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Rapid Identification of Mycobacterial Species by PCR Amplification of Hypervariable 16S rRNA Gene Promoter Region

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A total of 0.3 to 0.4 kb of the promoter region of the 16S rRNA genes from *Mycobacterium tuberculosis*, *M. gordonae*, *M. xenopi*, and *M. leprae* was PCR amplified, cloned, and sequenced. The observed number of substitutions, insertions, and deletions exceeded those found in previously used target sequences, including the entire 16S coding region. A simple and generally applicable restriction fragment length polymorphism method that can be used to distinguish between mycobacterial species is described.

The recent increase in infections caused by *Mycobacterium tuberculosis* as well as other so-called atypical mycobacteria has spurred the quest for more rapid alternatives to traditional, culture-based methods for mycobacterial species differentiation. In principle, this challenge can be met by the application of DNA (18) or RNA (4) amplification techniques. Of critical importance for the success of these techniques is the choice of the target sequence. Most approaches used to discriminate between the individual mycobacterial species and to establish phylogenetic relationships have thus far focused on interspecific polymorphisms of the 16S rRNA (25) or its gene (15, 16, 21, 23). However, since this gene product serves a vital function in the ribosome, the extent of permissible mutations is inherently limited. As a consequence, some species are either indistinguishable, for example, *M. kansasii* and *M. gastri*, or highly homologous, for example, *M. avium* and *M. paratuberculosis* (99.9%), even if the homology of 1.5 kb of DNA sequence is being determined (16).

In this report, we propose the use of a shorter (0.3- to 0.4-kb), noncoding, hypervariable DNA target sequence upstream of the 16S rRNA coding region which exhibits a much higher degree of interspecific polymorphisms, permitting the substitution of previously required cumbersome sequencing protocols by a more rapid and simple generation of restriction fragment length polymorphism (RFLP) patterns.

MATERIALS AND METHODS

Mycobacterial isolates. Clinical isolates of *M. tuberculosis*, *M. bovis*, *M. gordonae*, *M. xenopi*, *M. intracellulare*, and *M. smegmatis* from the Central Hospital Gauting, Gauting, Germany, were grown on a modified Löwenstein-Jensen medium (Germany Industry Norm DIN 58943, part 7). Species determination was performed by standard methods for the identification of mycobacteria (3). The *M. avium* culture and *M. leprae* cells grown on mouse footpads were kindly provided by P. Mursic, Munich, Germany, and V. Sticht-Groh, Würzburg, Germany.

DNA extractions. One to two loops of mycobacterial colonies were scraped from the agar surface, resuspended in 1 ml of 0.9% NaCl solution, and boiled for 10 min. After sonication for 1 min in a water bath sonicator, 0.5 ml of the suspension was extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) saturated with 10 mM Tris · HCl (pH 8.0) and 1 mM EDTA. After separation by a 4-min microcentrifuge spin, the DNA in the aqueous phase was adsorbed to 5 μl of a silica gel suspension (Geneclean; Bio 101, Inc., La Jolla, Calif.) and was eluted into 400 μl of water.

PCR amplifications. The construction of the upstream primer MYC-12 (5′-CGG AAT TCC ACA TCG ATC GCG G-3′) was based on a 17-bp region conserved between *M. tuberculosis* (10) and *M. leprae* (20), located 0.4 kb upstream of the start of the coding site for the 16S rRNA. The downstream primer MYC-13 (5′-CGG GAT CCT GAG CCA GGA TCA A-3′) is targeted to positions 31 through 15 in the 16S coding region. Additionally, the MYC-12 and MYC-13 primer sequences possess *Eco*RI and *Bam*HI restriction sites, respectively, to facilitate cloning of the PCR products. PCR amplifications were performed by using a "hot start" technique in which 39.5 μl of DNA solution and 1 μl each of both primers (50 μM each) were overlaid with two drops of mineral oil and were denatured at 96°C for 2 min. After cooling to 80°C, a mixture of 5 μl of 10× buffer (100 mM Tris · HCl [pH 8.3], 500 mM KCl), 2.5 μl of 50 mM $MgCl_2$, 0.5 μl of a deoxynucleoside triphosphate mixture (25 mM each), and 0.5 μl (5 U/μl) of *Taq* polymerase (Amersham, Braunschweig, Germany) was added. The most sensitive signal-to-background relation was observed after 50 cycles of denaturing at 92°C for 60 s, annealing at 60°C for 60 s, and extension at 72°C for 90 s. The reaction products were visualized in a 1.3% agarose gel containing 0.2 μg of ethidium bromide per ml.

