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Identification of *Streptomyces rochei* (*MMKK5*_1492R_S0068) by using 16S rDNA Sequencing Isolated from Coal Fields of Andhra Pradesh

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Actinomycetes are best known for their ability to produce antibiotics and are gram positive bacteria which comprise a group of branching unicellular microorganisms.In this study, we revealed rapid detection of streptomycin – producing *Streptomyces sp*. By extraction of total soil DNA from 16 soil samples using a modified lysis method followed by PCR amplification of a genus specific sequence in the *Streptomyces* 16S r DNA gene. DNA band of the expected size was seen with the sample (MMKK5_1492R_S0068 (850 bp). Additionally16S rDNA fragment was amplified by PCR from MMKK-5 genomic DNA using 16S rDNA universal primers:8F and 1492R. These results indicate that PCR amplification of Streptomyces specific genes could be used for direct detection of streptomycin – producing Streptomyces *rochei* from coal mine soil of Andhra Pradesh

Key words: Actinomycetes, 16 s rDNA, Amplification.

Traditional actinomycete taxonomy was dependent on morphological characteristics such as aerial spore mass colouration, diffusible pigments and spore chain morphology. The subjective nature of such tests, the high nature morphological diversity and intense economic interest in these organisms led to a massive over speciation of the genus (Trejo, 1970). Microbial nature products constitute one of the most important sources of lead compounds in the pharmaceutical industry. Despite a trend towards the use of alternative sources, including synthetic combinatorial libraries, computer-based molecular modeling and most recently combinational biology (or) the manipulation of biosynthetic gene clusters and the pharmaceutical pipeline remains crowded with traditional, microbial-derived natural products and their derivatives (Jensen *et al*., 2003). The taxonomic integrity of cluster analysis was further brought into doubt by the results of fatty acid analysis (Saddler *et al*., 1987) total protein pattern (Manchester *et al*., 1990), DNA: DNA relatedness (Labeda and Lyons, 1991), numerical phenetic survey (Goodfellow *et al*., 1992) and the LFRFA (Beyazova and Lechevalier, 1993) studies. Actinomycetes, phylogenetically defined as a number of a taxa within the high G+C sub-division of the gram-positive phylum (Embley and Stackebrandt, 1994) are involved in important

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processes in a wide range of habitats . Metabolites from actinomycetes continue to be an important source of antibiotics, enzymes and bioactive products. To overcome the severe limitations of culture-dependent methods in discovering bacterial diversity (Giovannoni *et al*., 1990), Molecular biological techniques have become increasingly popular (Muyzer and Ransing, 1996). Actinomycete groups have been detected and characterized by their 16S r RNA sequences in cases where cultivation has proved unsuccessful (Relman *et al*., 1992; Niner *et al*., 1996;). The well-known fact is that the identification of each isolate by using polyphasic taxonomy method is time-consuming, which cannot ever be undertaken when handling large numbers of new isolates in a natural product screening program dealing with bacteria especially. About 50% of these bacteria are known only by 16S r RNA gene sequences (Paster *et al*., 2001). PCR amplification of conserved regions of the 16S r RNA gene followed by cloning and sequencing of PCR products has been widely applied to study of the bacterial diversity in different human healthy and diseased sites. Several studies have devised PCR primers based on sequences from oral clones libraries to survey larger number of samples from periodontally diseased subjects for the presence of uncultivated bacteria In an attempt to establish correlations with disease etiology (Harper-Owen *et al*., 1999; Leys *et al*., 2002; Sakamoto *et al*., 2002 and Kumar *et al*., 2003) distance matrix methods may produce either rooted or unrooted trees depending on the algorithm used to calculate them.

Once the need for molecular identification is established, the method of identification needs to be determined. Gen-probe offers a number of culture identification tests that are based on hybridization of a probe to RNA extracted from a culture isolate. Each kit contains one probe for a specific organisms. These kits perform very well and are useful for specific applications, but their use is limited because an unknown isolate can be tested with only one probe at a time, and a limited number of probes are available. High-density probe arrays will allow for hybridization to many probes at the same time. This technology will be a valuable tool for bacterial and actinomycete identification, especially in clinical situations in which the types of bacteria present in a specimen are predictable. However, identification of hybridization only allows a microbiologist to ask whether an unknown isolate is one of these organisms. There will always be unpredictable or novel isolates. Sequencing is more powerful molecular identification method because it answers the question "What is the unknown isolate?". The fragment also includes two of the three regions of the 16S r DNA which are reported to be suited for species discrimination of streptomycetes. Another advantage of the second approach is that the group-specific pattern of the actinomycetes from one sample can be directly compared to the bacterial community pattern of the same sample because the same fragment is analyzed.

