JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1990, p. 1751–1759
0095-1137/90/081751-09$02.00/0

Vol. 28, No. 8

Detection and Identification of Mycobacteria by Amplification of rRNA

BORIS BÖDDINGHAUS,[1] TILL ROGALL,[1] THOMAS FLOHR,[1] HELMUT BLÖCKER,[2] AND ERIK C. BÖTTGER[1]*

Institut für Medizinische Mikrobiologie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, D-3000 Hannover 61,[1] and Gesellschaft für Biotechnologische Forschung, D-3300 Braunschweig,[2] Federal Republic of Germany

Received 21 February 1990/Accepted 23 May 1990

Oligonucleotides specific at a genus, group, or species level were defined by a systematic comparison of small-subunit rRNA sequences from *Mycobacterium tuberculosis*, *M. bovis*, *M. africanum*, *M. bovis* BCG, *M. avium*, *M. kansasii*, *M. marinum*, *M. gastri*, *M. chelonae*, *M. smegmatis*, *M. terrae*, *M. nonchromogenicum*, *M. xenopi*, *M. malmoense*, *M. szulgai*, *M. scrofulaceum*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. simiae*, *M. flavescens*, *M. paratuberculosis*, *M. sphagni*, *M. cookii*, *M. komossense*, *M. phlei*, and *M. farcinica*. On the basis of the defined oligonucleotides, the polymerase chain reaction technique was explored to develop a sensitive taxon-specific detection system for mycobacteria. By using *M. tuberculosis* as a model system, fewer than 10 bacteria could be reliably detected by this kind of assay. These results suggest that amplification of rRNA sequences by the polymerase chain reaction may provide a highly sensitive and specific tool for the direct detection of microorganisms without the need for prior cultivation.

The genus *Mycobacterium* consists of a diverse group of acid-fast bacilli which includes a number of human and animal pathogens (23). In addition to such overt human pathogens as *Mycobacterium tuberculosis* and *M. leprae*, there are a number of opportunistic pathogens (e.g., *M. avium*, *M. intracellulare*, *M. kansasii*, *M. simiae*, and *M. szulgai*) which can cause severe lung disease whenever the normal cellular defenses have been temporarily depressed (6). Furthermore, occasional clinical reports describe most of the mycobacterial species defined so far as potential pathogens (e.g., references 19 and 25). Despite progress in biochemical, biological, and immunological techniques, the reliable identification of mycobacteria remains a problem.

Commonly, two approaches are used in clinical practice for the diagnosis of mycobacterial infections. The direct identification of acid-fast organisms by microscopy is rapid but does not permit the identification of the mycobacterial species observed and lacks sensitivity because large numbers of organisms ($>10^4$/ml) must be present to be reliably detected (2). Culture, when positive, permits the subsequent identification of the mycobacterial species investigated, but the slow growth of mycobacteria in vitro entails a delay of 3 to 6 weeks.

Nucleic acid technology provides a radically different approach and may begin a new era in clinical microbiology provided that it can be established in clinical laboratories in addition to the traditional serological, cultural, and biochemical methods. Recently, a method which permits the amplification of specific DNA sequences and which can be used to multiply even a single copy of a given DNA sequence by a factor of 10^{12} has been developed (21). This technique has already been applied as an effective tool for bacteriological diagnosis (13, 14, 18).

Several reasons make rRNAs appealing targets for detection assays based on nucleic acid structure. (i) rRNA is an essential constituent of bacterial ribosomes (24). (ii) Comparative analysis of rRNA sequences reveals some stretches of highly conserved sequences and others with a considerable amount of variability (24). (iii) rRNA is present in large copy numbers, i.e., 10^3 to 10^4 molecules per cell, thus facilitating the development of sensitive detection assays (11, 17, 20). (iv) The nucleotide sequence of 16S rRNA can rapidly be determined without any cloning procedures (3, 10, 16). The rRNA sequence is characteristic for nearly every organism and has been used for establishing phylogenetic relationships (22, 24) as well as for identification of microorganisms (11, 12, 20).

In the present study, the feasibility of using rRNA-derived DNA probes to differentiate between highly related taxa within the genus *Mycobacterium* was investigated. Our results demonstrate that despite the high sequence conservation of rRNA cistrons, such probes can be used to differentiate between very closely related mycobacterial species. We have developed a sensitive and rapid technique for the detection of mycobacteria which is based on the amplification of nucleic acid sequences from 16S rRNA. The subsequent identification of the amplified DNA fragment is carried out by hybridization to oligonucleotides, the specificity of which is defined at a genus, group, or species level.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains whose 16S rRNA sequences were determined for this study. The complete sequences will be published elsewhere.

Extraction of nucleic acids. Cultures grown on Löwenstein-Jensen slants were harvested and washed in 10 mM Tris–1 mM EDTA (TE). The pellet was dissolved in 10 mM Tris–10 mM EDTA–0.1% Tween 80–2 mg of lysozyme (Boehringer GmbH, Mannheim, Federal Republic of Germany) ml^{-1}, incubated for 2 h at 37°C with shaking, and centrifuged. The bacterial pellet was lysed by redissolving in TE containing 100 μg of proteinase K (Boehringer) ml^{-1}–1% (wt/vol) sodium dodecyl sulfate and incubated for 1 h at 37°C. Nucleic acids were extracted by adding an equal volume of TE-saturated phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol). The aqueous phase was transferred to another tube and 0.1 volumes of cold 3 M sodium acetate (pH 5.2) were added. After being mixed by inversion, samples were placed on ice for 10 min before centrifugation in a microcentrifuge for 10 min. The supernatant fluid was transferred to

* Corresponding author.

