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

III. BACTERIA

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Random amplified polymorphic DNA (RAPD) analysis was evaluated for the selection and elimination of bacterial strains used in microbial screening. For this pilot study we used eight bacterial strains producing fragin and two *Pseudomonas fragi* strains, which are often isolated during the screening. A dendrogram constructed by the statistical analysis using parsimony, PAUP, based on the band patterns of RAPD with primer R28 was in good correlation with the results of DNA - DNA hybridization, HPLC analysis of metabolites, and conventional morphological and physiological characterization. RAPD was also applicable to a wide range of bacteria. This rapid selection system by RAPD was a very useful tool for excluding similar bacterial isolates encountered during screening.

Bacteria are an important source for new drug screening. It is essential for the discovery of new compounds to screen a large number of different strains. However, it is more difficult to select bacterial strains than fungal and actinomycete strains because of their simple morphology. Therefore, the selection of bacterial strains on the basis of traditional taxonomic methods is labour-intensive. A simpler and more effective selection method is necessary to perform high quality screening.

We have therefore applied the fingerprint identification system by the random amplified polymorphic DNA (RAPD) as a convenient procedure to evaluate strains using fragin producers that were isolated during our screening program. A wider range of bacterial genera were also evaluated by this method.

Materials and Methods

Strains

The bacterial strains used are listed in Tables 1 and 2. *Pseudomonas* strains were isolated from Japanese soil samples. Fragin was isolated from *Pseudomonas fragi* in 1969 by MURAYAMA *et al.*[1] Two reference strains of *Ps. fragi* were used for our study. There was no information of available on fragin production in the reference strains. Other reference strains, including type strains, were purchased from the Institute for Fermentation, Osaka, Japan, (IFO) and the American Type Culture Collection, Rockville, U.S.A., (ATCC).

Table 1. Bacterial strains studied.

Species	Strain	Source
Pseudomonas fragi	IFO 3458^T	
Ps. fragi	IFO 12049	
Pseudomonas sp.	NR 2996	Soil, Yamagata, Japan
Pseudomonas sp.	NR 2997	Soil, Gumma, Japan
Pseudomonas sp.	NR 2998	Soil, Gumma, Japan
Pseudomonas sp.	NR 2999	Soil, Kyoto, Japan
Pseudomonas sp.	NR 3000	Soil, Kagoshima, Japan
Pseudomonas sp.	NR 3001	Soil, Aichi, Japan
Pseudomonas sp.	NR 3002	Soil, Aichi, Japan
Pseudomonas sp.	NR 3003	Soil, Aichi, Japan

T, type strain.

Taxonomic Characterization

Morphological and physiological characteristics of *Pseudomonas* strains were examined according to PALLERONI.[2]

Table 2. Various bacterial strains used for RAPD with primer R28.

Species	Strain	Species	Strain
Acinetobacter calcoaceticus	IFO 13006	*Rhizobium leguminosarum*	IFO 14168
Aeromonas hydrophila	IFO 3820	*Serratia liquefaciens*	IFO 12979
Agrobacterium tumefaciens	IFO 3058	*Sphingobacterium spiritivorum*	ATCC 33861^T
Shewanella putrefaciens	IFO 3908^T	*Xanthomonas campestris*	IFO 13551
Azotobacter chroococum	IFO 12994	*Arthrobacter ramosus*	IFO 12958^T
Escherichia coli	NIHJ JC-2	*Bacillus laterosporus*	ATCC 64^T
Erwinia herbicola	IFO 12686	*B. licheniformis*	IFO 12200^T
Flavobacterium meningosepticum	IFO 12535^T	*B. pumilus*	IFO 12092^T
Proteus vulgaris	IFO 3167	*B. subtilis*	IFO 13719^T
P. vulgaris	ATCC 6898	*Corynebacterium glutamicum*	ATCC 13869
P. vulgaris	IFO 3851^T	*C. michiganens*	IFO 12471
P. vulgaris	ATCC 6380	*Micrococcus luteus*	IFO 3333^T
Pseudomonas aerginosa	A3	*M. roseus*	IFO 3768^T
Ps. caryophylli	IFO 12952	*Staphylococcus aureus*	209P JC-1
Comamonas testosteroni	ATCC 11996^T		

^{T}, type strain.