Cloning and sequencing. PCR products (0.3 to 0.4 kb) were excised from the agarose gel, and the DNA was purified by silica gel adsorption as described above. Since direct sequencing frequently resulted in stops, the products were ligated into *Eco*RI-*Bam*HI-cut pBluescript II SK$^-$ vectors (Stratagene, La Jolla, Calif.), taking advantage of the flanking primer restriction sites, and the constructs were used to transform XL1-Blue cells (Stratagene) by electroporation. Sequencing was performed as described by Sanger et al. (19) by using a Sequenase II kit (United States Biochemical Corporation, Cleveland, Ohio). To exclude polymerase errors, the DNA sequence for each species was determined by establishing the identities of nucleotides at each position from at least two independent PCR amplifications from the respective isolate. For *M. tuberculosis* and *M. bovis*, however, four and three isolates, respectively, were sequenced once to additionally account for the possibility of intraspecific polymorphisms. Each sequence originated from a separate PCR amplification.

Sequence analyses. Sequence alignments were obtained by using the program CLUSTAL in the PC/GENE software package (IntelliGenetics, Mountain View, Calif.) by a method developed by Higgins and Sharp (7). Computation parameters were set to a K-tuple value of 5, a gap penalty of 5, a window size of 10, and a filtering level of 2.5. Restriction enzyme site analyses were done by using the program RESTRI from the same PC/GENE software package.

Restriction endonuclease digests. After excision of the PCR product from the agarose gel and silica gel purification as described above, the DNA was eluted into 20 μl of water. For each digest 9.8 μl of DNA solution and 1.2 μl of the appropriate 10× buffer were incubated with 1 μl of either *Aci*I (5 U/μl) or *Sau*3AI (10 U/μl) at 37°C for 90 min. The digestion products were visualized in a 2.5% agarose gel containing 0.2 μg of ethidium bromide per ml.

RESULTS

The nucleotide sequences of the 16S rDNA promoter regions for *M. tuberculosis*, *M. gordonae*, *M. xenopi*, and *M. leprae* between the end of the primer MYC-12 and the start of the 16S coding region are shown in Fig. 1. Sequence lengths ranged from 332 bp in *M. tuberculosis* to 371 bp in *M. leprae*

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M_TUB   ATATCCGTTG TTCGTGGAGA ACCTGGTGAG TCTCGGTGCC GAGATCGAAC    50
M_GOR   CTACCCGTTG TTCGTTGAGA ACCTGGCGAT TTTGGGAGCG GAGATCGAGC    50
M_XEN   CTATCCGTTG TTCGTCGAGA ACCTGGTCAG TCTCGGCGCC GAGGTCGAGA    50
M_LEP   GTATCCATTG TTCGTGGAGA ACCTGGCGAA TTTAGGAGCC GAGATCGAGC    50
          ** ** *** ***** **** ******  *  * * ** **  *** ****

M_TUB   GGGTATGCT- ----GTTAGG CG-------- ---------- ACGGTCACCT    77
M_GOR   GGGTACAGTA GCCAAGTCA- CGC------- -CCGATCGGC GCGATCACCT    91
M_XEN   GGGTATGCT- -CTTAGATA- CGC------- -CCGGCAGCC CCGGTCATCT    89
M_LEP   GAGTATGCTT GTAGGTACTG CTCGTATAGT ACCG-CTGAC ACTGTCACCA    99
        * ***   *            *                     *  *** *

M_TUB   ATGGATATCT ATGGATGACC GAA-CCTGGT CTTGACTCCA TTGCCGGATT   126
M_GOR   C-CCTCAAGA AGATG--ATC GAAACCAGGG CTTGACTCTC CTGCCGGATC   138
M_XEN   T-C-TCAA-- AGTTG--GCC GGAACCGGGG CTTGACCAAG CCGCCAGAAT   133
M_LEP   GGCATTAAGA TGTTATAGTC -AACCCGGGA CTTGACTCCT CTGCTGGATC   148
           *             *   * ** **  ******       **  **