TABLE 1. Bacterial strains used in this study

Species and strains[a]	Source
M. tuberculosis	
M. bovis	Institute for Medical Microbiology, Hannover, Federal Republic of Germany
M. bovis BCG	
M. tuberculosis H37	
M. avium Chester DSM 43216	DSM
M. gastri ATCC 15754^T	ATCC
M. kansasii DSM 43224	DSM
M. marinum	K. Schröder, Forschungsinstitut Borstel, Borstel, Federal Republic of Germany
M. africanum	K. Schröder
M. chelonae ATCC 14472	DSM
M. smegmatis ATCC 14468	DSM
M. terrae ATCC 15755^T	DSM
M. gordonae ATCC 14470^T	ATCC
M. scrofulaceum ATCC 19981^T	ATCC
M. szulgai ATCC 25799^T	ATCC
M. intracellulare ATCC 15985	DSM
M. nonchromogenicum ATCC 19530^T	ATCC
M. xenopi ATCC 19250^T	ATCC
M. malmoense ATCC 29571^T	ATCC
M. simiae ATCC 25275^T	ATCC
M. flavescens ATCC 14474^T	ATCC
M. fortuitum ATCC 6841^T	ATCC
M. paratuberculosis ATCC 19698	B. Jørgensen, National Veterinary Laboratory, Copenhagen, Denmark
M. sphagni ATCC 33027^T	J. Kazda, Forschungsinstitut Borstel
M. cookii	J. Kazda
M. komossense ATCC 33013^T	J. Kazda
M. farcinica DSM 43294	DSM
M. phlei ATCC 35784	J. Kazda

[a] ATCC, American Type Culture Collection, Rockville, Md.; DSM, Deutsche Stammsammlung für Mikroorganismen, Braunschweig, Federal Republic of Germany. T indicates type strain.

another tube, and the nucleic acids were precipitated by the addition of 20 μg of acrylamide ml^{-1}, 0.05 volumes of 3 M sodium acetate, and 2.5 volumes of ethanol, washed, dried, redissolved in H_2O or TE, and stored at −20°C. Concentrations of nucleic acids were determined spectrophotometrically at 260 nm and verified by judging the intensities of bands after electrophoresis on 0.8% (wt/vol) agarose gels.

To produce specimens containing a known number of mycobacteria, a culture of *M. tuberculosis* H37 was obtained, and serial 10-fold dilutions were prepared from this culture. Samples of the dilutions were grown on Löwenstein-Jensen slants to determine the number of organisms present.

Reverse transcription and amplification reaction. Nucleic acid extracts were heated for 5 min at 65°C before rRNA was transcribed into cDNA in 20 μl of 50 mM Tris (pH 8.3)–30 mM KCl–8 mM $MgCl_2$ containing 100 pmol of synthetic oligonucleotide primer, 10 U of AMV reverse transcriptase (Seikaguku, Rockville, Md.), and 16 U of RNasin (Promega Corp., Madison, Wis.) by incubation for 1 h at 37°C.

The amplification of DNA sequences was performed as described elsewhere (10). Briefly, the polymerase chain reaction (PCR) was performed in a total volume of 100 μl with 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, Conn., or GIBCO BRL, Gaithersburg, Md.)–0.05% detergent W-1 (GIBCO BRL)–50 mM KCl–10 mM Tris hydrochloride (pH 8.3)–1.5 mM $MgCl_2$–0.01% (wt/vol) gelatin–100 pmol of each of the two primers and 200 μM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP). The 90-μl mixture was covered by 100 μl of light mineral oil (Perkin-Elmer Cetus). DNA was always added last in a 10-μl quantity while the reaction mixture was kept at 70°C. Before DNA was added, it was incubated for 2 min at 95°C and for 15 min at room temperature. Similarly, 10 μl of the reverse transcriptase reaction mixture was used in the PCR. The thermal profile involved 36 cycles of denaturation at 93°C for 1 min, primer annealing at 60 or 63°C for 2 min, and extension at 72°C for 6 min.

Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia) and purified with a shadow-casting polyacrylamide gel (1).

Hybridization and analysis of amplified samples. Aliquots of amplified samples (10 μl) were electrophoresed through 0.8% agarose gels, and the DNA was visualized by UV fluorescence after it was stained with ethidium bromide.

For slot blot analysis, 20-μl aliquots of amplified samples or nucleic acids from each organism were denatured by the addition of 100 μl of 0.5 M NaOH–25 mM EDTA, incubated for 30 min at room temperature, and neutralized with 120 μl of 2 M ammonium acetate. The samples were loaded into wells of a manifold (Minifold II; Schleicher & Schuell, Dassel, Federal Republic of Germany) fitted with a nitrocellulose membrane (BA 85; Schleicher & Schuell) previously wetted in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). Each well was rinsed with 0.4 ml of 10× SSC, and the membranes were heated for 2 h at 80°C. Hybridization was performed by standard techniques (1). The membranes were rinsed in 6× SSC and prehybridized in 6× SSC–5× Denhardt solution (1× Denhardt solution is 0.02% Ficoll–0.02% polyvinylpyrrolidone–0.02% bovine serum albumin)–0.1% sodium dodecyl sulfate–100 μg of tRNA (Boehringer) ml^{-1} for 1 to 2 h at 50°C. Hybridization was performed in the same solution containing 2×10^6 to 1×10^7 cpm of 5′-end-labeled oligonucleotide ml^{-1} overnight. The blots were washed three times for 20 min each at room temperature in 6× SSC and three times for 20 min each in 6× SSC–0.1% sodium dodecyl sulfate at the appropriate temperature given by the T_m in degrees Celsius of the oligonucleotide. The blots were exposed to XAR-5 film (Eastman Kodak Co., Rochester, N.Y.) in cassettes containing an amplifying screen for various lengths of time at −70°C.

The oligonucleotides were labeled in 15 to 20 μl of 50 mM Tris (pH 7.6)–10 mM $MgCl_2$–5 mM dithiothreitol–0.1 mM spermidine–0.1 mM EDTA with 8 pmol of synthetic oligonucleotide, 100 μCi of [γ-^{32}P]ATP (specific activity, >5,000 Ci/mmol; Amersham, Braunschweig, Federal Republic of Germany), and 10 U of T4 polynucleotide kinase (New England BioLabs, Schwalbach, Federal Republic of Germany) by incubation for 1 h at 37°C. The kinase was inactivated by incubation for 10 min at 75°C; the nucleotides were extracted by phenol, and the labeled oligonucleotide was purified and separated from residual [γ-^{32}P]ATP with push columns (Stratagene, Heidelberg, Federal Republic of Germany). The resulting specific activity of the labeled oligonucleotide was 2×10^8 to 5×10^8 cpm/μg.

RESULTS

Sequence data. Evolutionarily less-conserved regions of potential use as target sites for genus- or species-specific oligonucleotide probes were identified by comparison within the collection of determined mycobacterial 16S rRNA sequences as well as with published sequences (9). On the

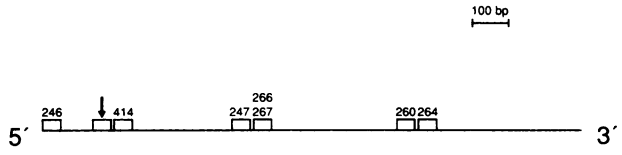

FIG. 1. Physical map of the oligonucleotides used with respect to their target sites in the 16S rRNA. The species-specific region in the rRNA molecule is indicated by an arrow.

basis of the extent of sequence variability, several regions were chosen; a selection of these is described here. Figure 1 illustrates a physical map of the selected oligonucleotide target sites on the 16S rRNA.

