.). Strains were

tested for their susceptibility against antibiotics (gentamicin (GM), lincomycin (LCM), oleandomycin (OM), benzylpenicillin (PCG)) using Tridisk 'Eiken' (Eiken chemical Co., Ltd.). Susceptibility to antibiotics was scored as positive. Antimicrobial activity of the strains against *Bacillus subtilis* PCI 219 was detected by a disk assay method using their broth cultured in a liquid medium consisting of 2.0% potato starch, 2.0% glucose, 0.5% yeast extract, 2.0% Toast soya, 0.25% NaCl, 0.005% $ZnSO_4 \cdot 7H_2O$, 0.0005% $CuSO_4 \cdot 5H_2O$, 0.0005% $MnCl_2 \cdot 4H_2O$ and $CaCO_3$ 0.32% (pH 7.0).

Table 1. Strains tested.

Strain	Culture collection reference No.
Streptomyces lavendulae subsp. *lavendulae*	IFO 3125
S. lavendulae subsp. *lavendulae*	IFO 3145
S. lavendulae subsp. *lavendulae*	IFO 3361
S. lavendulae subsp. *lavendulae*	IFO 12340
S. lavendulae subsp. *lavendulae*	IFO 12341
S. lavendulae subsp. *lavendulae*	IFO 12343
S. lavendulae subsp. *lavendulae*	IFO 12344
S. lavendulae subsp. *lavendulae*	IFO 13710
S. lavendulae subsp. *fuscus*	IFO 14028
S. lavendulae subsp. *lavendulae*	IFO 13709
S. lavendulae subsp. *lavendulae*	IFO 12789^T
S. virginiae subsp. *lipoxae*	JCM 4724
S. virginiae	IFO 3392
S. virginiae	IFO 3729
S. virginiae	ATCC 13013
S. virginiae	ATCC 13161
S. virginiae	ATCC 15894
S. virginiae	IFO 12827^T

DNA-DNA Hybridization

DNA was extracted from the cells by the method of MARMUR[8]. The extent of DNA homology between strains was determined by the fluorometric DNA hybridization method using photobiotin in microdilution wells described by EZAKI[9]. Hybridization was carried out for 2 hours at 55°C.

Analysis of Metabolites by HPLC

Each strain was cultured in a 500-ml baffled Erlenmeyer flask containing 100 ml of the same medium as described above on a rotary shaker at 27°C for 5 days at 220 rpm. The culture broth was extracted with ethyl acetate. The ethyl acetate layer was separated and concentrated under reduced pressure. The residue was dissolved in methanol and chromatographed according to the method of FRISVAD[10]. A Waters HPLC system 600E with a PDA detector was used. The UV detector was set to monitor at 200, 225, and 254 nm. Samples (20 μl) were injected with a Waters autosampler injection system. Analyses were performed on a 150 × 4.6 mm ODS C_{18} column (YMC pack A301). A gradient solvent system (with solvent A = water and solvent B = 0.05% trifluoroacetic acid in acetonitrile) was used. The gradient program was as follows: the initial percentage of solvent B was 10%, which was raised to 50% in 30 minutes, then to 90% in 10 minutes, held at 90% for 10 minutes and lowered to 10% again in 3 minutes, and held at 10% for 2 minutes at a flow-rate of 2.0 ml/minute until the next injection occurs.

LFRFA by PFGE

A slant culture of strain was inoculated into 100 ml YG broth consisting of 1% yeast extract and 1% glucose (pH 7.2) in a 500-ml baffled Erlenmeyer flask and incubated on a rotary shaker at 27°C for 3 days at 220 rpm. Two ml of this culture was transferred into 100 ml of YG broth supplemented with 0.1% glycine and incubated overnight on a rotary shaker under the same conditions as described above. Washed mycelia from the resulting culture were embedded in 0.5% agarose. The preparation of the agarose plug was carried out according to the methods of LEBLOND[1] and KIESER[11]. After treatment with proteinase K (Merck), the plug was incubated in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.1 mM (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride (Wako Pure Chemical Co., Ltd.) for 1 hour at 4°C to inhibit protease. The plugs were stored at 4°C in NDS buffer (0.5 M EDTA, 10 mM Tris-HCl (pH 9.0), 1% (w/v) lauroyl sarcosine).

To digest the DNA, the plug was soaked in 1 × restriction enzyme buffer (Takara) for 15 minutes. The buffer was removed, replaced with 200 μl of fresh buffer, and 5 μl of bovine serum albumin (0.1%; Takara) and a restriction enzyme were added. Fifty unit of *Dra*I (Takara) and *Vsp*I (Takara), and 2.5 unit of *Ssp*I (Takara) were incubated as described by KIESER[11].

