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Isolation of a DNA Probe for Identification of *Mycobacterium kansasii*, Including the Genetic Subgroup

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In order to develop a DNA-based assay to identify all *Mycobacterium kansasii* clinical isolates, a specific DNA probe was isolated in plasmid p6123. A total of 145 *M. kansasii* clinical isolates were collected from several countries and were examined with three probes by DNA hybridization. Of the 145 isolates, 115 (79%) were positive with the previously described probe pMK1-9 (Z. H. Huang, B. C. Ross, and B. Dwyer, J. Clin. Microbiol. 29:2125–2129, 1991), 129 (88%) were positive with the commercial Accu-probe assay (Gen-Probe), and 145 (100%) were positive with the p6123 probe. Southern blot analysis of *Eco*RI-digested *M. kansasii* chromosomal DNA with p6123 revealed that all Accu-probe-positive *M. kansasii* strains exhibited a 3-kb fragment, whereas all Accu-probe-negative *M. kansasii* strains displayed DNA fragments of variable molecular sizes. These results indicate that, unlike the previously described probes for *M. kansasii*, the fragment cloned into p6123 identified all 145 biochemically typical strains tested and provides an ideal target for future DNA-based speciation assays.

Mycobacterium kansasii is an opportunistic pathogen which usually causes infections in individuals with predisposing medical conditions (8). These include immune deficiencies such as AIDS (6, 7) or chronic obstructive pulmonary disease or pneumoconioses (1, 3, 4). Rarely, *M. kansasii* can infect healthy individuals. The standard laboratory identification of *M. kansasii* relies upon a variety of biochemical and growth tests, including photochromogenicity, catalase production, urease activity, Tween hydrolysis, and nitrate reduction. Recently, the genetic identification of *M. kansasii* has been made possible by the release of a commercial DNA test (Gen-Probe, San Diego, Calif.) and the isolation of a specific 2.2-kb DNA probe, pMK1-9 (2). Studies in our laboratory have determined that 20% of *M. kansasii* isolates were negative with probe pMK1-9 and 10% were negative with the Gen-Probe method (5). The pMK1-9 probe-negative isolates were shown to represent a distinct group by restriction fragment length polymorphism analysis and 16S rRNA gene sequencing. More recently, we have demonstrated the existence of a repeated sequence present only in the genome of probe-negative *M. kansasii* (9). Collectively, these results suggest the existence of a genetic subgroup of *M. kansasii* which is only partially detected by the available DNA probes.

In order to develop a DNA-based assay to detect all *M. kansasii* strains, we attempted to clone a DNA fragment common to probe-negative and probe-positive strains. In this paper, we describe a novel DNA probe derived from a clinical isolate which hybridizes to all *M. kansasii* strains.

MATERIALS AND METHODS

Mycobacterial strains. The mycobacterial reference strains and clinical isolates used in this study are listed in Table 1.

Extraction of mycobacterial DNA. Mycobacterial strains were grown and the DNA was extracted as described previously (5).

DNA cloning and sequencing. A clone library of the *M. kansasii* genome was established in bacteriophage lambda gt11. Briefly, DNA was extracted from a clinical *M. kansasii* 1652 isolate, digested with *Eco*RI, ligated into lambda gt11 DNA, and packaged into phage proteins according to the manufacturer's instructions (Promega, Madison, Wis.). Plaque hybridization was performed with digoxigenin-labelled DNA probes prepared from genomic DNA of *M. kansasii* 1652, *M. kansasii* NCTC, *M. gastri* NCTC, and *M. tuberculosis* NCTC strains. DNA inserts were subcloned into pT7T3 (Pharmacia, Uppsala, Sweden) and pGEM-7 (Promega) vectors. Sequencing was performed with a Taq-Track sequencing kit (Promega).

Labelling of nucleic acid probes. Both RNA and DNA were labelled with the Genius nonradioactive nucleic acid labelling and detection system supplied by Boehringer GmbH (Mannheim, Germany). For screening of the clone library in lambda gt11, four probes were prepared from chromosomal DNA by random primed labelling with digoxigenin-dUTP according to the manufacturer's recommendations. For Southern blot hybridizations, RNA probes were prepared from cloned fragments in plasmid pT7T3 (Pharmacia) by using either T3 or T7 RNA polymerase and digoxigenin-UTP according to the manufacturer's recommendations (Boehringer GmbH).