Analysis of Metabolites by HPLC

Extracts from *Pseudomonas* cultures were analyzed by HPLC. The seed and fermentation medium contained the following (w/v): 1% glucose, 1% dextrin, 1% Pharmamedia, 1% S-3 meat, 0.06% K_2HPO_4, 0.025% KH_2PO_4, and an antifoaming agent (Nissan Disfoam CA-115). The bacterial cell suspension was inoculated into 100 ml of the seed medium in a 500-ml baffled Erlenmeyer flask and cultured on a rotary shaker (220 rpm) at 27°C for one day. Then 0.1 ml of the seed culture was transferred into 100 ml of the fermentation medium in a 500-ml baffled Erlenmeyer flask and cultured for 3 days under the same conditions as described above. The culture broth was extracted with ethyl acetate and the extract was concentrated under reduced pressure. The residual sample was dissolved in 1 ml of methanol. Analyses were conducted on a 150×4.6 mm ODS C_{18} column (YMC pack A301) HPLC system 600 E with a photodiode array detector (Waters). 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 sample injection.[3]

DNA - DNA Hybridization

A loop of cell mass was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of nutrient broth (Kyokuto) or tripticase soy broth (BBL) supplemented with 0.4% glycine and cultured at 27°C on a rotary shaker at 220 rpm. The cells were harvested in exponential growth phase. DNA was extracted from cells according to the method of SAITO and MIURA.[4]

DNA - DNA hybridization was performed at 45°C for 90 minutes by the microplate method according to EZAKI *et al.*[5]

Amplification and Electrophoresis Conditions for RAPD

Bacterial cells were peeled off from a potato sucrose ager slant surface and completely suspended in 500 μl of 10 mM Tris-1 mM EDTA buffer. About 5 μl of 5N NaOH was added to the cell suspension and gently mixed. After centrifugation at 15,000 rpm at 4°C for 10 minutes, the aqueous phase (400 μl) was dispensed into another microtube. DNA was precipitated in 800 μl cold ethanol and centrifuged at 10,000 rpm at 4°C for 5 minutes. The crude DNA was dried, dissolved in 100 μl of TE buffer, and used as a template for polymerase chain reaction (PCR).

Primers R28 and B1 to B11 were used in this study.[6]

The PCR solution (50 μl) contained 1×reaction buffer (Pro Bio), 2.5 mM $MgCl_2$, 200 μM each of dNTPs (Takara), 25 ng template DNA, 2.5 μM primer and 4 units of *Taq* DNA polymerase (Pro Bio). Random DNA amplifications were run in a ZYMOREACTER II (Atto) programmed as follows: initial

and subsequent 30 cycles of denaturation at 94°C for 1 minute, annealing at 34°C for 1 minute, extension at 72°C for 1 minute, and extension at 72°C for 10 minutes as the final cycle. The PCR products with BPB-sucrose solution containing the front and origin markers,[7] were applied to a 5% polyacrylamide gel with TAE buffer (0.04 M Tris-acetate and 0.002 M EDTA); electrophoresis was carried out at 50 V for 90 minutes in Mupid-2 (Advance). The PCR products were stained with ethidium bromide (1 mg/ml) and detected by a transilluminator (302 nm, UVP).

Data analysis

The Rf values of each DNA band were measured by an image analyzer (Millipore). The Rf value was converted to a data matrix consisting of "0" or "1" according to FUJIMORI and OKUDA.[7] The statistical analysis using parsimony, PAUP (vers. 3.0 s),[8] was used to construct a dendrogram. The dendrogram was obtained using the bootstrap method with a heuristic program search provided by PAUP.

