# Fingerprinting Method for Phylogenetic Classification and Identification of Microorganisms Based on Variation in 16S rRNA Gene Sequences

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## ABSTRACT

*The paper describes a method for the classification and identification of microorganisms based on variations in 16S rRNA sequences. The 16S rRNA is one of the most conserved molecules within a cell. The nature of the variable and spacer regions has been found to be specific to a given organism. Thus, the method presented here can be very useful for the classification and identification of microorganisms for which very little information is available. To automate the method, a comprehensive computer program called FPMAP has been developed for the analysis of restriction fragment pattern data. The method involves the restriction digestion of genomic DNA, preferably using four-cutters that may recognize 6–9 sites within the 16S rDNA. The fragments are separated on a polyacrylamide gel along with a suitable marker, then transferred into a nylon membrane and hybridized with a radiolabeled 16S rDNA probe. After autoradiography, the fragment sizes are calculated, and the data are analyzed using the FPMAP software. We demonstrate that the method can be used for identification of strains of* Streptomyces *and* mycobacteria. *The software is available from our ftp site ftp://imtech.chd.nic.in/pub/com/fpmap/unix/.*

## INTRODUCTION

The identification and classification of microorganisms are of fundamental importance in microbiology. Classical methods are mainly emperical and depend on phenotypic data. They are also time consuming and expensive. More recently, various fingerprinting methods using some unique property of an organism have been described. The existing approaches to fingerprinting can be broadly classified as: *(i)* classical DNA fingerprinting such as restriction enzyme analysis of the genomic DNA on pulse field gel electrophoresis, *(ii)* the use of a repetitive DNA sequence as a probe for a particular region of the genome, *(iii)* DNA-DNA hybridization, *(iv)* PCR amplification of a particular section of the genome followed by restriction analysis of the amplified fragment and (v) the use of an insertion sequence.

In all of these methods, genomic DNA has been the subject of analysis (6,10,22). A serious limitation of these approaches is that alterations in the DNA structure and composition occuring during the processing of the DNA are completely overlooked. Further, it is a well-known fact that many organisms undergo large deletions in chromosomal DNA, or loss or gain of plasmids, acquisition of transposons or an *IS* element either during different growth condition or in response to environmental conditions. An organism may exhibit slightly different characteristics depending on the environment from which it was isolated. Thus, the properties related to the presence or absence of plasmids, transposons or *IS* elements are unsuitable as tools for phylogenetic identification. However, almost all of these methods have been used for epidemiological studies or to differentiate between strains of the same species or to confirm the species of the genus.

Ribosomes are a part of the translational machinery of a cell, and rRNA is vital for cellular growth, function and survival. Consequently, the primary, secondary and tertiary structures of rRNA molecules have been conserved during evolution (8). Analysis by classical methods of comparative oligonucleotide cataloguing and consideration of full or partially complete sequences of 16S rRNA have revealed that the primary structure of rRNAs consists of highly conserved regions interspersed by regions of moderate to low homology within related species (4). Despite the highly conserved nature of rRNAs, they vary in size and in the organization of the spacer as well as variable regions within the rRNA. This results in variation of the cleavage sites for restriction endonucleases. The small size of 5S rRNA and extensive secondary

and tertiary structures present in the 23S rRNA render these molecules unsuitable for sequencing and further analysis. However, the moderate size of the 16S rRNA, sequence conservation during evolution and proposed and confirmed importance in classification has proven that 16S rRNA sequences can be used effectively for phylogenetic identification of the microorganisms at various levels of heirarchy. Most of the earlier studies have used rRNA sequences for some kind of epidemiological studies or to confirm the earlier data of the phylogeny of a particular group. No earlier studies in the literature have concentrated on using the 16S rRNA sequence for the phylogenetic identification of organisms.