Hybridizations. Samples of mycobacterial DNA were subjected to Southern blot hybridization as described previously (5).

The Accu-probe assay for the identification of *M. kansasii* was performed according to the manufacturer's recommendations (Gen-Probe). This assay detects the presence of rRNA by in solution hybridization with a chemiluminescent probe. Because of the requirement for freshly grown bacteria, those strains negative by Accu-probe were recultivated and tested again before they were considered negative.

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TABLE 1. Origin of the mycobacterial strains used in this study

Species	Origin or strain no. (source)[a]
145 *M. kansasii* samples (*n*)	
Australia (33)	Clinical isolates
Australia (7)	Environmental isolates
Belgium (9)	Clinical isolates
Japan (11)	Clinical isolates
South Africa (29)	Clinical isolates
Switzerland (17)	Clinical isolates
United Kingdom (19)	Clinical isolates
United States (20)	Clinical isolates
M. africanum	ATCC 25420
M. asiaticum	CAP 21674
M. avium	NCTC 8559
M. bovis BCG	CSL (Australia)
M. bovis AN5	CSIRO (Australia)
M. chelonae	NCTC 10269
M. flavescens	ATCC 10271
M. fortuitum	NCTC 10394
M. gastri	Clinical isolate (SHLA)
M. gordonae	NCTC 10267
M. intracellulare	TMC 1403
M. kansasii	NCTC 10268
M. malmoense	ATCC 11298
M. marinum	NCTC 02275
M. neoaurum	No. 5780186
M. phlei	ATCC 11758
M. scrofulaceum	ATCC 19981
M. simiae	ATCC 25275
M. smegmatis	NCTC 08159
M. szulgai	NCTC 10831
M. terrae	NCTC 10856
M. thermoresistibile	NCTC 10831
M. tuberculosis H37RV	NCTC 10409
M. ulcerans	ATCC 94459
M. vaccae	NCTC 10916
M. xenopi	NCTC 10042

[a] Abbreviations: ATCC, American Type Culture Collection, Rockville, Md.; CAP, College of American Pathologists; CSIRO, Commonwealth Scientific and Industrial Research Organisation, Australia; CSL, Commonwealth Serum Laboratories, Australia; NCTC, National Collection of Type Cultures, London, United Kingdom; SHLA, State Health Laboratories, Brisbane, Australia; TMC, Trudeau Mycobacteria Collection.

RESULTS

Cloning of an *M. kansasii* specific DNA probe. To isolate a DNA fragment common to all *M. kansasii* strains, we established a genomic clone library from a clinical isolate of *M. kansasii* and screened it with whole chromosomal DNA probes of *M. tuberculosis*, *M. gastri*, and both the typical and the previously described genetic subgroups of *M. kansasii* (5). Plaques positive with both *M. kansasii* probes and negative for *M. tuberculosis* and *M. gastri* were selected, and the inserts from the phage were subcloned into plasmid pGEM-7 (Promega). The plasmid inserts were labelled with digoxigenin and used as probes in hybridization experiments with DNA from various mycobacteria. The insert from one of these plasmids, p612, showed strong hybridization with all types of *M. kansasii* and only weak hybridization with other mycobacteria (results not shown). In an attempt to define the most species-specific part of the 3-kb insert of p612, it was digested with *Sma*I and each of the four resulting fragments was subcloned into pGEM-7. A 0.5-kb fragment cloned into plasmid p6123 showed the highest degree of species specificity while displaying strong homology with all isolates of *M. kansasii*. This plasmid, p6123, contains an insert of 0.5 kb