A region known to be highly variable in eubacteria, i.e., positions 200 to 240 according to the IUB numbering of *Escherichia coli*, is partially contained within the part of 16S rRNA determined to be a possible species-specific region for mycobacteria (Fig. 2). Inspection of the aligned sequences revealed small but consistent sequence differences between the species investigated. In detail, unique sequence stretches can be defined for the *M. tuberculosis* group (including *M. bovis* and *M. africanum*), the *M. avium* group (including *M. paratuberculosis*), *M. kansasii* (including *M. gastri*, *M. simiae*, and *M. scrofulaceum*), *M. chelonae*, *M. smegmatis*, *M. terrae*, *M. gordonae*, *M. szulgai*, *M. malmoense*, *M. intracellulare*, *M. nonchromogenicum*, *M. xenopi*, *M. fortuitum*, *M. flavescens*, *M. marinum*, *M. sphagni*, *M. cookii*, *M. komossense*, *M. farcinica*, and *M. phlei*.

A few regions were initially characterized as potentially genus specific (Fig. 3). These regions showed mismatches of at least two bases when compared to published 16S rRNA sequences (4, 7–9), including sequences from closely related species belonging to the *Corynebacterium-Nocardia-Actinomyces* group.

Oligonucleotide 414 (Fig. 3A) is invariable in the mycobacterial species investigated, except for *M. tuberculosis* (one mismatch) and *M. xenopi* (characterized by an insertion of three nucleotides). The region of oligonucleotide 247 (Fig. 3B) is characterized by single mismatches of one base each with *M. xenopi* and *M. flavescens* as well as a mismatch of two bases with *M. nonchromogenicum*. Oligonucleotides 266 and 267 (Fig. 3C) target the same region in 16S rRNA. Except for *M. simiae*, the sequence of which is less conserved within this part of the small-subunit rRNA, mycobacteria show nucleotide stretches identical to oligonucleotide 266 or 267. Notably, *M. flavescens* shows a single mismatch to oligonucleotide 267. Oligonucleotide 260 (Fig. 3D), as well as oligonucleotide 264 (Fig. 3E), is well conserved within the mycobacterial species investigated.

Specificity of oligonucleotides. Selected oligonucleotides were investigated in more detail to evaluate their specificity.

Sufficient differences in 16S rRNA positions 161 to 215 to define oligonucleotide probes which should distinguish mycobacteria at the species level were present. Thus, as an example, synthetic 20-mers within this region which were complementary to nucleotides from the *M. tuberculosis* group (including *M. bovis* and *M. africanum*), *M. avium-M. paratuberculosis*, and *M. intracellulare* were ^{32}P labeled and hybridized with nucleic acid extracts. The probes complementary to *M. tuberculosis*, *M. avium*, and *M. intracellulare* hybridized only to the nucleic acids from the corresponding species but not to nucleic acids from any other mycobacterial species investigated (Fig. 4).

The complement of oligonucleotide 247 (reverse 247) was hybridized to nucleic acid extracts of various bacteria, including close relatives of the genus *Mycobacterium*, and

```
AAC TGG GTC TAA TAC CGG ATA GG-ACCA CGG GAT GCA TG--TCT-TGT GGT GGA AAG C    M.tuberculosis
... ... ... ... ... ..A ... T.-...G ..C AC. T.C ..G-.G.--.. ... ... ... .    M.chelonae
... ... ... ... ... ..A ... T.-.... ..C .C. T.. ..G-.G.--.. ... ... ... .    M.fortuitum
... ... ... ... ... ..A ... TT-C..T NT. .TC ...  .GCCTGG.A- ..- ... ... .    M.flavescens
... ... ... ... ... ..A ... CACC.TG .T. .TC ... ..GCCTGG.A- ..- ... ... .    M.smegmatis
... ... ... ... ... ... ... ..-.... .TT .GC ... ..--C..-... ... ... ... .    M.simiae
... ... ... ... ... ..A ... ..-...G .AT .C. ... ..--GTG-... ... ... ... .    M.nonchromogenicum
... ... ... ... ... ... ... ..-.... T.. ... ... ..-T...--.. ... ... ... .    M.terrae
... ... ... ... ... ... ... ..-.... TTC TGC ... ..--.GG-G.. ... ... ... .    M.xenopi
... ... ... ... ... ..A ... ..-.... .A. ..C A.. ..--..C-... ... ... ... .    M.gordonae
... ... ... ... ... ... ... ..-.... .TT .GC ... ..--C..-... ... ... ... .    M.scrofulaceum
... ... ... ... ... ... ... ..-...T .AA ..C ... ..--...-.C. ... ... ... .    M.avium
... ... ... ... ... ... ... ..-...T .AA ..C ... ..--...-.C. ... ... ... .    M.paratuberculosis
... ... ... ... ... ... ... ..-...T TTA .GC ... ..--...-.TA ... ... ... .    M.intracellulare
... ... ... ... ... ... ... ..-.... .TT .GC ... ..--C..-... ... ... ... .    M.gastri
... ... ... ... ... ... ... ..-.... .TT .GC ... ..--C..-... ... ... ... .    M.kansasii
... ... ... ... ... ..A ... ..-...C ..A .GC ... ..--C..-..G ... ... ... .    M.malmoense
... ... ... ... ... ... ... ..-...C ..A .GC ... ..--C..-..G ... ... ... .    M.szulgai
... ... ... ... ... ... ... ..-.... ... ... T.. ..--..C-... ... ... ... .    M.marinum
... ... ... ... ... ..A ... T.-.T.. T.C TC. T.. ..--GGG-... ... ... ... .    M.sphagni
... ... ... ... ... ... ... ..-..T. ... AC. ... ..G-...--.. ... ... ... .    M.cookii
... ... ... ... ... ... ... T.-.... T.. AC. T.. C.G-.T.--.. ... ... ... .    M.komossense
... ... ... ... ... ... ... ..-.... ..C .C. T.. ..G-.G.--.. ... ... ... .    M.farcinica
... ... ... ... ... ... ... CACC.TT .T. .T. ... ..G--..-G.G A.G ... ... .    M.phlei
```

FIG. 2. Alignment of mycobacterial 16S rRNA sequences. The first nucleotide in the figure corresponds to *E. coli* 16S rRNA position 161 (IUB nomenclature). The 16S rRNA sequences of *M. bovis*, *M. bovis* BCG, *M. tuberculosis* H37, and *M. africanum* are identical to that of *M. tuberculosis* and therefore are not shown. *M. tuberculosis* was used as the reference sequence. Nucleotides different from those of *M. tuberculosis* are indicated; dashes indicate deletions. The complementary strand of the underlined sequences was used as a species-specific probe in the experiment illustrated in Fig. 4.