In the present work, the variation within 16S rRNA is exploited for phylogenetic identification and classification of microorganisms. An important fingerprinting technique is the analysis of the restriction fragment length polymorphism (RFLP). Several computer programs have been developed to compare the RFLP of a test organism with the RFLP database generated in the same laboratory, but not in different laboratories (3,5,20,21). However, in this study, we have developed a comprehensive software named FPMAP (fingerprint mapping) that not only compares the RFLPs but also facilitates *(i)* the selection of suitable restriction enzymes for the sequences obtained from GenBank® or the ribosomal database project (RDP), *(ii)* the graphical presentation of the positions of restriction sites, *(iii)* the graphical presentation of the positions of the DNA fragments according to the molecular weights, *(iv)* RFLP analysis and comparison with the subject strain RFLP and *(v)* the creation of a PHYLIP format of the complete data and the use of the PHYLIP software package to generate an unrooted tree. The phylogenetic tree obtained indicates the identity of the test strain.

To demonstrate the use of a fingerprinting technique in the identification and classification of microorganisms, we selected two genera, *Streptomyces spp*. and *Mycobacterium spp*. The restriction enzyme sites of the 16S rRNA sequences of the type strains of these genera available in GenBank and RDP were analyzed using the FPMAP program. Using a 16S rRNA sequence as a probe and FPMAP as a pattern analysis tool, one can phylogenetically identify a strain. Because the maximum size of the 16S rRNA is 1500 bp, many restriction enzymes will have no sites. Methylase-sensitive enzymes may not be a good choice. An enzyme that does not cut frequently may also not be a good choice because conservation in the 16S rRNA sequences is high and only those enzymes that have small recognition sequences are likely to produce good RFLP analyses. Success of the method depends, therefore, on the selection of suitable restriction enzymes, suitable

molecular weight markers and good electrophoretic resolution. We recommend the selection of restriction enzymes that cut frequently and produce DNA fragments in the range of 20–700 bp, and the use of 7%–8% nondenaturing polyacrylamide gel for electrophoresis. The FPMAP software has been developed in standard C language so that it can be ported to a variety of other platforms. Executable and source codes of the FPMAP are available at ftp://imtech.chd.nic.in/pub/com/fpmap/unix/.

## MATERIALS AND METHODS

### Media, Chemicals and Strains

Type strains of *Mycobacterium smegmatis* (ATCC 14468), *M. vaccae* (DSM 43292) and *Streptomyces rimosus* (from M. Goodfellow's collection, University of Newcastle upon Tyne, UK) were obtained from the Microbial Type Culture Collection (IMTECH, Chandigarh, India) and *M. bovis* BCG was obtained from the Tuberculosis Vaccine Laboratory, Madras, India. *S. coelicolor* B385 was a gift from Prof. D.A. Hopwood (John Innes Centre, Norwich, UK). A new isolate of *Saccharomonospora sp.* (PA136, classified and identified by chemotaxonomy) and *Saccharomyces cerevisiae* DNA were used as controls. *M. bovis* BCG and *M. smegmatis* and *M. vaccae* were grown on Sauton's medium. (One liter contains 0.05 g ferric ammonium citrate, 4 g L-asparagine, 2 g citric acid, 0.5 g $K_2HPO_4$, 0.5 g $MgSO_4$, 60 mL glycerine, 2.5 mL 0.1% $ZnSO_4$ and 6 mL 5 M NaOH to make pH 7.2.) *Nocardia lurida* (DSM 43134) and *N. asteroides* (DSM 43757) were grown on the CM medium (2 g yeast extract, 10 g malt extract, 4 g glucose and *S. coelicolor* B385 and *Saccharomonospora sp.* were grown as described by Hopwood et al. (9). All chemicals were obtained from Sigma (St. Louis, MO, USA), and the medium components were obtained from Difco, Detroit, MI, USA. Restriction enzymes and labeling kits were purchased either from Promega (Madison, WI, USA) or from New England Biolabs (Beverly, MA, USA).