Results and Discussion

The taxonomic characteristics of our isolates and the reference strains are shown in Table 3.[2] These strains were Gram negative non-sporulating rods with a cell size of $0.5 \sim 0.7 \times 1.4 \sim 2.3\ \mu m$, aerobic, and motile with one or several polar flagella. The color of colonies was pale yellow. The oxidase and catalase productions were positive, and the OF test was oxidative. Growth factors were not required. Nitrate reduction was negative except for NR 2996. No PHB accumulation was observed in any strains. On the basis of these characteristics, therefore, the 8 strains belonged to RNA group 1 of the genus *Pseudomonas*. No fluorescent pigment was produced on KING's B medium. The strains were further divided into two groups, "*stutzeri* group" containing NR 2996 and NR 3000, which can use glucose for growth, and a group of *Ps. alcaligenes* containing NR 2997, NR 2998, NR 2999, NR 3001, NR 3002, and NR 3003, which cannot. Homology values based on DNA-DNA hybridization experiments are shown in Table 4. Three major groups were recognized using a criterion of more than seventy percent homology. Within the first group that contained NR 2996 and NR 3000, the strains exhibited a high homology value and were considered as the same species. The six strains that belonged to the group of *Ps. alcaligenes*, NR 2997, NR 2998, NR 2999, NR 3001, NR 3002, and NR 3003, were one taxon based on their high homology value of DNA hybridization (the second group of DNA homology). NR 2999 has a lower level of similarity to the other strains of the group when DNA of NR 2999 was used as a probe. The six strains, however, formed a compact group according to their DNA relatedness. The two strains of *Ps. fragi* belonging to the "*stutzeri* group" had physiological properties that were obviously different from those of the first group in the DNA homology results. These hybridization results were consistent with the morphological data, such as the number of flagella.

Metabolites of the *Pseudomonas* strains were analyzed by HPLC. Four profiles were observed in the 10 strains. Most production profiles corresponded well to the above taxonomic groups (Fig. 1). The profile of NR 2996, however, differed from that of NR 3000 in the same species. These two strains were distinct. No fragin production was detected from the two reference strains of *Ps. fragi*.

We compared the band patterns of the PCR products with primer R28 among our isolates and the reference strains (Fig. 2). After Rf values of each band were measured and converted to the "0-1" matrix, a dendrogram was generated by the PAUP (Fig. 3). Four clusters were formed. NR 2997, NR 2998, NR 2999, NR 3001, NR 3002, and NR 3003 showed very similar band patterns and were in the same cluster. The six strains were identical from the RAPD results, which were supported by the HPLC profiles. The RAPD pattern for NR 2996 was different from that of NR 3000. The results of PAPD were consistent

Table 3. Morphological and physiological characteristics of fragin producers and two *Pseudomonas strains*.

Strains	*NR 2996*	*NR 3000*	*NR 2997*	*NR 2778*	*NR 2999*	*NR 3001*	*NR 3002*	*NR 3003*	*Pseudomonas fragi* IFO 3458[T]	*Ps. fragi* IFO 12049
Gram stain	−	−	−	−	−	−	−	−	−	−
Morphology & cell size (μm)	R, 0.7×1.4	R, 0.5×2.3	R, 0.5×1.5	R, 0.5×1.4	R, 0.7×1.5	R, 0.6×1.7	R, 0.6×2.1	R, 0.7×2.2	R, 0.5~1.0 ×0.75~4.0[a]	R
Aerobic	+	+	+	+	+	+	+	+	+	+
Number of flagella	≥5	≥4	≥5	≥5	≥4	≥5	≥5	≥6	1	1
Oxidase	+	+	+	+	+	+	+	+	+	+
Catarase	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	+	−	−	−	−	−	−	−	−	−
PHB accumulation	−	−	−	−	−	−	−	−	−	−
Fluorescent pigments	−	−	−	−	−	−	−	−	−	−
Production of fragin	+	+	+	+	+	+	+	+	−	−
Utilization of fructose	+	−	−	−	−	−	−	−	+	+
glucose	+	+	−	−	−	−	−	−	+	+
arabinose	+	−	−	−	−	−	−	−	+	+
sucrose	+	−	−	−	±	−	−	−	+	+

[T], type strain. R, rods. [a], BERGEY's Manual of Determinative Bacteriology. 7th *Ed.*, pp. 110~111, Williams & Willkins Co., 1957.