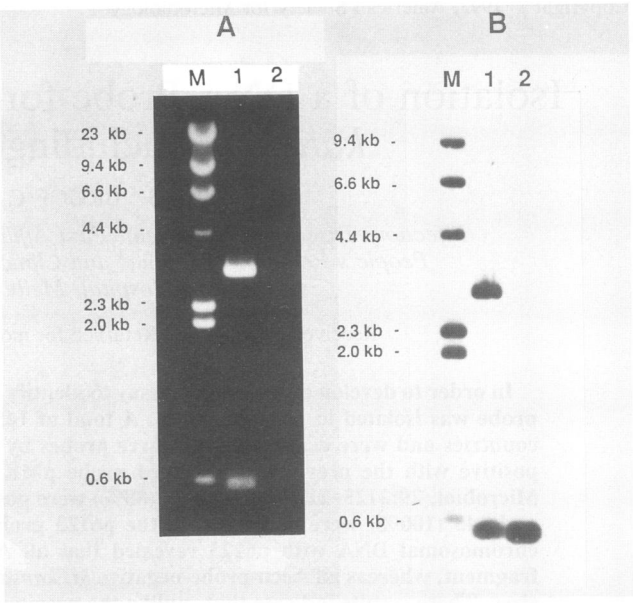

FIG. 1. Characterization of the cloned fragment by ethidium bromide-stained agarose gel (A) and Southern blot hybridization with a probe from the insert of plasmid p6123 (B). Lanes contain *Sma*I-digested DNA from plasmid p6123 (lane 1) and chromosomal DNA of *M. kansasii* (lane 2). The migration of the molecular size markers in lane M is indicated.

(Fig. 1A) representing an *Eco*RI-*Sma*I fragment which corresponds to a band with the same molecular size in *Eco*RI-*Sma*I-digested *M. kansasii* DNA (Fig. 1B). The specificity of the probe was confirmed by analysis of DNA from different mycobacterial species by Southern blot hybridization, which showed no species cross-hybridization (Fig. 2).

Analysis of *M. kansasii* isolates by Southern blot hybridization. A total of 138 clinical isolates and 7 environmental isolates were collected from Australia, Belgium, the United Kingdom, South Africa, Switzerland, Japan, and the United States (Table 1). Routine laboratory identification indicated that all strains possess typical *M. kansasii* characteristics, namely, that they were niacin negative, catalase positive, urease positive, nitrate positive, Tween hydrolytic, photochromogenic, and showed a temperature preference of 36°C. The isolates were examined for their reactivity by DNA hybridization with three probes: pMK1-9 (2), the Accu-probe *M. kansasii* identification kit (Gen-Probe), and p6123. Of the 145 isolates, 115 (79%) were positive with the pMK1-9 probe, 129 (88%) were positive with Accu-probe, and 145 (100%) of isolates were positive with the p6123 probe. Sample Southern blot hybridization results are depicted in Fig. 3 and 4. A 3-kb DNA band was observed in *Eco*RI-digested DNA of all *M. kansasii* strains which were pMK1-9 probe positive, with two strains sharing an extra 5.6-kb band. Interestingly, all Accu-probe-negative strains gave DNA bands of various sizes with molecular sizes of less than 3 kb.

Sequencing of p6123. The entire nucleotide sequence of plasmid p6123 was determined with sequencing primers to the SP6 and T7 promoters within the vector pGEM-7. The sequence is 514 bp long and contains two potential open reading frames in frames 1 and 3 (Fig. 5). However, analysis of the codon usage suggests that this sequence is unlikely to code for a protein. A search of genetic computer data bases