M_TUB   TGTATTAGAC TGGCAGGGTT GCCCCGAAGC GGGCGGAAAC AA-GCAAGC-   174
M_GOR   TGTATTAAGC TGGCAGGGTT GCCCCGAAAC GGGCGAGAAC ATAGCGAGAA   188
M_XEN   CGTATTAAGC TGGCAGGGTT GCCCCGAAGC GGGTGAAGAC A-AGCAAGC-   181
M_LEP   TGTATTAATC TGGCTGGGTT GCCCCGAAGC GGGGGAAGT- -AAGCTTGAA   196
         ******  * **** ***** ******** * *** *         **  *

M_TUB   GTGTTGTTTG AGAACTCAAT AGTGTGTTTG GTGGTTTCAC ATTTTTGTTG   224
M_GOR   GTGTTGTTTG AGAACTCAAT AGTGTGTTTG GTGAATT-GT TTTGTTGTTG   237
M_XEN   GTGTTGTTTG AGAACTCAAT AGTGTGTTTG GTGTAAT-TG TTTGTTGTTG   230
M_LEP   GTGTTGTTTG AGAACTCAAT AGTGTGTTTG GT----T-TT GTTGTTGTTG   241
        ********** ********** ********** **    *     ** ******

M_TUB   TTATTTTTG- ---GCCATGC TCTTGATGCC CCGT---TGT CGG----GGC   263
M_GOR   TTTTTTTGAC TACACCTAGC ACT-----CC CCGTGTGT-- CGGTGTAGTC   280
M_XEN   TTTTTTTGGC TACGCCGTTC ACT-----CC CCGTGTGG-- -GGCGTAGTC   272
M_LEP   ATTTTTTGAC TACATCTAGC ATT-----CC TCGTGTGTGT AGGTGTAGTT   286
         * ****         *   *   *     **  ***        **    *

M_TUB   ---------- ------GTGG CCG------- -------TTT GTTTTGTCAG   283
M_GOR   ------GCAT TTTTTGGATG CCAGTT-TTG GTGTCTTTTT GTTAGATCAG   323
M_XEN   ------GCAT ATTCAG-ATG CCAGTAATTG GCGTCTT--- -----GTCAG   307
M_LEP   TATTATGTTA TTTATAGATG CCAGTT-TTG GTGTCTT--- -----GTCAG   327
                          * **                        ****

M_TUB   GATATTTCTA AATACCTTTG GCTCCCTTTT CCAAAGGGAG TGTTTGGGT    332
M_GOR   ATTATCTCTG ATTGT----G AATTC----A CCTCGTTATC GA-GGGGTT    363
M_XEN   GT-ATCTCTG ATTGT----A AATTC----G CCTCGTTATC GA-GGGGTT    346
M_LEP   GT-ATCTCTA GAAATTGAAA ATTTC----G TCTAGTTATT GATGGAGTT    371
           ** ***              * *       *             * *
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FIG. 1. Alignment of 16S rDNA promoter regions between primer MYC-12 and the start of the 16S rRNA coding region from *M. tuberculosis* (M_TUB), *M. gordonae* (M_GOR), *M. xenopi* (M_XEN), and *M. leprae* (M_LEP). Hyphens indicate alignment gaps; asterisks identify conserved positions.

(Table 1). The sequences of all seven *M. tuberculosis* and *M. bovis* isolates were identical except for one position in one of the *M. tuberculosis* amplifications, which was regarded as either a polymerase error (error rate of $1/(7 \cdot 332) = 0.04\%$) or a representation of a relatively small extent of intraspecific polymorphisms detectable with the described PCR primers under the given PCR conditions. Another indication for a low rate of intraspecific polymorphism in *M. tuberculosis* is the high degree of homology of the sequences of our isolates compared with that of the *M. tuberculosis* H37Rv strain reported by Kempsell et al. (10), whose sequence is identical to that of our isolate except for six instead of four G nucleotides at positions −71 to −74 relative to the start of the 16S coding region.