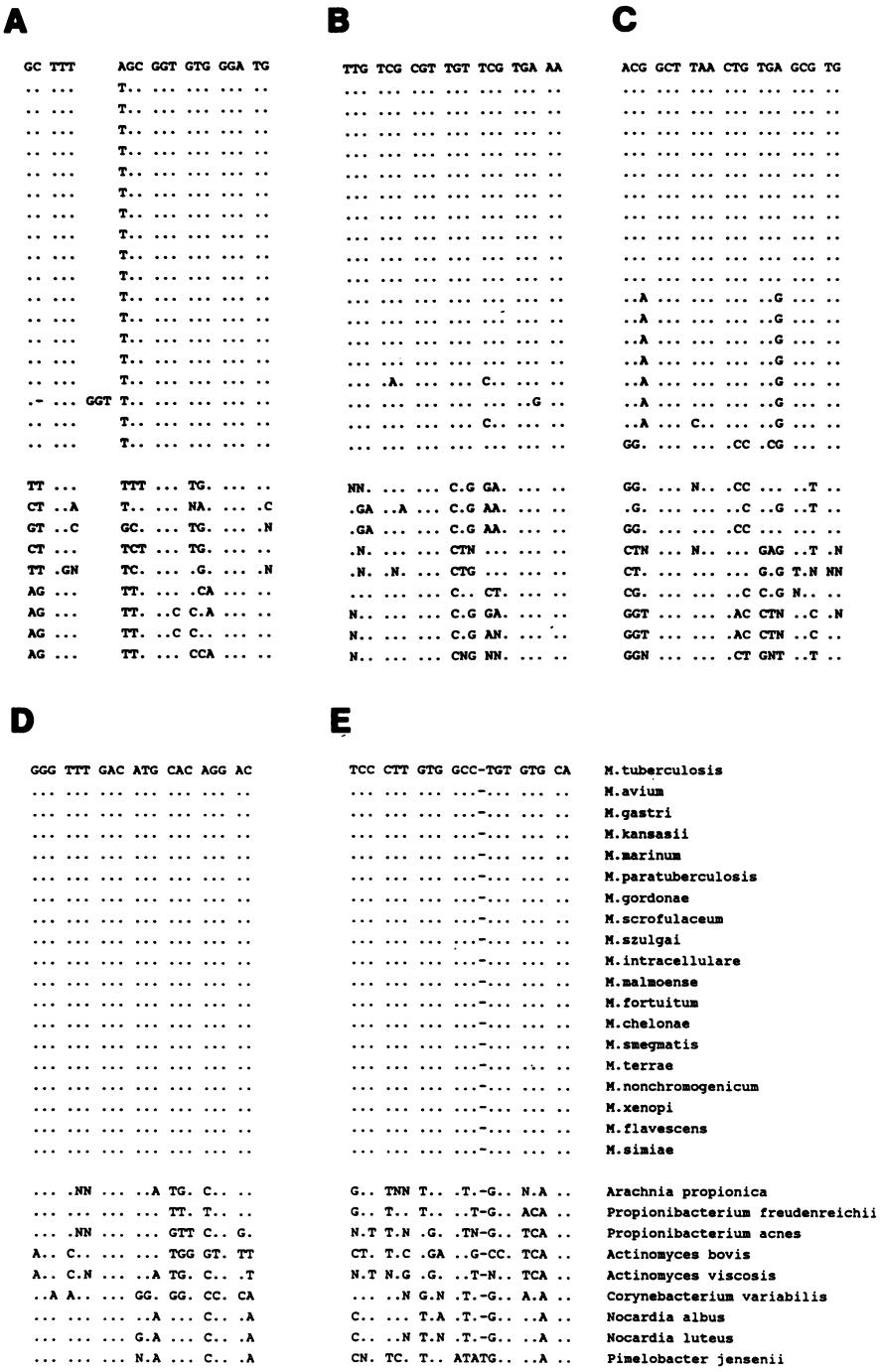

FIG. 3. Alignment of mycobacterial 16S rRNA sequences with selected published 16S rRNA sequences (4, 7, 8), including those from species belonging to the related *Corynebacterium-Nocardia-Actinomyces* group. N, Undetermined nucleotide. (A) Oligonucleotide 414 region, corresponding to *E. coli* positions 212 to 230; (B) oligonucleotide 247 region, corresponding to *E. coli* positions 590 to 609; (C) oligonucleotide 266-267 region, corresponding to *E. coli* positions 614 to 633; (D) oligonucleotide 260 region, corresponding to *E. coli* positions 988 to 1007; (E) oligonucleotide 264 region, corresponding to *E. coli* positions 1027 to 1046.

demonstrated an exclusive specificity for mycobacteria (Fig. 5). Bacterial species other than mycobacteria did not generate positive signals. As expected from the sequence comparisons, this probe did not hybridize under stringent conditions to nucleic acids extracted from *M. nonchromogenicum*, the sequence of which differs by two bases in this region.

Oligonucleotides complementary to oligonucleotide 260, 264, or 266 and 267 were used as primers in the PCR on several different template DNAs to evaluate the specificity of these potentially genus-specific regions for amplification. Thus, these primers should direct the preferential synthesis of the appropriate 16S rRNA gene fragment from mycobacteria but not from other species. Most importantly, human DNA, which is present in clinical samples, should not