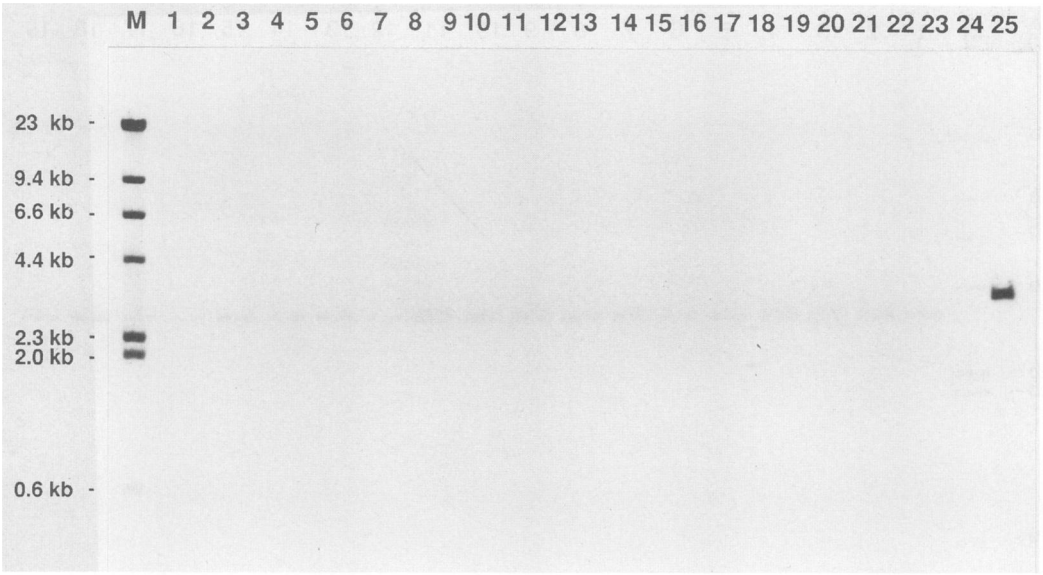

FIG. 2. Southern blot hybridization of genomic DNA from different mycobacterial species digested with *Eco*RI with plasmid p6123 as probe. Lanes 1 to 25 contain DNA from *M. africanum*, *M. asiaticum*, *M. avium*, *M. bovis* BCG, *M. bovis* AN5, *M. chelonae*, *M. flavescens*, *M. fortuitum*, *M. gastri*, *M. gordonae*, *M. intracellulare*, *M. malmoense*, *M. marinum*, *M. neoaurum*, *M. phlei*, *M. scrofulaceum*, *M. simiae*, *M. smegmatis*, *M. szulgai*, *M. terrae*, *M. thermoresistibile*, *M. tuberculosis*, *M. ulcerans*, *M. vaccae*, *M. xenopi*, and *M. kansasii*, respectively. The migration of DNA size markers in lane M is indicated.

revealed no significant homology with known nucleotide sequences.

DISCUSSION

Isolates of *M. kansasii* have previously been shown to consist of two distinct groups on the basis of DNA probing studies (5). A genetic subgroup was defined which had a distinct 16S rRNA sequence, a different RFLP profile, and failed to hybridize with a species-specific probe, pMK1-9 (5). Some overlap between these groups was demonstrated with the commercially available Accu-probe test, which detected some but not all of the genetic subgroup. The aim of the present study was to isolate a region of the *M. kansasii* genome which was common to both groups of isolates and which could be used for direct probing or as a template for