MATERIAL AND METHODS

Isolation of DNA

Cultures were grown in 20 ml of TSB in 50-ml conical tubes for 7 days and then centrifuged at 4,000 rpm (Centra 4 model centrifuge; International Equipment Company) for 5 min. The mycelial pellet was resuspended in 500 µl of 5 M NaCl and transferred to a 2-ml Eppendorf tube. The cells were centrifuged (Eppendorf model 5415 centrifuge) at 10,000 rpm for 30 s, and the pellet was resuspended in 1 ml of 10 mM Tris-Cl-1 mM EDTA (pH 7.5) (TE) containing 20 mg of lysozyme/ml and 20 mg of RNase A/ml and incubated at 37°C for 1 h. Following incubation, 250 µl of 0.5 M EDTA, 250 µl of TE containing 5 mg of proteinase K/ml, and 100 µl of 10% sodium dodecyl sulfate were added to each tube and incubated at 37°C for 1 h. The tubes were mixed by inversion after the addition of 250 µl of 5 M NaCl. Immediately thereafter, 200 µl of cetyltrimethylammonium bromide (CTAB) solution (10% CTAB plus 0.7 M NaCl) was added, and the tubes were heated in a 65°C water bath for 10 min. Cellular debris was removed by centrifugation (8,000 rpm for 5 min in an Eppendorf model 5415 centrifuge), and the supernatant solution was transferred to a new 2-ml microcentrifuge tube. Proteins and lipids were removed by the addition of 0.3 volume of phenol-chloroform, and the phases were mixed by inversion and centrifuged at 12,500 rpm for 5 min. The aqueous phase was transferred to a new tube, and the DNA was precipitated with an equal volume of isopropanol. After the genomic DNA was

centrifuged, the pellet was rinsed with 70% ethanol to remove traces of salt, dried, and redissolved in 200 µl of TE for immediate use or storage at -20°C.

16S rRNA gene amplification and DNA sequencing.

The 16S rRNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR Universal primers 8F 5'-AGAGTTTG ATCMTGG-3'1492 R5'ACCTTGTTA CGACTT-3 'were procured from Bangalore Genie, India. DNA amplifications were performed in a model PTC 200 thermal cycler). PCR was performed in a 25 µl reaction mixture with final concentration (per reaction) of 1PCR core buffer, 2.5 mM $MgCl_2$, 0.2 mM (each) dNTPs, 10 pmol of each primer, 1 U of AmpliTaq and 50 ng of template. First denaturation was carried out for 3 min at 95°C. Initial amplifications were performed as 5 cycles of 95°C for 30 s (denaturation), 52°C for 30 s (annealing) and 72°C for 1.5 min (extension). Further amplification was performed as 25 cycles of 95°C for 30 s (denaturation), 51°C for 30 s (annealing) and 72°C for 1.5 min (extension), followed by a 10 min final extension of 72°C. Negative controls were carried out without template to ensure there was no contamination. PCR products were resolved by electrophoresis in 2% low melting point agarose, and visualized by ethidium bromide staining. Sequencing of the PCR products was carried out by on an automated multi-capillary DNA sequencer, ABI Prism 3130xl Genetic analyzer using the Big Dye Terminator v.3.1 Ready Reaction BDT v3.1 Cycle Sequencing Kit. Sequences were then matched with those already known using the BLAST (Basic local alignment search tool) search option at NCBI Genbank (http:// www.ncbi.nlm.nih.gov) and Based on maximum identity score first ten sequences were selected for construction of the phylogenetic tree using MEGA4

RESULTS AND DISCUSSION

The genomic DNA MMKK5_1492R_S0068 of were subjected to PCR-amplification of the ITS region by using ITS1 and ITS4 primers. The amplified PCR product turned out to be approximately 700 bp in length as shown in the Fig.2.