### Preparation of Genomic DNA and Southern Hybridization

Genomic DNA from *Streptomyces sp*. was isolated essentially as described by Hopwood et al. (9) but with some modification. Fifty milligrams of wet mycelium was suspended in 0.5 mL lysozyme solution (2 mg/mL and 50 µg/mL RNase from Promega) and incubated at 37°C until the cells became translucent. In this mixture, 0.25 mL 2% SDS was added and vortex mixed until the viscosity of the solution decreased noticeably. To this solution, 0.25 mL neutral phenol-chloroform was added and vortex mixed for 30 s. The solution was centrifuged for 2 min, and the supernatant was collected in a fresh tube. The procedure was repeated twice more before final chloroform extraction. To this supernatant, 0.1 vol 3 M sodium acetate (pH 4.8) (0.3 M as a final concentration) was added and mixed. DNA was precipitated with 2 vol cold absolute alcohol by slow mixing and cooling in ice. We found that by intermittent cooling and slow mixing, it was possible to obtain high molecular weight DNA as a pellet that could be removed by a glass rod or pipet tip.

Mycobacterial DNA was prepared by a method developed in the author's laboratory. The 21-day-old culture was grown in Sauton's medium at 37°C at 200 rpm, Tween® 80 was added aseptically at a final concentration of 1%, and the culture was grown for another 24 h. The cells were harvested by centrifugation at 10 000× *g* at 4°C and washed twice in TE (10 mM Tris-HCl and 1 mM EDTA). The resuspended cells were again incubated at 45°C for 30 min in a Tris buffer (25 mM Tris-HCl, pH 7.5). This step seems to remove a lot of the waxy layer present outside the mycobacteria cell wall and made the cells more accessible to lysozyme treatment. The cells were resuspended in 25 mM Tris-HCl and 10 mM EDTA, pH 8.0. Lysozyme was added to the final concentration of 10 mg/mL (about 30 mg wet weight) of cells and incubated at 37°C for 2 h. To this mixture, 10 µg/mL proteinase K and 1 µg/mL RNase were added, and incubation was continued for another 2 h at 37°C. The preparation was extracted twice with phenol and then twice with chloroform. DNA was precipitated in the presence of 0.3 M sodium acetate (pH 4.5) and 2 vol cold absolute ethanol. The DNA pellet was washed twice in 70% ethanol, air-dried and dissolved in TE.

Three micrograms of genomic DNA was digested to completion by *Hha*I, *Mbo*I, *Rsa*I and *Bst*NI. Lambda phage DNA digests (*Hin*dIII, *Eco*RI + *Hin*dIII and *Pst*I) and the *Hin*fI and the *Bst*NI digest of the Bluescript® plasmid ($SK^+$, $SK^-$, Stratagene, La Jolla, CA, USA) were used as molecular weight markers. The digests were separated on a 7% polyacrylamide gel, and the separated fragments were electrotransferred onto a nylon membrane (HybondN®; Amersham Pharmacia Biotech, Little Chalfont, UK). Because of the high gel concentration and the nature of polyacrylamide, capillary transfer and gel pretreatment is not recommended. DNA was denatured in the membrane by placing it in 0.4 M NaOH for 10 min (DNA side up). The membrane was rinsed in 2× SSC for 2 min, air-dried and then fixed at 80°C for 30 min ( the DNA can also be UV fixed). The 16S rDNA from five different newly isolated and unidentified actinomycetes strains was amplified using the following primers: 27f-5′-AGAGTTTGATC**M**TGGCTCAG-3′ and 5′-AAGGAGGTG**W**TCCA-**R**CC-3′ (13) where M = A + C, W = A + T and R = A + G.

The PCR product (approximately 1.5 kb) was purified from the gel by using a DEAE-cellulose membrane (NA-45; Schleicher and Schuell, Keene, NH, USA) (19). The ends of the PCR products were phosphorylated by T4 polynucleotide Kinase, cloned into the dephosphorylated pGEM5Zf(+) vector (both from Promega) at the *Eco*RV site and transformed into the *E. coli* strain JM109. The white clones from ampicillin-X-Gal-IPTG plates were collected, and the plasmid was checked for the 16S rDNA inserts by *Nco*I and *Spe*I digestions. The entire 16S rDNA fragment was sequenced to confirm the chemotaxonomy results (to be published separately) and to use the cloned fragments as a probe. The GenBank accession numbers for these strains are as follows: AF 223346, AF 223347, AF 223348, AF 223349 and AF 223350. For use as a probe, the cloned fragment was recovered by digesting the recombinant vector with *Nco*I and *Spe*I enzymes and was purified from the agarose gel as described by Sambrook et al.