Table 4. Homology values among *Pseudomonas* strains by DNA-DNA hybridization.

Strain	% Homology with photobiotin-labeled DNA									
	IFO 3458[T]	IFO 12049	NR 2996	NR 3000	NR 2997	NR 2998	NR 2999	NR 3001	NR 3002	NR 3003
Pseudomonas fragi IFO 3458[T]	100									
Pseudomonas fragi IFO 12049	88	100								
Pseudomonas sp. NR 2996	11	20	100							
Pseudomonas sp. NR 3000	10	33	70	100						
Pseudomonas sp. NR 2997	18	26	42	42	100					
Pseudomonas sp. NR 2998	20	27	52	64	96	100				
Pseudomonas sp. NR 2999	9	21	47	50	82	106	100			
Pseudomonas sp. NR 3001	6	8	22	41	78	92	32	100		
Pseudomonas sp. NR 3002	5	7	28	44	70	74	34	97	100	
Pseudomonas sp. NR 3003	23	27	25	38	62	89	41	82	77	100

[T], type strain. NR 2996 and NR 3000, *Ps. stutzeri* like group; NR 2997, NR 2998, NR 2999, NR 3001, NR 3002 and NR 3003, *Ps. alcaligenes* like group. DNA homology values are expressed as % of labeled DNA reassociated with heterologous DNA compared with that reassociated with homologous DNA (100%).

Fig. 1. HPLC analysis of extracts of culture broth from *Pseudomonas* strains.

HPLC elution types of *Pseudomonas* strains were as follows: (A) *Ps. fragi* IFO 3458^T and *Ps. fragi* IFO 12049; (B) *Pseudomonas* sp. NR 2996; (C) *Pseudomonas* sp. NR 2997, *Pseudomonas* sp. NR 2998, *Pseudomonas* sp. NR 2999, *Pseudomonas* sp. NR 3001, *Pseudomonas* sp. NR 3002 and *Pseudomonas* sp. NR 3003; (D) *Pseudomonas* sp. NR 3000.

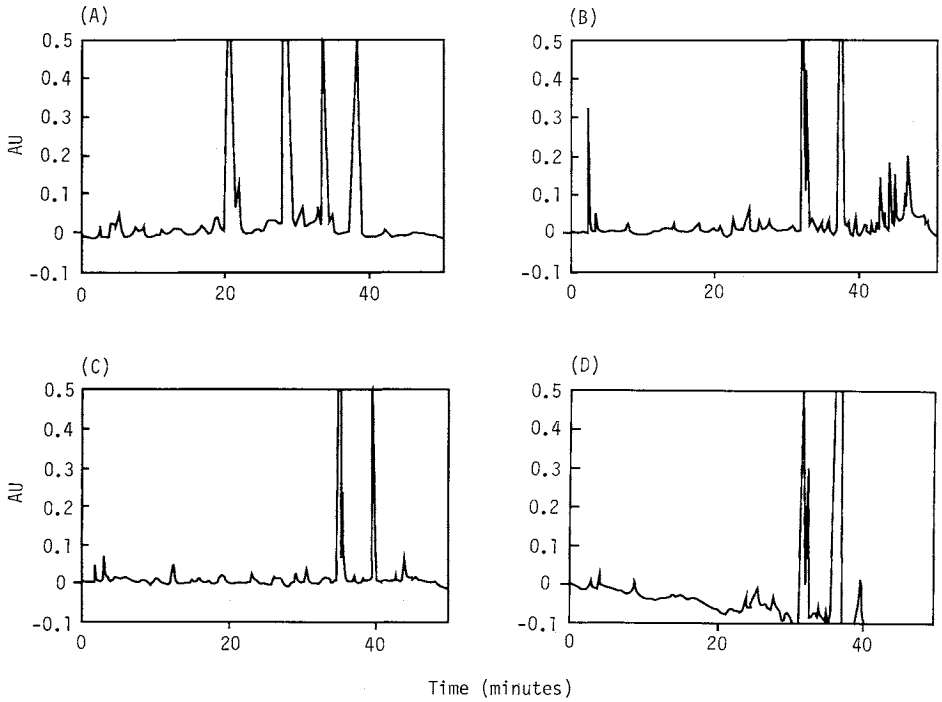


Fig. 2. RAPD patterns of *Pseudomonas* strains and fragin producers using primer R28.

