JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1992, p. 942–946
0095-1137/92/040942-05$02.00/0
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Vol. 30, No. 4

Molecular Cloning of a Highly Repeated DNA Element from *Mycobacterium tuberculosis* and Its Use as an Epidemiological Tool

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Received 29 August 1991/Accepted 14 January 1992

In order to develop a technique for distinguishing between isolates of *Mycobacterium tuberculosis*, we cloned two hypervariable DNA fragments from *Nde*II-digested genomic DNA. The cloned DNA fragments of 3.8 and 4.7 kb were found to contain the same repetitive element, which was different from previously characterized repetitive elements. It is present in at least 30 copies per genome and is distributed among mycobacterial species other than those of the tuberculosis complex, including *M. kansasii*, *M. gastri*, and *M. szulgai*. When used as a probe on restriction enzyme-digested DNA, it can distinguish between strains from unrelated cases of tuberculosis while demonstrating identical banding patterns for isolates from epidemiologically related cases.

Tuberculosis remains a major world health problem, and recent statistics suggest that in many countries control measures have been ineffective in limiting its spread. It is estimated that 1.7 billion people are infected with *Mycobacterium tuberculosis*; this number represents one-third of the world's population. *M. tuberculosis* is responsible for an estimated 3 million deaths per year, one quarter of all preventable deaths (20). Most cases are confined to developing countries, in which a number of factors contribute to the spread of infection, including limited access to health care, malnutrition, and coexistence with other diseases (19). In highly industrialized nations, public health measures, combined with the advent of radiographic screening programs and improvements in drug therapy, have greatly decreased the incidence of tuberculosis. However, the disease remains a significant problem in many of these countries, including the United States, where the incidence is highest among immigrants and the economically disadvantaged. Since the latter part of the 1980s, the number of reported cases has been increasing (15).

In common with most developed nations, the incidence of tuberculosis in Australia declined significantly, from 50 cases per 100,000 in 1950 to fewer than 10 cases in the 1980s (4). However, in the past 5 years the number of culture-confirmed cases has remained constant, at approximately 600 cases annually, although the frequency of drug-resistant strains is increasing (6). Currently, reference laboratories in Australia lack both the resources and the laboratory techniques to enable detailed epidemiologic studies on tuberculosis infection. Such studies are an important component of disease surveillance in defining the origin and spread of tuberculosis in the community that can facilitate effective disease prevention and control measures.

Epidemiologic investigations into the transmission of *M. tuberculosis* have been hampered by the lack of a simple and highly discriminative typing system. Apart from antimicrobial susceptibility tests, which are uniform for most strains, phage typing is the only widely available means of subdividing *M. tuberculosis* strains, but it has had only limited use in epidemiological studies (12, 18). Early attempts to detect DNA restriction fragment length polymorphisms (RFLPs) to distinguish among members of the tuberculosis complex (2, 3, 5) and among isolates of *M. tuberculosis* (7, 11, 17) were largely unsuccessful. Only one of these studies was able to demonstrate significant heterogeneity among isolates of *M. tuberculosis* by digesting chromosomal DNA with *Mbo*I, followed by Southern blotting with a whole genomic probe to visualize high-molecular-weight DNA fragments (15). This method has been extended to use other restriction enzymes and has been adapted to a nonradioactive technique (16).

Recently, there have been a number of studies that have demonstrated the existence of repeated DNA sequences within the genome of *M. tuberculosis* (9, 10, 14). When used as probes on restriction enzyme-digested genomic DNA, RFLPs were demonstrated among different isolates by using two of these sequences as probes (9, 10, 22). The nucleotide sequences of these two repetitive elements, IS*986* and IS*6110*, indicate that they are essentially the same, with only three nucleotide differences between them (13, 21). In addition, they share considerable homology with IS*3* insertion sequences from the enterobacteria.

In this study, we aimed to develop a means of distinguishing among isolates of *M. tuberculosis* by cloning high-molecular-weight DNA fragments from *Nde*II (*Mbo*I)-digested chromosomal DNA which have been shown to exhibit heterogeneity between strains (15, 16). Two fragments were cloned, each of which contained a highly repeated sequence that appeared distinct from previously reported repetitive elements and proved useful in discriminating between clinical isolates.

MATERIALS AND METHODS

Bacterial strains. The origins of the mycobacterial strains used in this study are listed in Table 1. The clinical isolates of *M. tuberculosis* are from four geographically and epidemiologically distinct clusters of infection consisting of two family outbreaks, a school outbreak, and a cluster of cases in a group of sewer workers. Phage typing was not available for these strains, but all of them exhibited uniform drug susceptibilities.