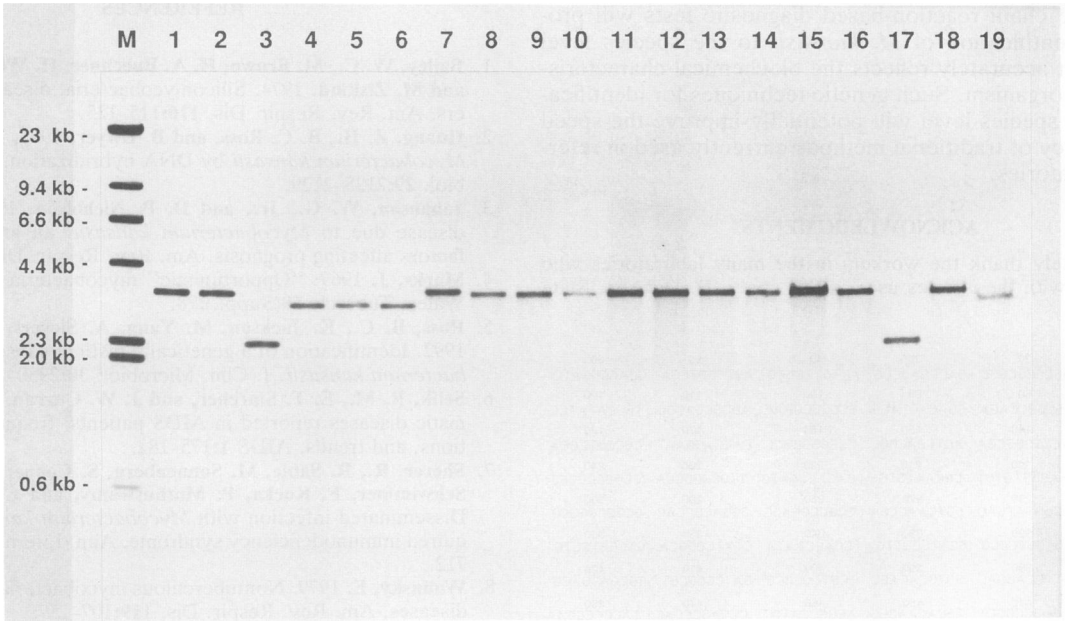

FIG. 3. Southern blot hybridization of DNA from *M. kansasii* subspecies strains digested with *Eco*RI with plasmid p6123 as probe. Lanes 1 to 19 contain pMK1-9-negative strains. Lanes 3 to 6, 17, and 19 were Accu-probe negative. The migration of DNA size markers in lane M is indicated.

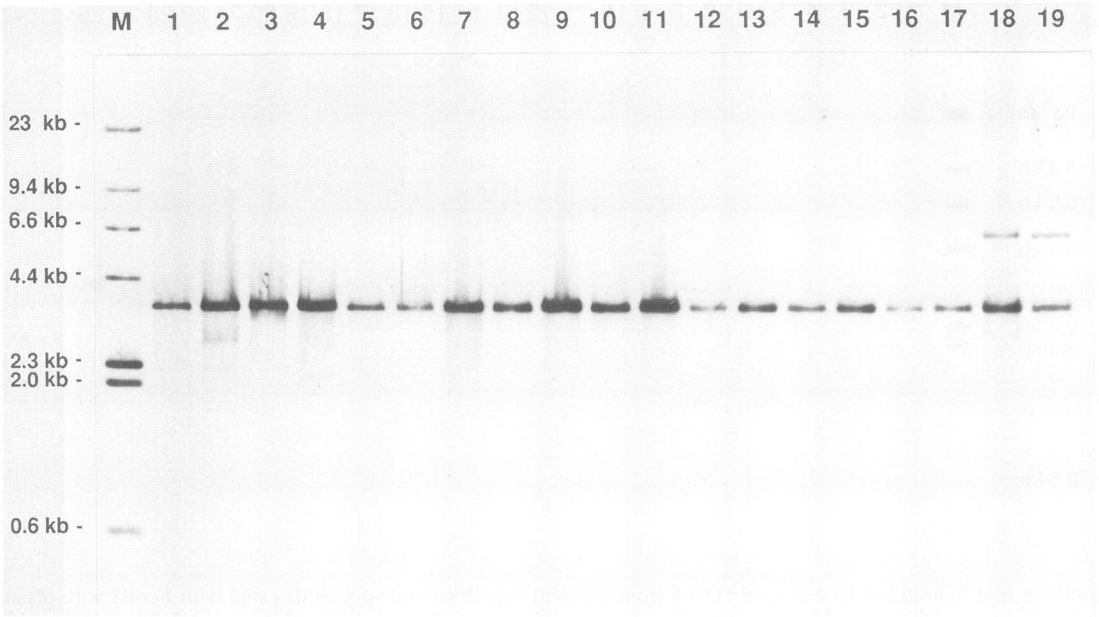

FIG. 4. Southern blot hybridization of DNA from genetically typical *M. kansasii* strains digested with *Eco*RI with plasmid p6123 as probe. All strains are pMK1-9 and Accu-probe positive. The migration of DNA size markers in lane M is indicated.

polymerase chain reaction. A 0.5-kb fragment was cloned which, when used as a probe, hybridized to all of the 145 *M. kansasii* isolates examined, including all types of the previously described genetic subgroup (5). This result is compared with those from the currently available probes pMK1-9 (2), which hybridized to only 79% of isolates, and the commercial Accu-probe assay, which detected 89% of the isolates. Interestingly, the strains negative by Accuprobe showed a different-sized fragment after *Eco*RI digestion and Southern blot hybridization with p6123 probe, further demonstrating genetic variability within *M. kansasii*.