The marks of arrowhead (a and b) are λ-DNA and 90 bp DNA, respectively.

1, NR 2996; 2, NR 2997; 3, NR 2998; 4, NR 2999; 5, NR 3000; 6, NR 3001; 7, NR 3002; 8, NR 3003; 9, *Pseudomonas fragi* IFO 3458^T; 10, *Ps. fragi* IFO 12049.

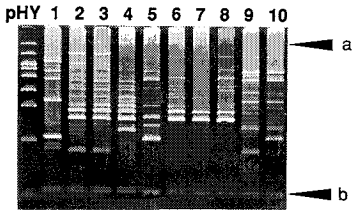
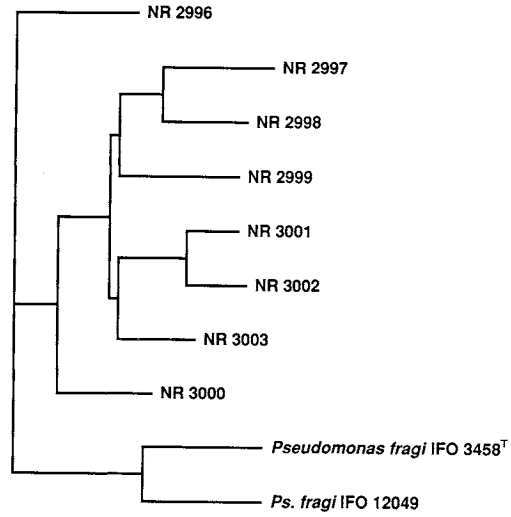


Fig. 3. Dendrogram of *Pseudomonas* strains and fragin producers by PAUP with the bootstrap method.



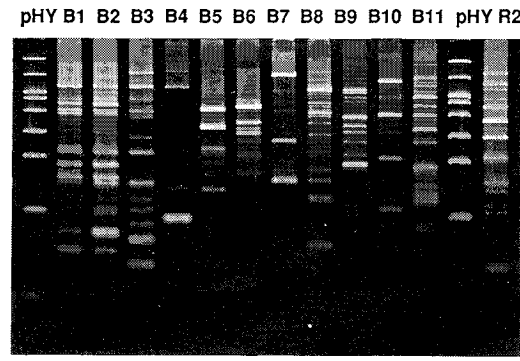
with the HPLC patterns and reflected the metabolites of strains.

Ps. fragi IFO 3458^T and IFO 12049 were in the same cluster and were regarded as one strain from the above reasoning. The results of the RAPD analysis agreed with those of morphological and physiological characteristics, DNA-DNA hybridization, and

HPLC analysis. NR 2997 and NR 2998 were isolated from the same soil sample, and NR 3001, NR 3002, and NR 3003 were isolated from a different single sample. These five strains were located in one RAPD cluster together with NR 2999, which was isolated from a third, different soil sample (Fig. 3). The strains isolated from the same place showed a closer relatedness than did the others. This method was able to demonstrate more accurately a difference between the strains such as isolation place (Table 1). From the dendrogram derived from the RAPD pattern, we identified three strains, NR 2999, NR 2996, and NR 3000, from among eight strains for microbial screening as independent. NR 2999 replaced the other strains in the same cluster. Although the three strains produced fragin, each strain exhibited a different HPLC pattern and had the potential to produce other active compounds in different screening procedures.