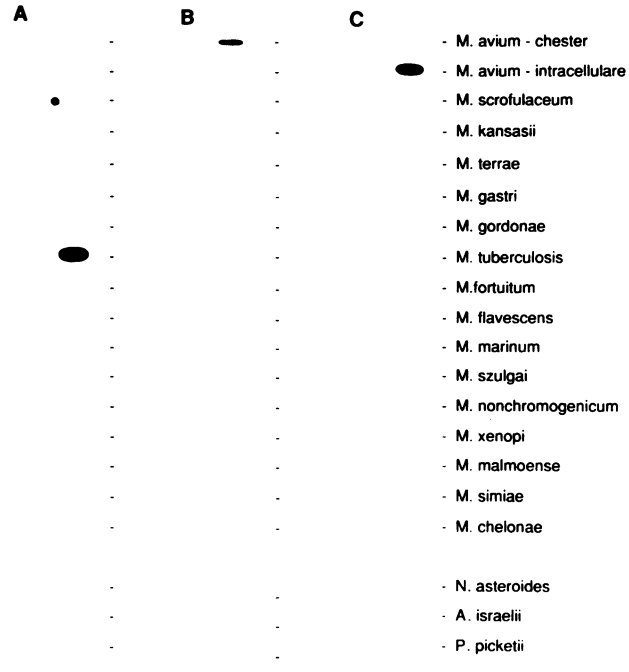

FIG. 4. Hybridization of nucleic acids (1 μg) from the indicated bacterial species to species-specific oligonucleotide probes. *P. pickettii*, *Pseudomonas pickettii*. (A) Sequence ACC ACA AGA CAT GCA TCC CG, specific for *M. tuberculosis*. Hybridization was performed at 52°C, and washings were performed at 61°C. (B) Sequence ACC AGA AGA CAT GCG TCT TG, specific for *M. avium-M. paratuberculosis*. Hybridization was performed at 52°C, and washings were performed at 60°C. (C) Sequence ACC TAA AGA CAT GCG CCT AA, specific for *M. intracellulare*. Hybridization was performed at 50°C, and washings were performed at 55°C.

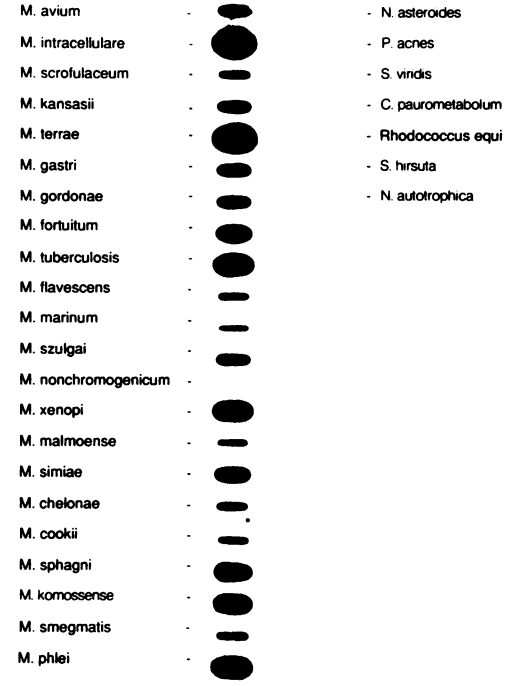

FIG. 5. Hybridization of nucleic acids (1 μg) from the indicated bacterial species to an oligonucleotide broadly reactive for mycobacteria at the genus level (reverse 247, with sequence TTT CAC GAA CAA CGC GAC AA; hybridization was performed at 52°C, and washings were performed at 55°C). *P. acnes*, *Propionibacterium acnes*; *S. viridis*, *Saccharomonospora viridis*; *C. paurometabolum*, *Corynebacterium paurometabolum*; *S. hirsuta*, *Saccharopolyspora hirsuta*; *N. autotrophica*, *Nocardia autotrophica*.

interfere with the amplification of mycobacterial DNA. As can be seen in Fig. 6A to C (lane 1), the combination of these primers with a second primer complementary to a common sequence within bacterial 16S rRNA (i.e., oligonucleotide 246 [10]) resulted in the synthesis of a prominent band of amplified DNA with the appropriate size in the presence of mycobacterial DNA. In contrast, no specific band of corresponding size was visible when amplification was performed with human DNA as a template (Fig. 6A to C, lane 6). In addition, the simultaneous presence of human DNA and mycobacterial DNA did not interfere with the specific amplification of mycobacterial DNA (Fig. 6A to C, lane 7). A faint band of amplified DNA was observed for the related species *Actinomyces israelii* when an oligonucleotide complementary to oligonucleotide 260 was used but not when reverse 264 or reverse 266–267 was used (Fig. 6A to C, lane 3). Amplification carried out on total DNA from *Nocardia asteroides*, another species closely related to the members of the genus *Mycobacterium* (24), did not result in visible amplified fragments (Fig. 6A to C, lane 2). Likewise, performing the PCR on DNA from more distantly related members of the family *Enterobacteriaceae* did not yield any band (Fig. 6A to C, lanes 4 and 5).

Extraction of nucleic acids, reverse transcription, and sensitivity of the amplification reaction. A simple procedure based on chemical and enzymatic methods was established to yield DNA as well as RNA from mycobacteria. This method results in extraction of intact, undegraded rRNA molecules (Fig. 7A). We reasoned that the high copy number of rRNA should facilitate the development of sensitive assays based on detection of nucleic acids. In order to make rRNA accessible to the amplification procedure, reverse transcriptase is used to prepare cDNA before the conventional PCR. We generated cDNA with a primer which does not interfere with the specificity of the PCR by defining a short 12-mer oligonucleotide for the reverse transcriptase reaction (Fig. 7B and C). Because of its shortness, this primer does not form a stable hybrid with its target DNA at 60°C, the temperature under which annealing was performed during the amplification cycles; therefore, the use of this primer in the PCR does not result in an amplified DNA fragment (Fig. 7D).

The sensitivity of the amplification procedure was investigated with *M. tuberculosis* as a model system by amplification of the probe site in a 1,030-base-pair (bp) fragment with primers 246 and reverse 264. The effect of using the high copy number of rRNA on the sensitivity of the assay was investigated by first performing reverse transcription of rRNA into cDNA.

By using serial dilutions of purified nucleic acids, 10 pg of purified nucleic acids added to the reaction mixture could be amplified sufficiently to yield a detectable band in an agarose gel (Fig. 8). When the presence of amplified mycobacterial DNA was identified by hybridization with a ^{32}P-labeled oligonucleotide by slot blot analysis the sensitivity was improved approximately 100-fold. Interestingly, when reverse transcription of the nucleic acid extracts into cDNA was performed first, sensitivity was increased 10^3-fold.