The use of this fragment as the template for probe or polymerase chain reaction-based diagnostic tests will provide an identification of *M. kansasii* to the species level which most accurately reflects the biochemical characteristics of this organism. Such genetic techniques for identification to the species level will potentially improve the speed and accuracy of traditional methods currently used in reference laboratories.

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10         20         30         40         50         60
CCCGGGCAAC GCCGCGCGGC GACCCAGCGG ACTGTTGGCG AGTTGGTCGA CCAGCGGATC
70         80         90         100        110        120
GTCACGGTGC TGCGGCCAGA CGACAGTACG GCCGGCCAAC AGCGCGACGC TGGGATATCC
130        140        150        160        170        180
GTCGAACAGC ACCTCGGCGA ACTCGACGGC CTCGGGCGCC CACCAGGAAT CGCCGTCGCA
190        200        210        220        230        240
GAACGCGACA AACGGTGTGT CGCAATGTGC CACACCGACG TTGCGGGCCA CCGGCCCTGG
250        260        270        280        290        300
TTACGGATCA GCGGTATGAC GGTCAACCGA CCGCCGGAGC GGGACGCGAC GCGGCCGGCG
310        320        330        340        350        360
GCGGCCACCG AGTGGTCGCG GGATGCGTTG TCCACCAATA CTATCGGGCA GCCGGTGGTG
370        380        390        400        410        420
TCGAGCAGAC GTTCCAGGAC GGTGCACAGC TCGTCGGCCC GATCGCGCGT CGCGATGACG
430        440        450        460        470        480
AAAGAAGTCC GGGGCCGGCT TGCAGAAGGG ACCGCCATAT CCGAGCCCAC TACCCCGGAA
490        500        510
TCCCGCTGGC AAAACACGTT TCATCATCGA ATTC
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FIG. 5. Nucleotide sequence of the insert from plasmid p6123.

REFERENCES

1. **Bailey, W. C., M. Brown, H. A. Buechner, H. Weill, H. Ichinose, and M. Ziskind.** 1974. Silicomycobacterial disease in sandblasters. Am. Rev. Respir. Dis. **110**:115–125.
2. **Huang, Z. H., B. C. Ross, and B. Dwyer.** 1991. Identification of *Mycobacterium kansasii* by DNA hybridization. J. Clin. Microbiol. **29**:2125–2129.
3. **Johanson, W. G., Jr., and D. P. Nicholson.** 1969. Pulmonary disease due to *Mycobacterium kansasii*: an analysis of some factors affecting prognosis. Am. Rev. Respir. Dis. **99**:73–85.
4. **Marks, J.** 1969. "Opportunistic" mycobacteria in England and Wales. Tubercle **50**(Suppl.):78.
5. **Ross, B. C., K. Jackson, M. Yang, A. Sievers, and B. Dwyer.** 1992. Identification of a genetically distinct subspecies of *Mycobacterium kansasii*. J. Clin. Microbiol. **30**:2930–2933.
6. **Selik, R. M., E. T. Starcher, and J. W. Curran.** 1987. Opportunistic diseases reported in AIDS patients: frequencies, associations, and trends. AIDS **1**:175–182.
7. **Sherer, R., R. Sable, M. Sonnenberg, S. Cooper, P. Spencer, S. Schwimmer, F. Kocka, P. Muthuswamy, and C. Kallick.** 1986. Disseminated infection with *Mycobacterium kansasii* in the acquired immunodeficiency syndrome. Ann. Intern. Med. **105**:710–712.
8. **Wolinsky, E.** 1979. Nontuberculous mycobacteria and associated diseases. Am. Rev. Respir. Dis. **119**:107–159.
9. **Yang, M., B. C. Ross, and B. Dwyer.** 1993. Identification of an insertion sequence-like element in a subspecies of *Mycobacterium kansasii*. J. Clin. Microbiol. **31**:2074–2079.