Labelling of nucleic acid probes. Both RNA and DNA probes were labelled by using the nonradioactive DIG nucleic acid labelling and detection system supplied by Boehringer GmbH (Mannheim, Germany). Chromosomal DNA was labelled by random priming with digoxigenin-dUTP, and RNA probes were prepared from cloned DNA with T3 RNA

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TABLE 1. Mycobacterial strains used in this study

Strain	Origin
M. tuberculosis H37Rv	ATCC 94459[a]
M. bovis BCG	CSL,[b] Australia (Weybridge strain)
M. bovis AN5	CSIRO,[c] Australia
M. africanum	Clinical isolate, MRL[d]
M. microti	NCTC 08710[e]
M. asiaticum	CAP[f] survey strain 21674
M. avium	NCTC 8559
M. chelonae subsp. *chelonae*	NCTC 10269
M. flavescens	NCTC 10271
M. fortuitum	NCTC 10394
M. gastri	Clinical isolate, D. Dawson[g]
M. gordonae	NCTC 10267
M. intracellulare	TMC 1403[h]
M. kansasii	TMC 1201
M. malmoense	NCTC 11298
M. phlei	ATCC 11758
M. simiae	ATCC 25275
M. szulgai	NCTC 10831
M. tuberculosis C1–C3	Isolates from school outbreak, MRL
M. tuberculosis C4–C6	Isolates from sewer workers, MRL
M. tuberculosis C4–C6	Isolates from family outbreak, MRL
M. tuberculosis C10–C11	Isolates from family outbreak, MRL

[a] American Type Culture Collection, Rockville, Md.
[b] Commonwealth Serum Laboratories, Melbourne, Australia.
[c] Commonwealth Scientific and Industrial Research Organization, Canberra, Australia.
[d] Mycobacterium Reference Laboratory, Fairfield Infectious Diseases Hospital, Melbourne, Australia.
[e] NCTC, National Collection of Type Cultures, London, United Kingdom.
[f] College of American Pathologists, Sulphur, La.
[g] State Health Laboratories, Brisbane, Australia.
[h] Trudeau Institute, Saranac, N.Y.

polymerase by using digoxigenin-UTP according to the manufacturer's recommendations. After being labeled, the RNA was precipitated with ethanol and added directly to hybridization solution.

Probes labelled by the polymerase chain reaction (PCR) were synthesized in a buffer supplied by the *Taq* polymerase manufacturer (Promega, Madison, Wis.) (total volume, 40 μl) containing 100 ng of heat-denatured *M. tuberculosis* DNA (strain H37Rv), 1 μM primers, 1 unit of *Taq* polymerase, 100 μM dATP, 100 μM dCTP, 100 μM dGTP, 65 μM dTTP, and 35 μM digoxigenin-dUTP (Boehringer GmbH). The primers synthesized for amplification of IS*6110* have been described previously (8). The reactions were performed by using an automated thermal cycler (Thermal Reactor, Hybaid, United Kingdom). DNA samples were initially heat denatured at 100°C for 5 min before amplification for 30 cycles of 95°C for 1 min, 68°C for 1 min, and 72°C for 1 min. Following the PCR, labelled DNA was purified by extraction with phenol-chloroform and precipitation with ethanol before heat denaturation for hybridization.

Preparation of mycobacterial DNA. Mycobacterial strains were grown in 50-ml cultures of Middlebrook 7H9 medium at 37°C with shaking until mid-logarithmic stage. D-Cycloserine (Sigma Chemical Co., St. Louis, Mo.) was then added to a final concentration of 1 mg/ml, and the cultures were incubated for an additional 48 h. Bacteria were then pelleted by centrifugation at 3,000 × *g* for 10 min and washed in 20 ml of 0.1 M NaCl–0.1 M Tris (pH 8.0)–0.01 M EDTA. After centrifugation, the cells were suspended in 4 ml of lysis solution (15% sucrose, 50 mM Tris (pH 8.5), 50 mM EDTA, 1 mg of lysozyme per ml) and incubated for 30 min at 37°C. After sodium dodecyl sulfate (SDS) was added to a final concentration of 4% and proteinase K was added to 100 μg/ml, the solution was incubated for 30 min at 37°C and for 5 min at 75°C. The lysate was then purified by extraction with phenol-chloroform, and the DNA was precipitated by adjusting the solution to 0.2 M NaCl and adding 2 volumes of ethanol. After centrifugation at 3,000 × *g* for 20 min, the ethanol was decanted and the dried DNA pellet was suspended in 0.5 ml of 10 mM Tris (pH 8)–1 mM EDTA (TE buffer). The DNA was purified further by extraction with phenol-chloroform, followed by chloroform extraction, and the DNA was precipitated as described before. After resuspension of the DNA pellet in TE buffer, the concentration of the DNA samples was estimated by electrophoresis of the DNA through agarose gels stained with ethidium bromide. DNA samples were stored at −20°C until required.