PFGE was performed by using a 1% agarose gel in 0.5 × Tris-borate buffer (1 × TBE contains 89 mM Tris, 89 mM boric acid, and 2 mM EDTA (pH 8.0)) in the Bio-Rad CHEF DR-II System. The running buffer was 0.5 × TBE held at 14°C. The following program was used for 200- to 2,000-kb fragments:

15 hours at 200 V with 60 second pulses, and 9 hours at 200 V with 90 second pulses. For 50- to 1,000-kb fragments the following program was used: 24 hours at 200 V with 50 to 90 second gradient pulses. The size standards used for the DNA bands at the higher molecular weights were *Saccharomyces cerevisiae* (strain YNN295) chromosomes, and lambda concatemers were the standards used for the lower molecular weight range, both from Bio-Rad. After electrophoresis, gels were stained with ethidium bromide.

Amplification and Electrophoresis Condition for RAPD

Amplification conditions were modified from those of WILLIAMS[12] and GOODWIN[13]. The amplification reaction mixture (50 μl) consisted of 1 × *Taq* DNA polymerase buffer (Pro Bio), 200 μM each of dATP, dCTP, dGTP and dTTP (Takara), 2 mM $MgCl_2$ 50 ng of genomic DNA, 2.5 μM primer and 4 unit of *Taq* DNA polymerase (Pro Bio). Amplifications were performed in the ZYMOREACTER II (Atto Co., Ltd.) with following program: initial denaturation at 94°C for 2 minutes, 30 or 35 cycles of denaturation at 94°C for 1 minute, annealing at 34°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 10 minutes. We used 12 primers, R28 (5'-ATGGATCCGC), B1 (5'-TCACGATGCA), and B2 to B11 which differed from B1 by substitution of a single nucleotide at a successive position in the sequence. The G + C content of primers B2 to B11 was maintained at 50%. All primers were synthesized on an Applied Biosystems Model 392 DNA synthesizer.

Electrophoresis of polymerase chain reaction (PCR) products was carried out according to FUJIMORI and OKUDA[14].

Data Analysis

Image analysis of the electrophoretic band patterns of RAPD and the conversion of Rf values of each band to the 0-1 matrix data were carried out according to the method of FUJIMORI and OKUDA[14]. Cluster analysis was achieved by the use of the phylogenetic inference package, PAUP (phylogenetic analysis using parsimony)[15].

Results

Taxonomic Characteristics

Morphological, cultural and physiological characteristics of 18 strains are shown in Table 2. All strains except for *S. lavendulae* subsp. *fuscus* IFO 14028 formed pale pinkish gray aerial mycelium. All strains hydrolyzed starch and showed antibiosis against *B. subtilis* PCI 219. All strains except for *S. lavendulae* subsp. *lavendulae* IFO 3361 produced melanoid pigments. The carbohydrate utilization and the enzymatic activity are shown in Tables 3 and 4, respectively. Regarding physiology, therefore, *S. lavendulae* subsp. *lavendulae* IFO 3361 and IFO 12341 and *S. virginiae* ATCC 13013 showed different characteristics from those of other strains.

The data in Tables 2, 3, and 4 were used for the cluster analysis with the PAUP. The dendrogram was generated by using the branch and bound algorithm (Fig. 1).

S. lavendulae subsp. *lavendulae* IFO 12343, IFO 12344, IFO 13710 and IFO 12789^T were located in the same cluster. *S. virginiae* subsp. *lipoxae* JCM 4724, *S. virginiae* IFO 3392 and ATCC 15894 formed another cluster. *S. virginiae* IFO 3729 and IFO 12827^T were also placed in the third cluster. While, *S. lavendulae* subsp. *lavendulae* IFO 3361 and IFO 12341 and *S. virginiae* ATCC 13013 were located in a distinct cluster far from the other strains.

DNA Relatedness

The DNA relatedness between *S. lavendulae* subsp. *lavendulae* IFO 12789^T and the other strains, and between *S. virginiae* IFO 12827^T and the other strains are shown in Table 5.

The homology value between *S. lavendulae* subsp. *lavendulae* IFO 12789^T and *S. virginiae* IFO 12827^T

Table 2. Morphological, cultural and physiological characteristics of 18 strains of *Streptomyces lavendulae* and *Streptomyces virginiae*.