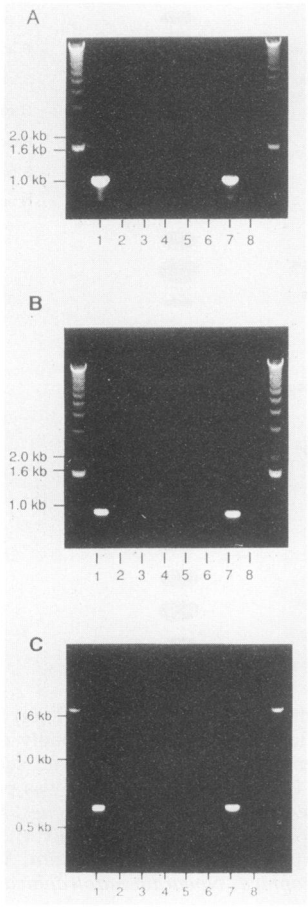

FIG. 6. Specificity of selected oligonucleotides in the PCR. The PCR was performed as described in Materials and Methods with 100 ng of bacterial DNA and 1 μg of human DNA. Shown is an analysis of amplified DNA by electrophoresis on a 0.8% agarose gel with 10 μl of the amplification reaction mixture. Lanes 1, Mycobacterial DNA; lanes 2, *N. asteroides*; lanes 3, *A. israelii*; lanes 4, *Pseudomonas* species; lanes 5, *E. coli*; lanes 6, human DNA; lane 7, human DNA and mycobacterial DNA; lanes 8, no DNA. *M. avium* was used as the source of mycobacterial DNA. (A) PCR with primers 246 (AGA GTT TGA TCC TGG CTC AG) and reverse 264 (TGC ACA CAG GCC ACA AGG GA) directing the synthesis of a 1,030-bp gene fragment; (B) PCR with primers 246 and reverse 260 (GTC CTG TGC ATG TCA AAC CC) directing the synthesis of a 990-bp gene fragment; (C) PCR with primer 246 in combination with a mixture of reverse 266 (CAC GCT CAC AGT TAA GCC GT) and reverse 267 (CAC GCC CAC AGT TAA GCT GT) directing the synthesis of a 620-bp gene fragment. The molecular mass marker is the 1-kb ladder (GIBCO BRL).

Thus, 100 fg of nucleic acids yielded a clearly detectable band in an agarose gel, and even 0.1 fg of purified nucleic acids yielded a clear-cut positive signal when the PCR mixture was hybridized with a ^{32}P-labeled oligonucleotide (Fig. 8). Given that the size of the *M. tuberculosis* genome is 2.2×10^9 daltons (5), 0.1 fg of nucleic acids corresponds to approximately 2% of the total nucleic acid content of a single bacterium.

As a further test to determine the sensitivity of the amplification procedure, nucleic acids extracted from sequential dilutions of a suspension containing a known number of *M. tuberculosis* cells were added to the reaction mixtures. When aliquots of the amplification mixture were electrophoresed through a 0.8% agarose gel, a discrete band of approximately 1.0 kilobases (kb) was visible in samples containing 500 mycobacteria when nucleic acids were used without prior reverse transcription as a substrate for the *Taq* polymerase (Fig. 9). However, reverse transcribing the rRNA into cDNA before the PCR resulted in a clear-cut band of the appropriate size in samples containing as few as approximately 30 mycobacteria. Combining the amplification procedure with a slot blot hybridization format resulted in a detection limit of three mycobacteria in a sample (Fig. 9).

DISCUSSION

The purpose of a clinical microbiology laboratory is to rapidly and accurately provide clinicians with information about the presence or absence of a microorganism that may be involved in an infectious disease process. Traditionally, this involves the isolation of a suspected pathogen from a clinical specimen followed by identification of the organism. Most of the tests necessary for identification require the isolation of a pure culture of the suspected bacterium. Consequently, the isolation and identification of slowly growing pathogens like mycobacteria entails a period of several weeks.

In this paper, we describe a method for the rapid detection and identification of mycobacteria based on the in vitro amplification of mycobacterial nucleic acids. Any detection system based on nucleic acids must fulfill two criteria: (i) it must result in reliable release of nucleic acids from cells, and (ii) it must be capable of definition of oligonucleotides of a characterized specificity.

The complex structure and impermeability of the cell walls of mycobacteria makes the lysis of mycobacteria for the extraction of DNA as well as RNA difficult. Thus, common methods for isolating DNA or RNA use physical procedures of cell disruption, e.g., the use of French presses (26). However, such methods are impractical in clinical laboratories. We have therefore established a simple and versatile procedure for isolating DNA and RNA from mycobacteria by chemical and enzymatic methods (Fig. 7).

rRNA, with its species-specific variable regions, may provide a general target for developing taxon-specific oligonucleotide probes for virtually any species of interest. The identification of species-specific regions by comparison of newly isolated 16S rRNA sequences with published 16S rRNA sequences (9), the number of which is constantly increasing, is relatively straightforward. To establish the use of rRNA-derived oligonucleotide probes to differentiate between closely related taxa, we have investigated the genus *Mycobacterium* as a model system. The results presented here indicate that there are small but consistent sequence differences in certain regions of the rRNA molecule which allow the definition of highly specific oligonucleotides, i.e., at the genus, group, or species level. We were able to identify a highly variable region which is extremely useful for definition of species-specific probes in mycobacteria. Thus far, the feasibility of this region for developing species-specific DNA probes has been experimentally demonstrated with *M. tuberculosis*, *M. avium*, and *M. intracellulare*.

Our aim was to outline a strategy which should be comparable in specificity and sensitivity to traditional culture techniques in mycobacteriology, i.e., a method which can detect virtually any mycobacterial organism present in low numbers in a sample irrespective of the species affiliation. For this purpose, we selected regions in the mycobacterial

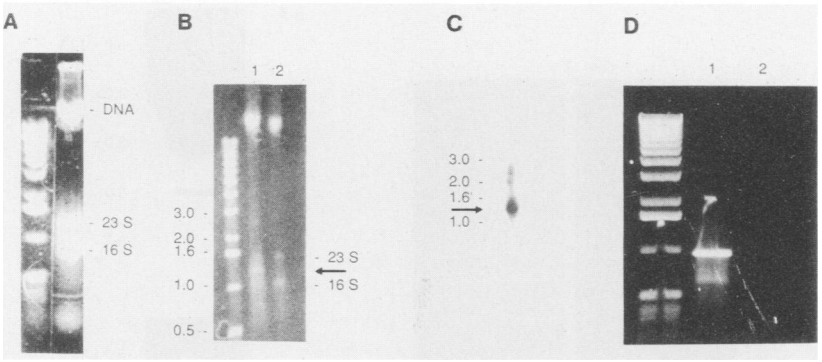

FIG. 7. Extraction of nucleic acids, cDNA synthesis, and amplification. (A) Extraction of nucleic acids from *M. tuberculosis*, resulting in chromosomal DNA and intact rRNA bands. (B) cDNA synthesis with oligonucleotide GGC AGT CTC TCA (corresponding to *E. coli* 16S rRNA positions 1162 to 1151) as primer; an ethidium bromide-stained agarose gel is shown. Lane 1, cDNA synthesis (the appropriate shift of the 16S rRNA band is indicated by an arrow); lane 2, input nucleic acids with the 16S and 23S rRNA bands. Numbers on the left indicate sizes in kilobases. (C) Autoradiogram made when [α-^{32}P]dCTP was incorporated during the cDNA synthesis reaction. The arrow points to the newly synthesized cDNA strand; numbers on the left indicate sizes in kilobases. (D) Inability of oligonucleotide GGC AGT CTC TCA to function as a primer in the PCR. The PCR was performed as described in Materials and Methods with primers 246 and reverse 264 (TGC ACA CAG GCC ACA AGG GA) (lane 1) or primer 246 in combination with GGC AGT CTC TCA (lane 2). Purified nucleic acids (100 ng) from *M. avium* were used as a template. The molecular mass marker is the 1-kb ladder (GIBCO BRL).