(19) using DEAE-cellulose membrane. The purified 16S rDNA fragment was radiolabelled with α[$^{32}$P]dCTP using a NEBlot® random priming kit (New England Biolabs) as described by the manufacturer. Hybridization was carried out at 50°C using a hybridization bag in a buffer containing 30% formamide, 5× SSPE containing 0.9 M NaCl, 50 mM sodium phosphate, 5 mM EDTA (pH 7.7), 0.5% SDS and 5× Denhardt's reagent containing 0.05% BSA, 0.05% Ficoll® Type 400 (Sigma) and 0.05% polyvinylpyrrolidone. The membrane was incubated in a hybridization buffer at 50°C for 2 h before adding the radiolabeled (α[$^{32}$P]dCTP) and denatured probe in the same solution. After removing all the air bubbles and sealing the bag, hybridization was carried out for 22 h. The membranes were washed in 2× SSC with 0.1% SDS at 67°C for 30 min and in 1× SSC with 0.1% SDS at 40°C for another 30 min before autoradiography with Kodak® X-Omat™ films (Eastman Kodak, Rochester, NY, USA) for 10–24 h. The sizes of the hybridizing bands were calculated with the program DNASIZE (16).

## RESULTS

Figure 1 suggests that a good-quality, high-molecular-weight DNA can be obtained from mycobacteria without using a commercial kit or guanidine hydrochloride.

To test the FPMAP identification software, we selected species of the genera *Mycobacteria* and *Streptomyces*. Both genera are medically, industrially and phylogenetically important. Genomic DNA of various species of these genera were digested with the restriction enzymes *Hha*I, *Mbo*I, *Rsa*I and *Bst*NI. Figure 2 shows the result of *Hha*I digests obtained after hybridizing with 16S rRNA as probe. The selection of enzymes was based on the restriction enzyme analysis of the published 16S rRNA sequences (17). The DNA fragments present in the Southern blot were measured using the program DNASIZE (16). The RFLPs of test strains were compared with those of databases created for the strains. The result is shown in the Figure 3, A and B, in which, for *S. rimosus* and *M. bovis*, the RFLP generated by the restriction enzymes *Rsa*I and *Hha*I, respectively, is identical to the one generated using the published sequence. The RFLP data was then processed with PHYLIP, and an unrooted evolutionary tree was generated (Figure 4, A and B). Figure 5 shows, however, the phylogenetic tree that would be obtained from the sequence data. It is evident from the tree that the subject strains have formed a closed cluster with the type strain of *S. rimosus* and *M. bovis* and other related species of *Streptomyces* and *Mycobacteria*. The restriction enzymes *Mbo*I and *Bst*NI produced distinctive RFLPs during sequence analysis, but neither of the enzymes worked well during digestions. Both *Mbo*I and *Bst*NI enzymes are methylase sensitive. Perhaps methylation of the

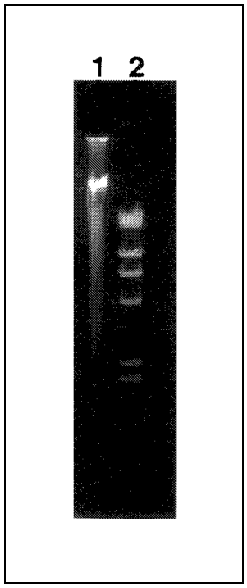


**Figure 1. DNA prepared from *M. bovis* BCG using in-house method.** (1) Undigested *M. bovis* BCG DNA. (2) λ *Hin*dIII marker.