Test	Strain No.[a]																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Spore chain morphology	RA	RA	RA	RA	RF	RA	RA	RA	RA	RA	RA	RA	RA	RA	RA	RA	RA	RA
Spore mass color	PG	PG	PG	PG	PG	PG	PG	PG	B	PG	PG	PG	PG	PG	PG	PG	PG	PG
Gelatin liquefaction at 20°C	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
at 27°C	−	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Milk coagulation at 27°C	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
at 37°C	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−
Milk peptonization at 27°C	+	+	+	+	+	+	+	−	−	−	+	−	−	+	−	−	−	+
at 37°C	+	−	+	−	+	−	−	−	−	−	−	−	−	+	+	−	−	+
Nitrate reduction	+	+	−	+	−	−	−	−	−	+	−	−	−	−	−	+	−	−
Melanin production: ISP 1	+	+	−	±	+	±	+	+	+	+	±	±	±	+	+	+	+	+
ISP 6	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
ISP 7	+	+	−	±	+	+	+	+	+	+	+	+	±	+	+	+	+	+
NaCl tolerance (%)	5	4	7	4	7	5	5	5	1	4	5	5	3	5	7	5	5	4
Growth at 5°C	−	−	−	−	+	+	+	−	−	−	+	+	+	−	−	+	+	−
at 40°C	+	+	+	−	−	−	−	−	+	−	−	−	−	−	+	−	−	−
Antibiotic susceptibility:																		
GM (2 μg/ml)	+	−	+	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+
LCM (15 μg/ml)	+	+	−	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+
OM (5 μg/ml)	+	+	−	+	−	+	+	+	+	+	+	+	+	+	−	+	+	+
PCG (10 u/ml)	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−
Antibiosis ag. *B. subtilis* PCI 219	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

RA, *Retinaculiaperti*; RF, *Rectiflexibiles*; PG, pinkish gray; B, brown.

[a] Numbers 1 to 11 are *S. lavendulae* strains and numbers 12 to 18 are *S. virginiae* strains. 1, IFO 3125; 2, IFO 3145; 3, IFO 3361; 4, IFO 12340; 5, IFO 12341; 6, IFO 12343; 7, IFO 12344; 8, IFO 13710; 9, IFO 14028; 10, IFO 13709; 11, IFO 12789^T; 12, JCM 4724; 13, IFO 3392; 14, IFO 3729; 15, ATCC 13013; 16, ATCC 13161; 17, ATCC 15894; 18, IFO 12827^T.

Table 3. Carbohydrate utilization of 18 strains of *Streptomyces lavendulae* and *Streptomyces virginiae*.

Carbohydrate	Strain No.[a]																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
L-Arabinose	+	+	+	+	−	−	−	±	−	−	−	+	+	−	+	−	+	−
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−
D-Mannitol	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−
Melezitose	−	−	−	−	−	+	+	+	−	−	+	−	−	−	−	−	−	−
Melibiose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Raffinose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Rhamnose	−	−	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−
Sucrose	+	−	−	−	±	−	−	−	+	−	−	−	−	−	±	−	−	−
D-Xylose	+	−	+	+	+	−	−	−	−	−	−	+	−	−	+	−	−	−

[a] Numbers 1 to 11 are *S. lavendulae* strains and numbers 12 to 18 are *S. virginiae* strains. 1, IFO 3125; 2, IFO 3145; 3, IFO 3361, 4, IFO 12340; 5, IFO 12341; 6, IFO 12343; 7, IFO 12344; 8, IFO 13710; 9, IFO 14028; 10, IFO 13709; 11, IFO 12789^T; 12, JCM 4724; 13, IFO 3392; 14, IFO 3729; 15, ATCC 13013; 16, ATCC 13161; 17, ATCC 15894; 18, IFO 12827^T.

Table 4. Enzymatic activity in 18 strains of *Streptomyces lavendulae* and *Streptomyces virginiae*.

Enzyme assayed for	Strain No.[a]																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Alkaline phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esterase (butyrate)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Esterase (caprylate)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Lipase (myristate)	+	+	+	+	−	+	+	+	+	+	+	−	−	+	+	+	−	+
Leucine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Valine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cystine arylamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Trypsin	+	+	+	+	+	−	−	−	+	+	−	+	+	+	+	+	+	−
α-Chymotrypsin	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid phosphatase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phosphoamidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-D-Galactosidase	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
β-D-Galactosidase	−	−	+	−	+	−	−	−	−	−	−	+	+	+	+	−	+	+
β-D-Glucuronidase	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
α-D-Glucosidase	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
β-D-Glucosidase	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+
N-Acetyl-β-D-glucosaminidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
α-D-Mannosidase	−	+	+	+	−	+	+	+	−	−	+	+	+	+	+	+	+	+
α-L-Fucosidase	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−

