Restriction Fragment Length Polymorphism Analysis (RFLP) of Some Streptomyces Strains from Soil

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Abstract: To separate the unknown Streptomyces strains isolated from soil samples, the interspacer regions of 16S-23S rDNA of 14 isolates were amplified with PCR (polymerase chain reaction) and digested with three restriction endonucleases, namely, Bsp143I, HaeIII and MnlI. The restriction patterns were used for RFLP (restriction fragment length polymorphism) analysis. A dendrogram were constructed using the unweighed pair group method using arithmetic averages algorithm (UPGMA) after analysis restriction patterns. Five RFLP groups were obtained and one test strain was left as a single member group. RFLP profile indicated that unknown strains could be identified with data based on the interspaced region of 16S-23S DNA rapid and quickly.

Key Words: Fingerprinting, intergenic rDNA, RFLP, Streptomyces

Toprakta İzole Edilen Bazı Streptomyces Suşlarının Restriksiyon Fragment Uzunluk Polimorfizm (RFLP) ile Analizi

Özet: Toprakta izole edilen ve bilinmeyen Streptomyces suşların b...
Introduction

A number of nucleic acid fingerprinting studies have been designed to separate closely related species and strains of the genus \textit{Streptomyces}. Strains belonging to different \textit{Streptomyces} species were subjected to restriction endonuclease digestion of genomic DNA by Crameri et al. (1) to determine the relationships between them. Since the generation of complex fingerprints contained a large number of low molecular weight fragments, it did not provide sufficient resolution for determining taxonomic relationships at species level. Beyazova and Lechevalier (2) applied the low-frequency restriction fragment analysis technique to differentiate between strains of six \textit{Streptomyces} species. Doering-Saad et al. (3) used RFLP to evaluate the potato scab-inducing and non-pathogenic streptomycetes and concluded that it was of little importance in the classification of \textit{Streptomyces} species. In contrast, Kim et al. (4) reported that RFLP data can distinguish between species assigned to various \textit{Streptomyces} genera. Gürtler and Stanisich (5) stated that conserved regions in the 16S and 23S rRNA genes flanking the 16S-23S rDNA spacer region is a reliable segment for distinguishing between strains. Clarke et al. (6) observed considerable variation in RFLP patterns of representatives of various \textit{Streptomyces} species although several common fragments were also examined and it was concluded that RFLP analysis of ribosomal RNA genes appeared to be an accurate and rapid strain identification tool for establishing relationships between closely related \textit{Streptomyces} species. Sadowsky et al. (7) used PCR-fingerprinting to differentiate between closely related strains of plant pathogenic \textit{Streptomyces} and show that the method might provide a useful and rapid way of determining organism identity. Hain et al. (8) examined the 16S-23S intergenic spacer region of identified strains of \textit{Streptomyces albidoflavus}. Electrophoresis and fragment size analysis of these products revealed extensive variability in the number and size of the spacer regions and this led to the recognition of 19 distinct banding patterns. This method was considered to be useful for discriminating between streptomycetes at strain level.

For the purpose of characterisation and classification, representative strains of different groups of \textit{Streptomyces} were subjected to the DNA fingerprinting technique (RFLP). This technique has been in use to differentiate bacteria, especially streptomycetes (9). Other methods of DNA fingerprinting such as Random Amplified Polymorphic DNA (RAPD) have also been applied to eliminate duplicate strains of actinomycetes (10) as well as to identify conserved regions of actinomycete genomes (11). In the present study, the representative strains of multimember colour groups were subjected to RFLP study and we report the usefulness of 16-23S rDNA intergenic spacer analysis for discrimination of unknown strains of the \textit{Streptomyces} genus.

Materials and Methods

Cultivation and DNA extraction and purification of strains: Fourteen representatives strains isolated by Sembring (12) were obtained from the Department of Environmental Agriculture
and Biological Sciences in Newcastle University and were cultivated in non-sporulation agar for 3 days at 25°C (Table 1).

All solutions used in the experiments were prepared from dilutions of stock solutions of the main reagents. Molecular biology grade reagents and enzymes were obtained from commercial suppliers (Sigma and Gibco). Stock solutions were prepared according to Sambrook et al. (14). Genomic DNA from strains of *Streptomyces* were prepared according to the guanidine thiocyanate extraction method (15).