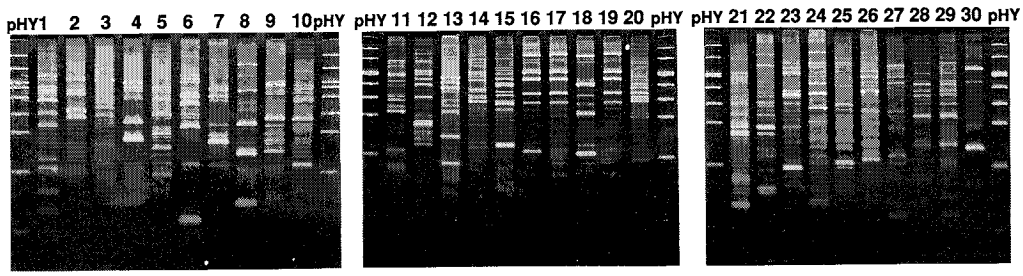
We examined twelve primers to select the optimum primer for bacterial strains using *Bacillus pumilus* IFO 12092^T (Fig. 4); primer R28 was also suitable for *Bacillus pumilus* IFO 12092^T (Fig. 5). R28 generated the greatest number of bands of all the primers tested, and they could be readily analyzed automatically. This result was also reproduced in other species, *Arthrobacter ramosus* IFO 12958^T, *Escherichia coli* NIHJ JC-2, *Flavobacterium meningosepticum* IFO 12535^T, and *Ps. fragi* IFO 3458^T (data not shown). Therefore, primer R28 was adopted for RAPD of bacterial strains.

Fig. 4. RAPD patterns of *Bacillus pumilus* IFO 12092^T using primers B1 ~ B11 and R28.



We applied to RAPD with primer R28 a wide variety of thirty strains, including both Gram-positive and -negative genera (Fig. 5). A number of characteristic band patterns sufficient for analysis was detected on acrylamide gels. Three different

Fig. 5. RAPD patterns of various bacterial strains using primer R28.



1, *Acinetobacter calcoaceticus* IFO 13006; 2, *Aeromonas hydrophila* IFO 3820; 3, *Agrobacterium tumefaciens* IFO 3058; 4, *Shewanella putrefaciens* IFO 3908^T; 5, *Azotobacter chroococum* IFO 12994; 6, *Escherichia coli* NIHJ JC-2; 7, *Erwinia herbicola* IFO 12686; 8, *Flavobacterium meningosepticum* IFO 12535^T; 9, *Sphingobacterium spiritiborum* ATCC 33861^T; 10, *Rhizobium leguminosarum* IFO 14168; 11, *Pseudomonas aerginosa* A3; 12, *Ps. caryophylli* IFO 12952; 13, *Ps. fragi* IFO 3458^T; 14, *Comamonas testosteroni* ATCC 11996^T; 15, *Serratia liquefaciens* IFO 12979; 16, *Proteus vulgaris* IFO 3167; 17, *P. vulgaris* ATCC 6898; 18, *P. vulgaris* IFO 3851^T; 19, *P. vulgaris* ATCC 6380; 20, *Xanthomonas campestris* IFO 13551; 21, *Arthrobacter ramosus* IFO 12958^T; 22, *Bacillus laterosporus* ATCC 64^T; 23, *B. licheniformis* IFO 12200^T; 24, *B. pumilus* IFO 12092^T; 25, *B. subtilis* IFO 13719^T; 26, *Corynebacterium glutamicum* ATCC 13869; 27, *Corynebacterium michiganens* IFO 12471; 28, *Micrococcus luteus* IFO 3333^T; 29, *M. roseus* IFO 3768^T; 30, *Staphylococcus aureus* 209P JC-1.

strains of *Proteus vulgaris* IFO 3167, ATCC 6898, and ATCC 6380, showed similar band patterns. They had a high DNA similarity level, and their homology values were in the 80 to 92% range, although the level of similarity between *P. vulgaris* IFO 3851^T and these three *P. vulgaris* strains was relatively low (25~50%).

As shown in this study, RAPD analysis is a valuable technique to differentiate bacteria. The RAPD patterns provide an adequate means for identification and reveals information relatedness, because the dendrogram based on RAPD band patterns using fragin producers with primer R28 correlated well with the results of DNA hybridization, HPLC analysis, isolation source, and other traditional taxonomic study. The method was also applicable to a wide range of bacteria. Rapid identification by this method can exclude similar bacterial isolates by the comparison of band patterns.

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