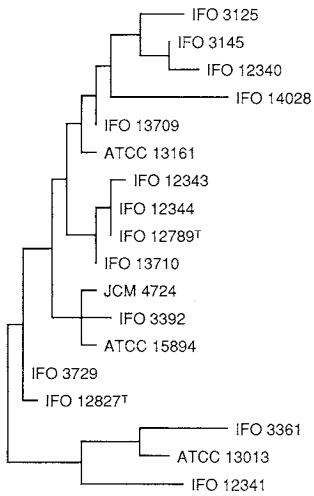
[a] Numbers 1 to 11 are *S. lavendulae* strains and numbers 12 to 18 are *S. virginiae* strains. 1, IFO 3125; 2, IFO 3145; 3, IFO 3361; 4, IFO 12340; 5, IFO 12341; 6, IFO 12343; 7, IFO 12344; 8, IFO 13710; 9, IFO 14028; 10, IFO 13709; 11, IFO 12789^T; 12, JCM 4724; 13, IFO 3392; 14, IFO 3729; 15, ATCC 13013; 16, ATCC 13161; 17, ATCC 15894; 18, IFO 12827^T.

was low (31～36%).

The homology values between *S. lavendulae* subsp. *lavendulae* IFO 12789^T and IFO 12343, IFO 12344 and IFO 13710 were very high (97%, 67% and 103%, respectively). Furthermore, the homology values between *S. virginiae* IFO 12827^T and *S. lavendulae* subsp. *lavendulae* IFO 13709, and *S. virginiae* IFO 3729 were also very high (84% and 107%, respectively). The DNA relatedness results between these strains

except for *S. lavendulae* subsp. *lavendulae* IFO 13709 agreed well with those of the previous cluster analysis. On the other hand, the homology values between 2 type strains and *S. lavendulae* subsp. *lavendulae* IFO 3361, IFO 12341 and *S. virginiae* ATCC 13013 were very low. These strains were located in a cluster far from the other strains (Fig. 1).

Fig. 1. Dendrogram for phenotypic data of 18 strains of *Streptomyces lavendulae* and *Streptomyces virginiae*.



This dendrogram was based on the data of Tables 2, 3 and 4 and solved by the branch and bound method contained within the PAUP (phylogenetic analysis using parsimony) phylogenetic inference package.

HPLC Profiles of Metabolites

The metabolites of each strains were analyzed by HPLC. A similar chromatogram was observed for *S. lavendulae* subsp. *lavendulae* IFO 12343 and IFO 12344 (Fig. 2). The UV spectra (190 to 400 nm) of the major peak were identical. HPLC elution profiles of *S. virginiae* IFO 3392 and ATCC 15894 were also similar to each other. These strains generated two distinct clusters according to the phenotypic data.

LFRFA

LFRFA using rare-cutting enzymes *Dra*I, *Vsp*I and *Ssp*I was conducted to discriminate 18 strains.

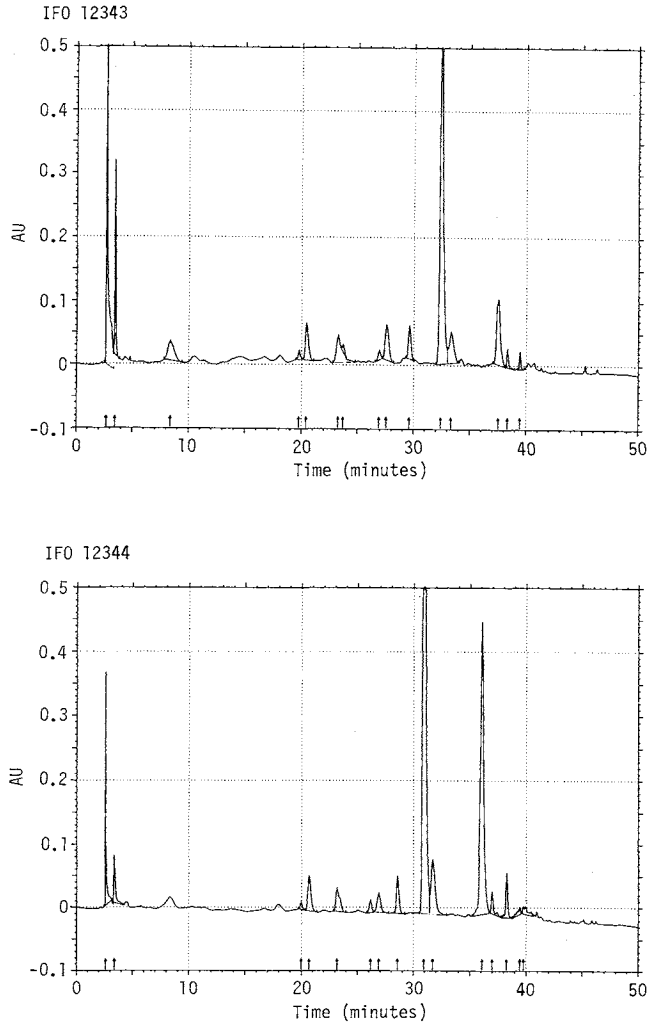
When undigested DNA samples were electrophoresed, extrachromosomal DNA molecules were observed in some strains. Therefore, these DNAs were removed from the agarose plugs by pre-