**Figure 2. 16S rDNA *Hha*I restriction pattern hybridizing with 16S rDNA probe.** (1 and 4) pBluescript plasmid SK$^+$ and SK$^-$ digested with *Hin*fI and *Bst*NI, respectively, as molecular size markers. Lane 1, 1074, 517, 456, 396 and 75 bp; lane 4, 1849, 314, 288 and 121 bp. Lane 2, *M. bovis* BCG and lane 3, *S. rimosus*. For experimental detail, please see Materials and Methods.

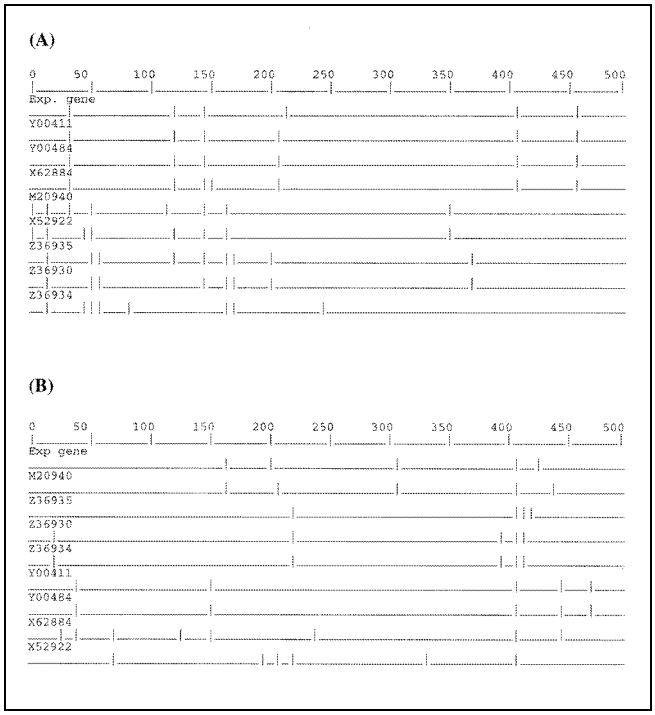


**Figure 3. Screen output of FMAP comparing the fragment maps of the experimental genes as obtained by the Southern hybridization with that obtained from the 16S rDNA sequence present in the database.** (A) Fragment map of *S. rimosus* (Exp. gene) and experimental gene for restriction enzyme *Rsa*I. (B) Fragment map of *M. bovis* (Exp. gene) and experimental gene for restriction enzyme *Hha*I where GenBank accession numbers X62884, Y00411, Y00484, Z36934, Z36935, Z36930, M20940 and X52922 represent *S. rimosus*, *S. coelicolor*, *S. lividans*, *N. asteroides*, *N. brasiliensis*, *N. nova*, *M. bovis* and *M. smegmatis*, respectively.

**Table 1. FPMAP Menu as Displayed on the Computer Screen**

| FPRINT 1.0 |
|---|
| Search restriction sites in nucleodide sequence |
| Display restriction sites in a sequence |
| Display of size of fragments in a sequence |
| Comparison of restriction sites in sequences |
| Comparison of fragments of sequences |
| Identification of an unknown gene |
| Generate fragment mapping in PHYLIP format (input) |
| Exit from program |

DNA at the *Mbo*I and *Bst*NI sites is the cause of a slightly different RFLP of the test strain than expected. Most of the strains tested produced good RFLP, but it is beyond the scope of this report to show large data.

## DISCUSSION

Evolutionary conservation and the uniqueness of the ribosomal RNA allow a broad spectrum of applications. Grimont and Grimont (6) described the importance of the 16S rRNA-based probes as taxonomic tools. The method is known as ribotyping (the generation of characteristic fragment patterns by hybridization of restriction endonuclease fragments of total DNA with labeled standard rRNA). This method has shown considerable discriminatory power in the identification of organisms up to the species level and in the typing of a number of bacteria, for example, *Aeromonas spp*. (1), *Campylobacter spp*. (15), *Gordona spp*. (18), *Legionella spp*. (7), *Mycobacterium spp*. (12), *Mycoplasma spp.* (23), *Rhodococcus spp.* (14) *Saccharomonospora spp*. (24), *Staphylococcus spp.* (11) and *Streptomyces spp*. (2).