**PCR amplification and RFLP analysis:** Strains were studied by the RFLP of the interspace regions of rDNA genes and by constructing a similarity matrix and dendrogram to show the relationship among the samples. PCR amplifications of the interspacer region of 16S-23S rDNA were carried out in a Perkin Elmer DNA Thermal Cycler 480, using 0.5 ml PCR microfuge tubes. Taq DNA polymerase, MgCl₂ solution and Taq buffer were from Biotaq™ DNA Polymerase Bioline. Deoxyribonucleotides (dNTPs) were obtained from Boehringer Mannheim GmbH, Germany, at a concentration of 100 mM. Working stock solution of dNTPs was made by mixing in a master stock in equimolar ratio to produce the final concentration of individual dNTP at 25 mM. The forward primer GP1 (\(5’-\text{GCGATTGGGACGAAGTCG}3’\)) and reverse primer GP2 (\(5’-\text{TATCGTGGC CTCCCACGTCC}3’\)) were used for PCR amplification. The reagents were mixed by vortexing and collected at the bottom of the tubes by a short pulse centrifugation (5 seconds). The tubes were kept in ice until they were placed in the Thermocycler. After the addition of the DNA sample, the reaction mixture was placed in the Thermocycler block and heated at 96°C for 5 min in an initial denaturation, and then Taq polymerase (0.5 ml) was

<table>
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<th>Strain Code</th>
<th>Sample source</th>
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<td>Ectorhizosphere</td>
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<tr>
<td></td>
<td>A5P2</td>
<td>Rhizoplane</td>
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<td></td>
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* from Seimbung 2000

Table 1. *Streptomyces* strains used in the RFLP in this study.*
added. The PCR amplification was performed according to the following temperature profile: an initial denaturation at 95°C for 5 min, 35 cycles of denaturation (1 min at 95°C); annealing (1 min at 55°C); extension (1 min at 72°C) and final extension at 72°C for 10 minutes.

The PCR products were kept at 4°C and then checked by agarose electrophoresis (1%, w/v in 0.5XTBE) on 0.5XTBE running buffer, containing 0.5 µg/ml ethidium bromide. Approximately 5 µl of the PCR reaction was mixed with 1 µl of gel loading buffer (blue juice) and loaded into wells in the agarose gel slab. Electrophoresis was run at 100V for 1h, and the size of amplified fragments (interspacer region of 16S and 23S rDNA) were identified by comparison with molecular size marker (Gene Ruler™ 100 bp DNA Ladder Plus, MBI Fermentas) at the position of 500-bp. PCR amplified 16S rDNA was separated by preparative agarose electrophoresis (14). After separation, it was eluted and purified from the agarose gel by a kit (Nucleospin Extract, Biogene Limited, Macherey-Nagel GmbH & Co. KG).

Digestion with the restriction enzyme: The PCR products (8.5 ml) were digested with three restriction endonucleases, *Bsp143I, HaeIII* and *MnlI* (Boehringer, France), at 37°C for 3 hours. Digested DNAs were analysed by horizontal electrophoresis in 4% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, ME, USA). Electrophoresis was carried out at 100V for 220 minutes with a gel electrophoresis apparatus in TBE buffer. The superladder-low 20-bp ladder (GenSura Laboratories Inc., Del Mar, California) was used as a DNA marker. After electrophoresis, scanning images analysis of the gel and estimation of fragment size were carried out under UV light and documented on a BioRad illuminator connected to a computer program called the Gel Compare Program. Restriction fragments shorter than 99bp were not considered in the analysis. Restriction patterns of each enzyme for whole test strains were transferred as TIF files for analysing in the Molecular Analysts Program. Informative bands derived from restriction enzyme digestion were scored by their presence or absence, and similarity and divergence were calculated. A similarity percentage distance matrix and dendrogram were constructed. The unweighed pair group method using arithmetic averages (UPGMA; 16) was used to construct the dendrogram.