TABLE 1. Analyzed lengths of target sequences

Organism	Length (bp) of target sequence		
	16S	*hsp65*	16S promoter
M. tuberculosis	1,087[a]	360[b]	332
M. gordonae	1,074[a]	360[c]	363
M. xenopi	1,077[a]	360[d]	346
M. leprae	1,099[e]	360[f]	371

[a] Data are from Rogall et al. (16).
[b] Data are from Kapur et al. (9) (GenBank accession number U17957).
[c] Data are from Kapur et al. (9) (GenBank accession number U17935).
[d] Data are from Kapur et al. (9) (GenBank accession number U17959).
[e] Data are from Liesack et al. (12).
[f] Data are from Mehra et al. (13).

The 16S rDNA promoter sequences have also been reported for *M. leprae* by Sela and Clark-Curtiss (20), and a partial 0.2-kb sequence has been reported by Ji et al. (8). These sequences are identical except for those at three loci: a guanosine to cytidine substitution at position −15, a thymidine insertion between positions −104 and −105, and an insertion of two C nucleotides between positions −199 and −200. In our isolate, the nucleotides at the first two sites were identical to those in the former sequence, and the nucleotide at the third site was identical to that in the latter sequence. No other polymorphisms were observed.

The number of polymorphisms (substitutions, deletions, and insertions) between *M. tuberculosis*, *M. gordonae*, *M. xenopi*, and *M. leprae* was compared with those derived from the 16S coding region (12, 16) as well as those from an often used 0.36-kb fragment of the 65-kDa heat shock protein (*hsp65*) gene (9). The extent of polymorphisms in the described 16S promoter region was greater than that in any of the two mentioned target sequences for all combinations of the four species investigated (Table 2).

TABLE 2. Polymorphism types of the 16S, *hsp65*, and 16S promoter regions

Organisms	No. of changes in the following target sequence[a]:					
	16S		*hsp65*		16S promoter	
	Subst.	Del./ins.	Subst.	Del./ins.	Subst.	Del./ins.
M. tuberculosis-M. gordonae	29	4	40	0	85	19
M. tuberculosis-M. xenopi	55	9	41	0	85	11
M. tuberculosis-M. leprae	22	4	40	0	82	19
M. gordonae-M. xenopi	55	8	31	0	65	11
M. gordonae-M. leprae	38	3	49	0	66	16
M. xenopi-M. leprae	66	8	54	0	77	14

[a] Subst., substitutions; Del./ins., deletions or insertions.

Restriction enzyme site analysis of the *M. tuberculosis*, *M. gordonae*, *M. xenopi*, and *M. leprae* sequences revealed an extensive restriction site polymorphism, from which *Aci*I and *Sau*3AI were selected to produce the RFLP patterns shown in Fig. 2. The RFLP pattern of *M. bovis* was identical to that of *M. tuberculosis*. The use of *Aci*I and *Sau*3AI also resulted in distinct RFLP patterns for *M. avium*, *M. intracellulare*, and *M. smegmatis* (Fig. 2).

DISCUSSION

In this report we propose the use of the 5′ noncoding promoter region of the 16S rRNA gene for the identification and differentiation of mycobacterial species because it offers several advantages over the target sequences used thus far. The 16S promoter region is more polymorphic than the entire 16S rDNA coding region or the previously reported portion of the *hsp65* gene (Table 2), resulting in an increased discriminatory power. For the mycobacterial species characterized, its length of 0.3 to 0.4 kb displays more base substitutions, deletions, and insertions than 1.5 kb of the 16S rDNA sequence, the sequences of which from some species, for example, *M. tuberculosis*, *M. gordonae*, and *M. kansasii*, exhibit homologies of 98 to 99% among each other (16). The resulting low degree of sensitivity of the 16S coding region, which has nevertheless found the widest application for mycobacterial species discrimination, has resulted in inconsistent phylogenetic conclusions, with the resulting phylogenetic trees being described as corresponding (23) as well as not corresponding (16) to the traditional Runyon group classification. Similarly, the use of phylogenetic trees derived from either 16S or 5S rRNA sequences results in distinct differences between the inferred phylogenetic relationships between the investigated mycobacterial species (5). Therefore, the analysis of noncoding leader or spacer regions has been suggested as an improvement over the analysis of the traditionally used coding regions of the rRNA gene (8).