rRNA that are useful as target sites for genus-specific oligonucleotides. The selection was based on the extent of sequence variability as revealed by comparison with published 16S rRNA sequences (9). The characterized oligonucleotides exhibited genus specificity in a hybridization format (Fig. 5) and directed the synthesis of the appropriate 16S rRNA gene fragment when used as primers in the amplification reaction preferentially when amplification was carried out on mycobacterial DNA (Fig. 6). Following generic amplification of mycobacterial nucleic acids after optional synthesis of cDNA, a DNA probe was used to detect and identify the amplified product at the species level. We show in this paper that samples containing fewer than 10 intact mycobacterial cells can give positive results (Fig. 9).

The ultimate use of this procedure to detect mycobacteria in clinical specimens will depend on factors which were not evaluated in this study, i.e., handling, versatility, sensitivity, and specificity in clinical practice. For example, because of the favorable kinetics of oligonucleotides when compared with cloned probes, hybridization and washing times have reportedly been reduced to 15 to 20 min and 2 to 3 min, respectively (15). We expect that the application of DNA probes coupled with specific amplification of target sequences will contribute to a more rapid diagnosis of mycobacterial infections.

In summary, we have provided evidence that amplification of rRNA sequences allows the identification and differentiation of mycobacteria at a genus, group, or species level. Based on rRNA as a general structure which permits the rapid generation of specific DNA probes for eubacteria without any cloning procedures (3, 10), a similar approach would probably succeed with many other bacteria, i.e., it would determine the presence of genera of bacteria in clinical specimens by the PCR followed by identification at

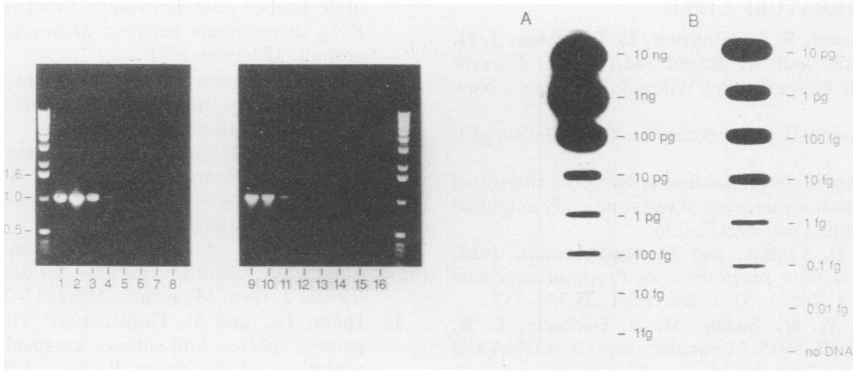

FIG. 8. Sensitivity of the detection of mycobacterial nucleic acids following amplification with primers 246 and reverse 264. The two gels on the left show an analysis of amplified DNA by electrophoresis on a 0.8% agarose gel with 10 μl of the amplification reaction mixture. A stock solution of purified nucleic acids from *M. tuberculosis* was diluted to give the following amounts in the samples: lane 1, 10 ng; lane 2, 1 ng; lane 3, 100 pg; lane 4, 10 pg; lane 5, 1 pg; lane 6, 100 fg; lane 7, 10 fg; lane 8, 1 fg; lane 9, 10 pg; lane 10, 1 pg; lane 11, 100 fg; lane 12, 10 fg; lane 13, 1 fg; lane 14, 0.1 fg; lane 15, 0.01 fg; and lane 16, no DNA. Reverse transcription was performed on the nucleic acids in lanes 9 to 15 before the PCR. The molecular mass marker is the 1-kb ladder (GIBCO BRL). The two panels on the right illustrate an experiment in which 20-μl samples of the amplified DNA were applied to nitrocellulose and hybridized to ACC ACA AGA CAT GCA TCC CG. (A) Purified nucleic acids were used directly in PCR; (B) reverse transcription of purified nucleic acids into cDNA with GGC AGT CTC TCA as the primer was performed first.

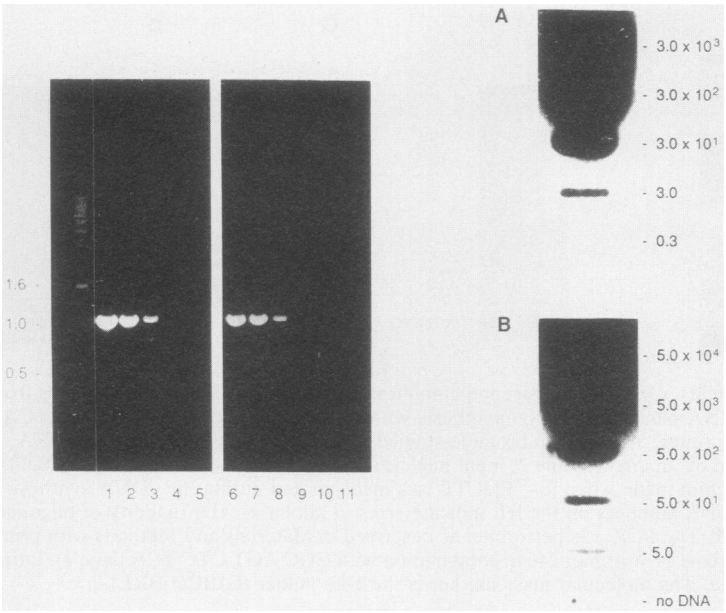

FIG. 9. Sensitivity of the PCR. The PCR was performed with primers 246 and reverse 264. On the left is an analysis of amplified DNA by electrophoresis on a 0.8% agarose gel. Nucleic acids were extracted from samples containing 5×10^4 (lane 1), 5×10^3 (lane 2), 5×10^2 (lane 3), 5×10^1 (lane 4), 5 (lane 5), 3×10^3 (lane 6), 3×10^2 (lane 7), 3×10^1 (lane 8), 3 (lane 9), or 3×10^{-1} (lane 10) *M. tuberculosis* bacilli. Reverse transcription was performed on the nucleic acids in lanes 6 to 10 before the PCR. Lane 11 is the negative control. On the right, 20-μl samples of the amplified DNA were applied to nitrocellulose and hybridized to ACC ACA AGA CAT GCA TCC CG. (A) Reverse transcription of the extracted rRNA into cDNA was performed first, with GGC AGT CTC TCA as the primer (B) Extracted nucleic acids were used directly in the PCR.

the desired taxon level with appropriate taxon-specific probes.