The recent review by Gurtler and Stanisich (8) emphasizes the importance of the spacer regions of 16S rDNA and 23S rDNA genes for typing and identification. In their method, the spacer regions of the 16S–23S rDNA are amplified and then the strain differentiations are made either by digesting the PCR fragments with RFLP or by single strand conformation polymorphism (SSCP). However, these methods rely on rather complicated procedures with large resource requirements that involve several steps before one obtains a RFLP. It is apparent that the complete sequence of the 16S rRNA of an organism is enough to determine the phylogenetic position of a strain. Despite automation of DNA sequencing, it is beyond the means of many laboratories to sequence large numbers of microorganisms. However, the need to protect biodiversity from the negative impact of globalization has revived the demand for simple yet comprehensive and reliable methods of identification. In the present study, we approach the use of the properties of the 16S rDNA for phylogenetic identification from a different perspective.

We argue that if one selects a set of restriction enzymes that cut frequently within the 16S rRNA genes and use them to digest the genomic DNA of a strain, the variability in the spacer regions and in the whole operon can be detected by hybridization using 16S rDNA as a probe. Since the size of the 16S rRNA is about 1.5 kb, many restriction enzymes will have either no recognition sites or less than three. Conservation

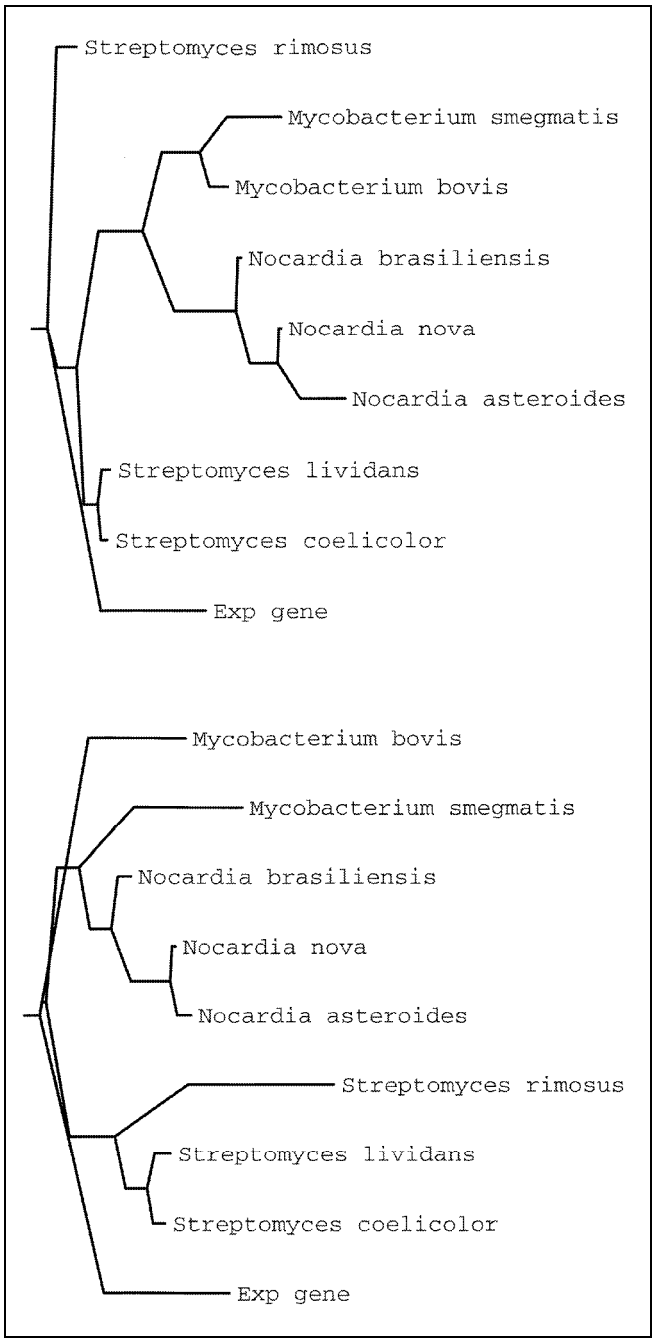


**Figure 4. Phylogenetic tree was generated by using FPMAP and PHYLIP (restml and drawgram).** (A) *S. rimosus* (Exp. gene) and experimental genes for restriction enzyme *Rsa*I. (B) Fragment map of *M. bovis* (Exp. gene) and experimental genes for restriction enzyme *Hha*I.