DNA sequencing

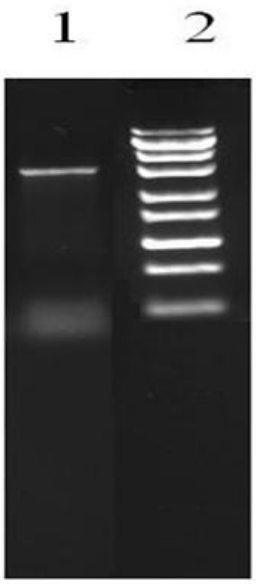
The amplicon DNA was gone to the Sequencing using DF (Direct Forward) DF: 5 ' A C C C C G C T G A A C T T A A G G - 3 ' a n d (D i r e c t R e v e r s e) D R : 5'GGTCCGTGTTTCAAGACGG3'Primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.

Comparison and constructing the phylogenetic tree

D2 region of LSU seq was compared with database using the BLAST (Basic Local Alignment Search Tool) search tool, based on maximum identity, E value ; the first ten (DQ327661.1, AY855912.1, AY351921, FJ372693.1, AF261536.1,

Table 2. Blast report showing the accession no, description, max score, total score query coverage max identity, Evalue of EU551710.1, FJ792552.1, EU741219.4, F799184.1, FJ792771. FJ532468.1, EU741237.1, FJ532445.1, FJ547121.1, FJ792558.1

Alignment View	ID	Alignment Results	Sequence Description
	MMKK5	0.98	Sample Studied (MMKK5)
	EU741237.1	0.96	*Streptomyces mutabilis* strain 13676F 16S rRNA
	EU741219.1	0.95	*Streptomyces spiralis* strain 13668B 16S rRNA
	EU551710.1	0.96	*Streptomyces* sp. C47 16S ribosomal RNA gene
	FJ792577.1	0.94	*Streptomyces rochei* strain cfcc3165 16S rRNA
	FJ792558.1	0.93	*Streptomyces rochei* strain cfcc3131 16S rRNA
	FJ792552.1	0.92	*Streptomyces rochei* strain cfcc3115 16S rRNA
	FJ799184.1	0.94	*Streptomyces anandii* strain MJM7840 16S rRNA
	FJ532468.1	0.95	*Streptomyces enissocaesilis* strain HBUM174967
	FJ532445.1	0.96	*Streptomyces mutabilis* strain HBUM174166 16S rRNA
	FJ547121.1	0.96	*Streptomyces rochei* strain HBUM175171 16S rRNA



Lane 1 D2region of LSU
Lane 2 DNA markers

Fig. 1. The Gel electrophoresis showing the result of amplified PCR product of 700bps

AY586703.1, J488128, AY684160.1, AF518625.1, AF393074.1) Identities were taken and constructed the phylogenetic tree using MEGA 4 software .The closest sequence was Pycnoporous sp. DIS 343f was conformed as show in the table

The evolutionary history was inferred using the Neighbor-Joining method[25]. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed[26]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method[27] and are in the units of the number of base substitutions per site.Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 573 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4.

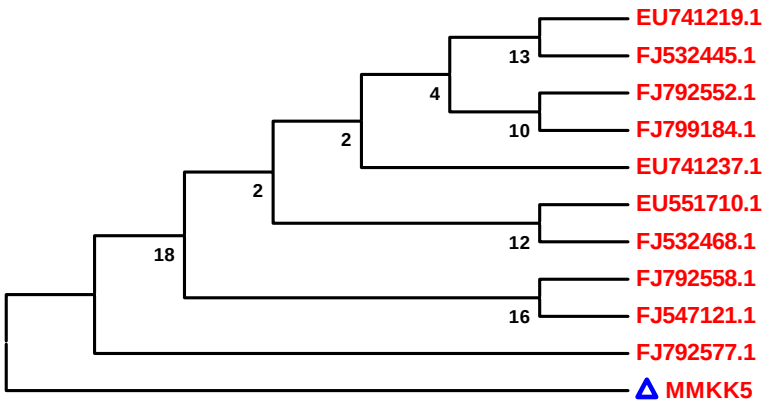


Fig. 2. Evolutionary relationships of 11 taxa

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