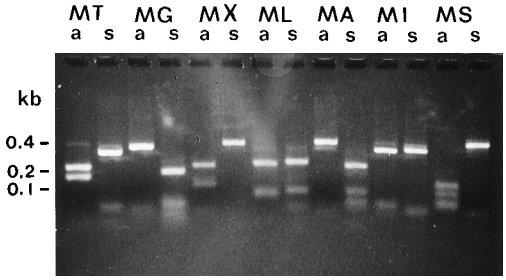

FIG. 2. Restriction endonuclease digests of the PCR products with *Aci*I (lanes a) and *Sau*3AI (lanes s) from *M. tuberculosis* (MT; expected: lane a, 0.21 kb + 0.16 kb + 0.02 kb + 0.01 kb; lane s, 0.31 kb + 0.05 kb + 0.01 kb [twice]), *M. gordonae* (MG; expected: lane a, 0.35 kb + 0.04 kb + 0.02 kb + 0.01 kb; lane s, 0.18 kb + 0.06 kb + 0.05 kb + 0.03 kb [twice] + 0.02 kb + 0.01 kb [twice]), *M. xenopi* (MX; expected: lane a, 0.22 kb + 0.13 kb + 0.04 kb + 0.02 kb; lane s, 0.37 kb + 0.01 kb [twice]), and *M. leprae* (ML; expected: lane a, 0.23 kb + 0.09 kb + 0.08 kb + 0.02 kb; lane s, 0.24 kb + 0.10 kb + 0.05 kb + 0.01 kb [twice]) as well as *M. avium* (MA), *M. intracellulare* (MI), and *M. smegmatis* (MS). Note that in addition to the sequence lengths given in Fig. 1, the PCR products also include the first 14 nucleotides of the 16S rRNA gene as well as 22 nucleotides each of both primers.

In contrast to its promoter region, the low number of polymorphic sites within the 16S coding region precludes the substitution of cumbersome sequencing methods by a more rapid and simple RFLP analysis of the PCR product. Conversely, by using the 16S rDNA promoter region, RFLP can be expected to eliminate the need for sequencing in most cases, for two reasons. First, the identity of the reported *M. tuberculosis* and *M. bovis* 16S rDNA promoter regions and the high degree of homology of previously reported *M. tuberculosis* and *M. leprae* sequences with those of our isolates suggest a rather low level of intraspecific polymorphisms. Second, RFLP analysis is insensitive to mutations between the restriction sites, which is by far the larger portion of the target sequence (e.g., 342/350 = 98% for a restriction enzyme with a four-base recognition sequence recognizing two sites in a 0.35 kb DNA target). Even in the rare cases in which the RFLP patterns could be expected to be inconclusive, however, the option of sequencing the PCR product would still be available.

Species determination by RFLP analysis has also been reported to be applicable with the *hsp65* gene (24), but compared with the coding and noncoding rDNA sequences, the interspecific genetic diversity of the *hsp65* gene does not reflect phylogenetic relationships quite as well as rDNA sequences typically do. For example, from their *hsp65* sequences, some isolates of *M. kansasii* (e.g., isolate 85-961) appear to be more homologous to other species (*M. avium*) than to other isolates (e.g., isolate 91-627) of their own species (9). The deduced phylogenetic tree (9) is significantly dissimilar to those derived from rDNA sequences (15, 16, 23).

In summary, further application of the 16S rDNA leader region to the differentiation of mycobacterial species and the analysis of their phylogenetic relationships is likely to prove to be advantageous over the use of previously used target genes, because the higher numbers of polymorphic sites provide an increased interspecific discriminatory power. The resulting sensitivity is likely to allow the generation of RFLP patterns in a matter of hours to differentiate not only between the species investigated in the present study but others as well, in most cases obviating the need to sequence the PCR product, which might require days. In contrast to the use of more or less specific hybridization probes (1), it also offers the possibility of identifying previously unidentified mycobacterial species and subspecies (2, 6, 11, 14, 17, 22) by the possible detection of novel RFLP patterns. In those cases, the PCR product could then still be characterized by subsequent sequencing.

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