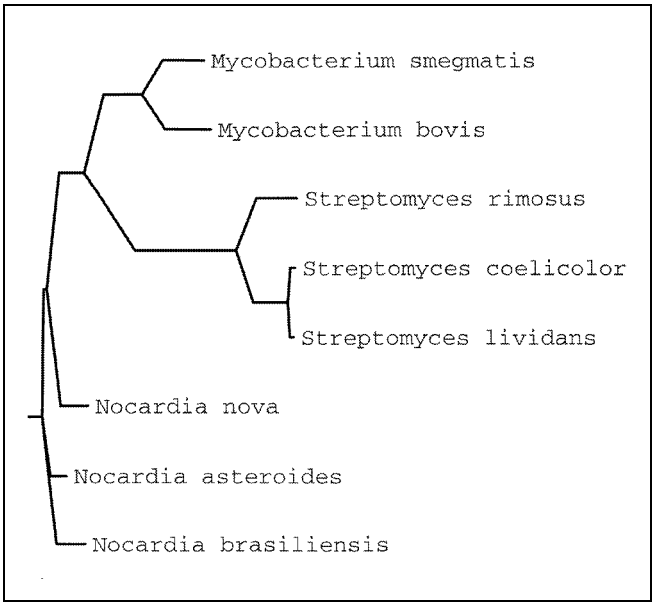


**Figure 5. Phylogenetic tree obtained using 16S rDNA sequences available in the database.** All sequences were compared using the multiple sequence alignment program CLUSTAL W. The phylogenetic tree was obtained using the program drawgram of the PHYLIP.

within 16S rRNA is such that long streches of homologous sequences are common among species of the same genus, and the GC content of the rDNA does not vary much between species. Therefore, enzymes that have short recognition sequences have more sites and can cut the DNA more frequently to reveal the RFLP within a small region of variability.

The two important factors in classification and identification using the method described here are the selection of restriction enzymes and the availability of correct and complete 16S rRNA sequences. The restriction enzymes should be such that *(i)* they cut frequently to produce distinctive RFLPs, *(ii)* the DNA fragments produced by the enzymes should separate well during electrophoresis (should not generate doublets or triplets) and *(iii)* the fragments produced should be within the working limits of the methods of electrophoresis. The partial sequence of many organisms that are available in the databases are not useful because the partial sequence does not provide a true picture of the variation within the system. The size of DNA fragments generated by the restriction enzymes is not really a limitation of this method; however, a fragment size of 6–10 bp and 1 kb and above creates some practical problems such as poor resolution, very faint signals even with the radioisotopes and difficulties obtaining appropriate molecular weight markers and selecting the optimal electrophoresis gel

concentration. It is important to use a molecular weight marker that covers the entire range of fragments produced by the restriction enzyme. Because FPMAP presents restriction sites and fragment maps graphically, the selection of appropriate restriction enzymes is facilitated. However, the identification of a strain may not be straightforward. As stated earlier, it is important to obtain distinct RFLPs. In a situation when there is some doubt, the identification option of the software allows one to visualize the fragment maps of the test and similar strains, which may aid the user in identification.

The objective of the present study was to provide a quick identification tool but not to carry out a taxonomic study. It is therefore beyond the scope of this article to present large amounts of data that could provide a real feeling for mismatches between the RFLP patterns inferred from the sequence data and the patterns obtained through experiments. Integration of FPMAP with PHYLIP permits processing of the RFLP data by PHYLIP to produce a phylogenetic tree.

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