Table 5. DNA relatedness between type strain and the other strains.

Strain	% Homology with photobiotin-labeled DNA	
	S. lavendulae subsp. *lavendulae* IFO 12789^T	*S. virginiae* IFO 12827^T
Streptomyces lavendulae subsp. *lavendulae* IFO 3125	38	32
S. lavendulae subsp. *lavendulae* IFO 3145	28	26
S. lavendulae subsp. *lavendulae* IFO 3361	21	15
S. lavendulae subsp. *lavendulae* IFO 12340	54	30
S. lavendulae subsp. *lavendulae* IFO 12341	22	15
S. lavendulae subsp. *lavendulae* IFO 12343	97	34
S. lavendulae subsp. *lavendulae* IFO 12344	67	28
S. lavendulae subsp. *lavendulae* IFO 13710	103	38
S. lavendulae subsp. *fuscus* IFO 14028	43	29
S. lavendulae subsp. *lavendulae* IFO 13709	49	84
S. lavendulae subsp. *lavendulae* IFO 12789^T	100	31
S. virginiae subsp. *lipoxae* JCM 4724	39	48
S. virginiae IFO 3392	42	49
S. virginiae IFO 3729	30	107
S. virginiae ATCC 13013	19	18
S. virginiae ATCC 13161	44	47
S. virginiae ATCC 15894	47	46
S. virginiae IFO 12827^T	36	100

DNA homology values are expressed as % of labeled DNA reassociated with heterologous DNA compared with that reassociated with homologous DNA (100%).

Fig. 2. HPLC profiles of metabolites produced by *S. lavendulae* subsp. *lavendulae* IFO 12343 and IFO 12344 (at 254 nm).



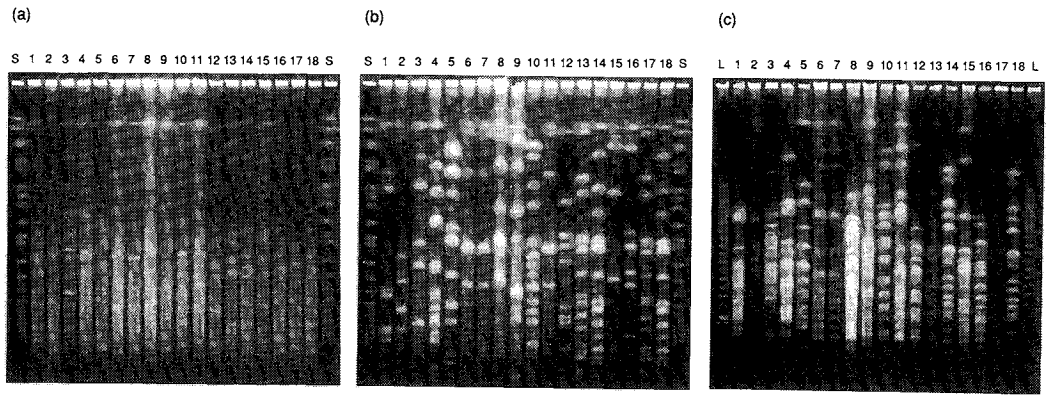
electrophoresis prior to digestion with a restriction enzyme. Fig. 3 shows the restriction patterns of 18 strains using *Dra*I, *Vsp*I and *Ssp*I. The restriction patterns of *S. lavendulae* subsp. *lavendulae* IFO 12343, IFO 12344 and IFO 12789^T by 3 restriction enzymes were similar. Those of *S. virginiae* IFO 3392 and ATCC 15894, and *S. virginiae* IFO 3729 and IFO 12827^T were also similar to each other. These results supported the previous phenotypic data, the DNA relatedness and the HPLC profiles of metabolites.