ACKNOWLEDGMENTS

We thank W. Heikens for synthesis of oligonucleotides; K. Schröder, B. Jørgensen, and J. Kazda for providing strains; S. Maibom for typing the manuscript; and D. Bitter-Suermann for continuous encouragement.

This work was supported by a grant from the Bundesministerium für Forschung und Technologie (01 K1 89117) to E. C. Böttger.

LITERATURE CITED

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Bates, J. H.** 1979. Diagnosis of tuberculosis. Chest **76**(Suppl.): 757–763.
3. **Böttger, E. C.** 1989. Rapid determination of bacterial ribosomal RNA sequences by direct sequencing of enzymatically amplified DNA. FEMS Microbiol. Lett. **65**:171–176.
4. **Charfreitag, O., M. D. Collins, and E. Stackebrandt.** 1988. Reclassification of *Arachnia propionica* as *Propionibacterium propionicus* comb. nov. Int. J. Syst. Bacteriol. **38**:354–357.
5. **Clark-Curtiss, J. E., W. R. Jacobs, M. A. Docherty, L. R. Ritchie, and R. Curtiss III.** 1985. Molecular analysis of DNA and construction of genomic libraries of *Mycobacterium leprae*. J. Bacteriol. **161**:1093–1102.
6. **Collins, F. M.** 1988. AIDS-related mycobacterial disease. Springer Semin. Immunopathol. **10**:375–391.
7. **Collins, M. D., M. Dorsch, and E. Stackebrandt.** 1989. Transfer of *Pimelobacter tumescens* to *Terrabacter* gen. nov. as *Terrabacter tumescens* comb. nov. and of *Pimelobacter jensenii* to *Nocardioides* as *Nocardioides jensenii* comb. nov. Int. J. Syst. Bacteriol. **39**:1–6.
8. **Collins, M. D., J. Smida, and E. Stackebrandt.** 1989. Phylogenetic evidence for the transfer of *Caseobacter polymorphus* (Crombach) to the genus *Corynebacterium*. Int. J. Syst. Bacteriol. **39**:7–9.
9. **Dams, E., L. Hendriks, Y. Van de Peer, J.-M. Neefs, G. Smits, J. Vandenkempt, and R. De Wachter.** 1988. Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res. **16**: r87–r175.
10. **Edwards, U., T. Rogall, H. Blöcker, M. Emde, and E. C. Böttger.** 1989. Isolation and direct sequencing of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res. **17**:7843–7853.
11. **Göbel, U. B., A. Geiser, and E. J. Stanbridge.** 1987. Oligonucleotide probes complementary to variable regions of ribosomal RNA discriminate between *Mycoplasma* species. J. Gen. Microbiol. **133**:1969–1974.
12. **Grimont, F., and P. A. D. Grimont.** 1986. Ribosomal nucleic acid gene restriction patterns as potential taxonomic tools. Ann. Inst. Pasteur (Paris) **137B**:165–175.
13. **Hance, A. J., B. Grandchamp, V. Lévy-Frébault, D. Lecossier, J. Rauzier, D. Bocart, and B. Gicquel.** 1988. Detection and identification of mycobacteria by amplification of mycobacterial DNA. Mol. Microbiol. **3**:843–849.
14. **Hartskeerl, R. A., M. Y. L. de Wit, and P. R. Klatser.** 1989. Polymerase chain reaction for the detection of *Mycobacterium leprae*. J. Gen. Microbiol. **135**:2357–2364.
15. **Haun, G., and U. Göbel.** 1987. Oligonucleotide probes for genus-, species- and subspecies-specific identification of representatives of the genus *Proteus*. FEMS Microbiol. Lett. **43**: 187–193.
16. **Lane, D. J., B. Pace, G. J. Olsen, D. A. Stahl, M. L. Sogin, and N. R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. Proc. Natl. Acad. Sci. USA **82**:6955–6959.
17. **Musial, C. E., L. S. Tice, L. Stockman, and G. D. Roberts.** 1988. Identification of mycobacteria from culture by using the Gen-Probe rapid diagnostic system for *Mycobacterium avium* complex and *Mycobacterium tuberculosis* complex. J. Clin. Micro-

biol. **26**:2120–2123.
18. **Olive, D. M.** 1989. Detection of enterotoxigenic *Escherichia coli* after polymerase chain reaction amplification with a thermostable DNA polymerase. J. Clin. Microbiol. **27**:261–265.
19. **Petrini, B., G. Svastengren, S. E. Hoffner, G. Unge, and U. Wildström.** 1989. Tenosynovitis of the hand caused by *Mycobacterium terrae*. Eur. J. Clin. Microbiol. Infect. Dis. **8**:722–724.
20. **Rossau, R., E. van Mechele, J. de Ley, and H. van Heuverswijn.** 1989. Specific *Neisseria gonorrhoeae* DNA-probes derived from ribosomal RNA. J. Gen. Microbiol. **135**:1735–1745.
21. **Saiki, R. K., D. M. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–491.
22. **Stackebrandt, E., and C. R. Woese.** 1981. The evolution of prokaryotes, p. 1–31. *In* M. C. Carlile, J. F. Collins, and B. E. B. Moseley (ed.), Molecular and cellular aspects of microbial evolution. Cambridge University Press, Cambridge.
23. **Wayne, L. G., and G. P. Kubica.** 1986. Family *Mycobacteriaceae* Chester 1897, 63^{AL}, p. 1436–1457. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
24. **Woese, C. R.** 1987. Bacterial evolution. Microbiol. Rev. **51**: 221–271.
25. **Wolinsky, E.** 1984. Nontuberculosis mycobacteria and associated diseases. Microbiol. Ser. **15**:1141–1207.
26. **Yoshimura, H. H., and D. Y. Graham.** 1988. Nucleic acid hybridization studies of mycobactin-dependent mycobacteria. J. Clin. Microbiol. **26**:1309–1312.