Results and Discussion

DNAs of all 14 strains tested were amplified with the ribosomal primers used in this study. All the strains produced a single band of about 300-400 bp. This size corresponds to the predicted size of the interspace region of 16S-23S rRNA gene from the primer pair used here. The three endonucleases (*MnlI, HaeIII* and *Bsp143I*) tested in the present study produced identical fragment patterns (Figure 1). Electrophoresis of these products revealed extensive variability in the number of spacer regions. In the restriction patterns of these three enzymes, clear bands were observed from the following whole test strains: A1P1, A5P2, A1P3, B1P2, B4P3, B1P1, C7P1, C3P1, C1P3, D2P1, D5R1, E2P1, E3P1, E1P1; and they produced identical bands. The restriction patterns of *Bsp143I* are shown in Figure 1 and combined
restriction patterns of the three enzymes are shown in Figure 2. To estimate the genetic relationships, scoring by fragment presence or absence was performed. A genetic distance dendrogram constructed from RFLP data using the UPGMA algorithm and the group compositions of each grouping are shown in Figure 2. Levels of similarities of 16S-23S spacer regions of rDNA genes digested with Bsp143I, HaeIII and MnlI endonuclease enzymes are seen in Table 2.

Group I (<72% similarity) included 2 out of the 14 strains studied. They were assigned to colour group 1 (A1P1, A5P1). Group II (<78% similarity) included 4 test strains; these belonged to colour group 3 (C1P3, C7P1, C3P1) and colour group 2 (B4P3). The RFLP profiles of this group indicated that the stains in colour group 3 are closely related each other. However, group II consists of strains isolated from rhizoplane, non-rhizosphere and ectorrhizosphere. Group III (<74% similarity) included 2 test strains which were colour group 4 strains (D2P1 and D5R1). Group IV (<74% similarity) involved 2 test strains (B1P2, B1P1)
which belonged to colour group 2. Strains belonging to colour group 5 were assigned to group V (E2P1, E1P1, E3P1). Group V strains (<74% similarity) belonged to colour group 5 and were isolated from ectorhizosphere soils. One test strain (A1P3) belonging to colour group I was in a single member group. However, this strain was grouped with its original group I strains at 55% similarity. It is important to determine the minimum number of restriction enzymes for examining the phylogenetic affiliation of the many isolates from environments using RFLP analysis of PCR amplified interspacer rDNA (17). Laguerre and colleagues (18) reported that the four tetrameric restriction enzyme combinations were the minimum needed to discriminate among *Rhizobium*. However, Moyer et al. (19) reported that a combination of tetrameric restriction enzymes had good resolution on the phylogeny of their computer-stimulated groups. Three or four endonuclease combinations are necessary for estimating approximate phylogenetic relationships among interspacer rDNA amplified from various environmental samples. In the present study, PCR-RFLP analysis using three restriction enzymes was demonstrated to approximate phylogenetic relationships among unknown isolates of *Streptomyces* from soil samples. Thus, this PCR-RFLP method could serve as a rapid tool for estimating the approximate phylogenetic relationship of isolates, without the need for

![Dendrogram obtained by unweighed pair group method using arithmetic averages (UPGMA; Sneath and Sokal, 1973) based on RFLP data.](image)
interspacer rDNA or 16S rDNA sequencing. It has been reported that PCR-RFLP analysis on the basis of the 16S-23S spacer region has been increasingly used to resolve closely related isolates in recent years, since the spacer region is more variable than the 16S rDNA genes (17, 20).

It is time consuming and difficult to identify unknown Streptomyces strains by phenotypic characters. PCR-RFLP analysis of the intergenic spacer regions of 14 strains show promise as a rapid tool for distinguishing the strains of Streptomyces. In the present study, many strains did not belong to previously described species. Hain et al. (8) concluded that this method is reproducible and useful for discriminating between streptomycetes at strain level since the spacer regions have extensive variability in number and in size. This method is very simple and does not need other supporting procedures. RFLP analysis linked with DNA databases should be particularly helpful in studies requiring rapid examination of Streptomyces isolates from different environments. It will be a rapid tool for distinguishing unknown strains of the genus Streptomyces and should be useful in routine identification systems.

References


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