Fingerprinting by RAPD Method

Genomic fingerprints were generated by the RAPD method. Fig. 4-A shows RAPD patterns of 18 strains using primer R28. Apparently three similar band patterns were observed in several strains. *S. lavendulae* subsp. *lavendulae* IFO 12343, IFO 12344 and IFO 12789^T possesed almost identical DNA bands. *S. virginiae* IFO 3392 showed a very similar pattern to that of *S. virginiae* ATCC 15894. Another almost identical feature was observed in *S. virginiae* IFO 3729 and IFO 12827^T. Rf value of each band

Fig. 3. PFGE of 18 strains of *Streptomyces lavendulae* and *Streptomyces virginiae*.

(a) DNAs of strains cut with *Dra*I. S, *Saccharomyces cerevisiae* chromosomes. (b) DNAs of strains cut with *Vsp*I. S, *S. cerevisiae* chromosomes. (c) DNA of strains cut with *Ssp*I. L, lambda concatemers. Lanes 1 to 11 are *S. lavendulae* strains and lanes 12 to 18 are *S. virginiae* strains. Lane 1, IFO 3125; lane 2, IFO 3145; lane 3, IFO 3361; lane 4, IFO 12340; lane 5, IFO 12341; lane 6, IFO 12343; lane 7, IFO 12344; lane 8, IFO 13710; lane 9, IFO 14028; lane 10, IFO 13709; lane 11, IFO 12789^T; lane 12, JCM 4724; lane 13, IFO 3392; lane 14, IFO 3729; lane 15, ATCC 13013; lane 16, ATCC 13161; lane 17, ATCC 15894; lane 18, IFO 12827^T.



was converted to the 0-1 matrix for analysis with the PAUP. Fig. 5-A shows a dendrogram generated by the bootstrap search option. These strains within 3 groups were located near each other. This result supported the cluster analysis of the phenotypic data, the DNA relatedness, the HPLC profiles of metabolites and the LFRFA.

Fig. 6 shows the RAPD patterns of *S. lavendulae* subsp. *lavendulae* IFO 12341 using primers B1 to B11. Similarly crowded band patterns were observed in polymerase chain reaction (PCR) with primers B1 to B3. Primers B4 to B7 decreased the number of bands. Primers B8 to B11 produced a large number of bands and different polymorphisms as compared to the original primer B1. This tendency was also confirmed in the other strains. Fig. 4-B and 4-C show the RAPD patterns of 18 strains using primers B1 and B9. The dendrogram obtained from primer B9 was similar to that from primer R28. Although the strains within a cluster showed the same band patterns as shown in Fig. 4-B, the dendrogram derived from primer B1 (Fig. 5-B) was different from that of primers R28 and B9. *S. lavendulae* subsp. *lavendulae* IFO 12343 and IFO 12344 were located near each other. On the other hand, *S. lavendulae* subsp. *lavendulae* IFO 12789^T was located a far distance from *S. lavendulae* subsp. *lavendulae* IFO 12343 and IFO 12344. Furthermore, *S. lavendulae* subsp. *lavendulae* IFO 3361 and IFO12340 which showed obviously different band patterns were located near each other.

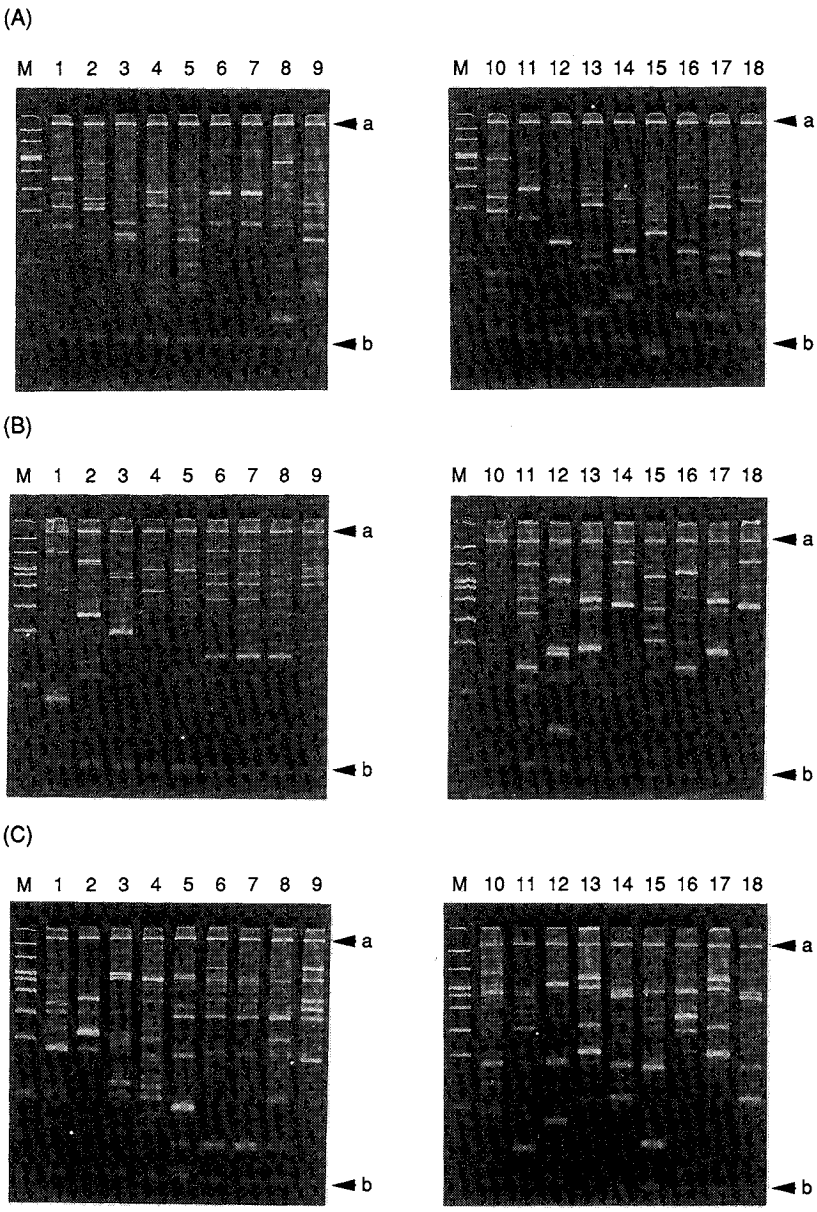
Discussion

We evaluated the RAPD method to eliminate duplicate actinomycete strains in this study. Reproducible DNA patterns were produced with all primers. In addition, the dendrograms with the PAUP derived from primers R28 and B9 corresponded well with other analyses (the phenotypic characteristics, the DNA relatedness, the HPLC profiles of metabolites and the LFRFA).

The number of DNA bands observed in each lane and the distribution of DNA bands should be taken into consideration in the selection of an appropriate primer. For example, the numer of DNA bands by primer B1 was less than those by primers R28 and B9, and DNA bands in each lane by primer

Fig. 4. RAPD patterns of 18 strains of *Streptomyces lavendulae* and *Streptomyces virginiae*.

(A), primer R28; (B), primer B1; (C), primer B9. M is pHY marker. The marks of arrowhead (a and b) are λ-DNA and 90 bp DNA, respectively. Lanes 1 to 11 are *S. lavendulae* strain and lanes 12 to 18 are *S. virginiae* strains. Lane 1, IFO 3125; lane 2, IFO 3145; lane 3, IFO 3361; lane 4, IFO 12340; lane 5, IFO 12341; lane 6, IFO 12343; lane 7, IFO 12344; lane 8, IFO 13710; lane 9, IFO 14028; lane 10, IFO 13709; lane 11, IFO 12789^T; lane 12, JCM 4724; lane 13, IFO 3392; lane 14, IFO 3729; lane 15, ATCC 13013; lane 16, ATCC 13161; lane 17, ATCC 15894; lane 18, IFO 12827^T.



B1 were closely packed together in the upper part of gel ($\geq$500 bp, $\leq$0.4 of Rf); thus, it might be difficult to measure precise Rf values so that an accurate dendrogram was not generated. Therefore, the dendrogram by primer B1 might not correspond to that of primer R28 and B9.

RAPD patterns were altered when a single nucleotide of primer B1 was substituted at successive

Fig. 5. Dendrograms for RAPD patterns of 18 strains of *Streptomyces lavendulae* and *Streptomyces virginiae*.

(A) primer R28, (B) primer B1. These dendrograms were based on the 0-1 matrices obtained from Rf values of RAPD patterns (Fig. 4-A and B) and solved by the bootstrap search option contained within the PAUP (phylogenetic analysis using parsimony) phylogenetic inference package.

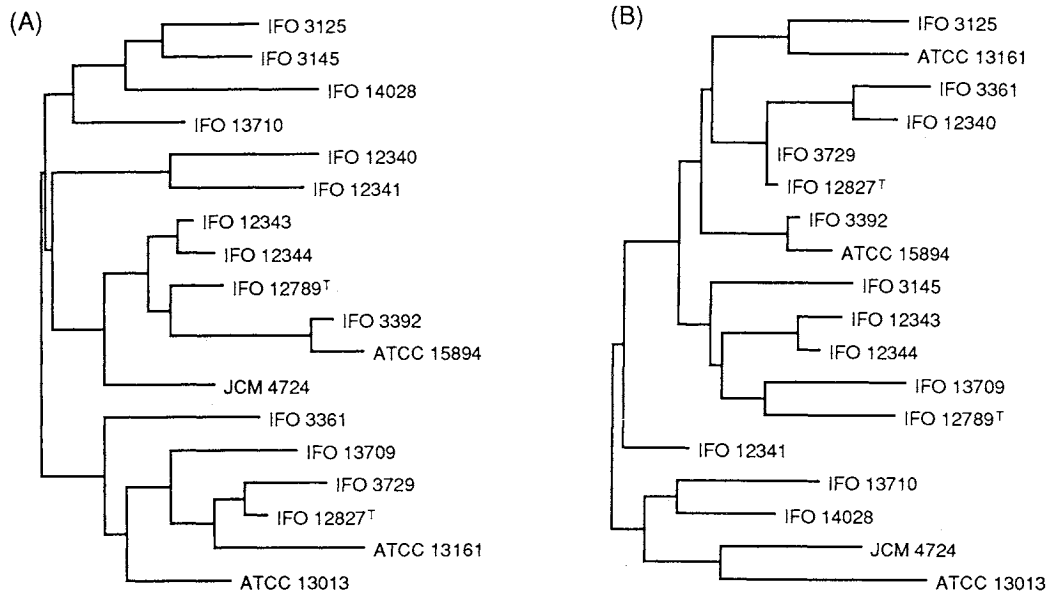
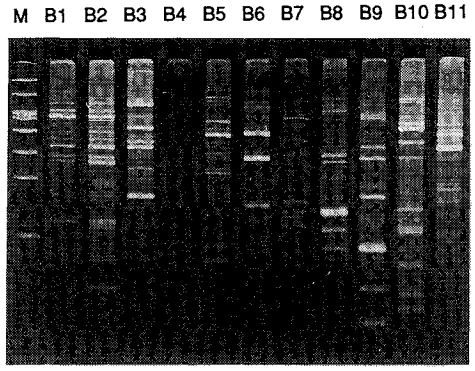


Fig. 6. RAPD patterns of *S. lavendulae* subsp. *lavendulae* IFO 12341 with primers B1 to B11.

M is pHY marker. Primer sequences: B1 (5'-TCACGATGCA), B2 (5'-ACACGATGCA), B3 (5'-TGACGATGCA), B4 (5'-TCTCGATGCA), B5 (5'-TCAGGATGCA), B6 (5'-TCACCATGCA), B7 (5'-TCACGTTGCA), B8 (5'-TCACGAAGCA), B9 (5'-TCACGATCCA), B10 (5'-TCACGATGGA), B11 (5'-TCACGATGCT).



positions in the sequence with a constant GC %. Furthermore, a dramatic change was observed on the substitution from the third nucleotide, and primer B9 was found to be more suitable for RAPD analysis than primer B1. Therefore, the 3' end sequences may be important for random amplification using the ten nucleotides primer.

In conclusion, since the following strains within each of the 3 groups were considered to be similar by RAPD analysis: *S. lavendulae* subsp. *lavendulae* IFO 12343, IFO 12344 and IFO 12789^T; *S. virginiae* IFO 3392 and ATCC 15894; *S. virginiae* IFO 3729 and IFO 12827^T, it was possible to select one strain from all similar strains.

Interestingly, it has been reported that *S. lavendulae* subsp. *lavendulae* IFO 12343, IFO 12344 and IFO 12789^T produces streptothricin and *S. virginiae* IFO 3729 and IFO 12827^T originated from the same strains. In addition, although WILLIAMS placed *S. virginiae* to a subjective synonym of *S. lavendulae*[6], and AKIMOV has reported that *S. lavendulae* group formed a single "genospecies"[16], the homology value between *S. lavendulae* subsp. *lavendulae* IFO 12789^T and *S. virginiae* IFO 12827^T was low in our study.

Therefore, we conclude that RAPD analysis with PCR is a simple, efficient, and reliable method. This method is a powerful tool for the elimination of duplicate actinomycete strains in microbial screenings.

Purified DNA was used in all these experiments, however, the same results were obtained with crude or purified DNA.

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