Southern blotting. Restriction enzyme digests of mycobacterial DNA were prepared by using approximately 1 μg of chromosomal DNA and 10 units of restriction enzyme for 5 h at 37°C in incubation buffers supplied by the enzyme manufacturer (Boehringer GmbH). The digested fragments were separated by electrophoresis through 0.7% agarose gels at 50 V for 16 h (20-cm gel) in 90 mM Tris-base–90 mM boric acid–2 mM EDTA. The DNA was transferred to a positively-charged nylon membrane (Boehringer GmbH) by using a vacuum transfer apparatus (Hybaid) at 80-cm H_2O vacuum for 1 h with 0.5 M NaOH and 1.5 M NaCl. The DNA was fixed to the nylon membrane by baking at 120°C for 15 min. The membranes were prehybridized for 2 h at 42°C in hybridization solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% *N*-lauroylsarcosine, 1% SDS, 200 μg of herring sperm DNA per ml, and 5% blocking reagent (Boehringer GmbH). After prehybridization, either the heat-denatured DNA probe or the RNA probe was added and the membrane was incubated overnight at 42°C. After hybridization, the membrane was washed twice for 5 min each time at room temperature with 2× SSC–0.1% SDS and twice for 30 min each time at 50°C with 0.1× SSC–0.1% SDS. The presence of digoxigenin-labelled DNA probe was determined with an alkaline phosphatase-conjugated antibody as described previously (Boehringer GmbH), except that 1% casein was used in the blocking step and as the conjugate diluent.

Cloning of *M. tuberculosis* DNA fragments. Previously, RFLPs have been demonstrated in high-molecular-weight DNA fragments after digestion of the *M. tuberculosis* genome with enzymes that recognize four base sequences (15, 16). To obtain cloned DNA probes capable of distinguishing between strains of *M. tuberculosis*, DNA fragments representing the high-molecular-weight bands were cloned. DNA from *M. tuberculosis* H37Rv was digested with *Nde*II and size fractionated by sucrose gradient ultracentrifugation (10 to 40%) as described previously (1). After identification of fractions containing high-molecular-weight DNA by Southern blot analysis (16), the DNA was subjected to further size fractionation by ion-exchange high-performance liquid chromatography (HPLC) using a Gen-Pak FAX column (Millipore, Milford, Mass.). The gradient for ion-exchange HPLC consisted of 0 M to 0.5 M NaCl over 5 min, 0.5 M to 0.7 M NaCl over 60 min, and 0.7 M to 1 M NaCl over 60 min at a flow rate of 0.75 ml/min. High-molecular-weight DNA fragments were then precipitated with ethanol and ligated into *Bam*HI-digested, alkaline phosphatase-treated pUC-8 (Pharmacia, Uppsala, Sweden). The ligated DNA was then transformed into *Escherichia coli* NM522 by electroporation, and transformants were selected on ampicillin-containing nutri-

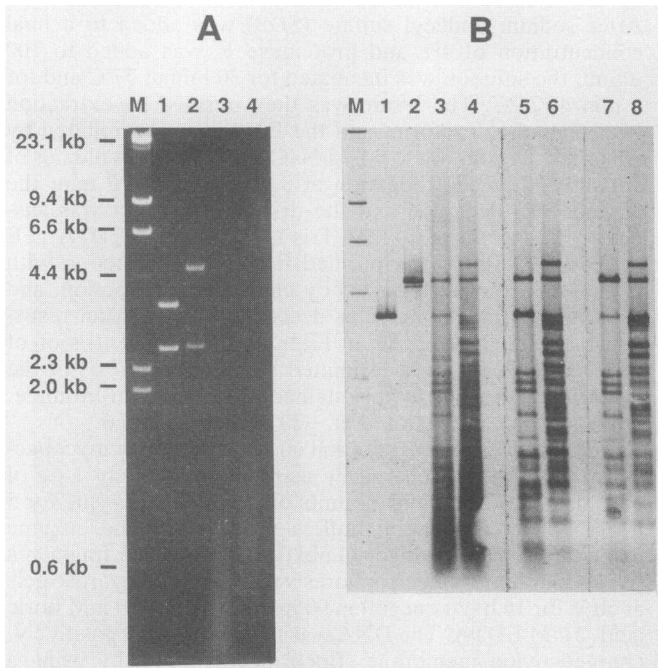

FIG. 1. Characterization of the cloned fragments by agarose gel electrophoresis and Southern blotting. After electrophoresis, the gel was stained with ethidium bromide (A) and then subjected to Southern blot analysis with various probes (B). Lanes: 1, *Hin*dIII-*Eco*RI-digested pTBN12; 2, *Hin*dIII-*Eco*RI-digested pTBN52; 3, 5, and 7, *Nde*II-digested *M. tuberculosis* DNA; 4, 6, and 8, *Alu*I-digested *M. tuberculosis* DNA. Lanes were hybridized with probes prepared from total *M. tuberculosis* DNA (lanes 1 to 4), pTBN12 (lanes 5 and 6), and pTBN52 (lanes 7 and 8). The molecular weights of DNA markers in lane M are indicated.

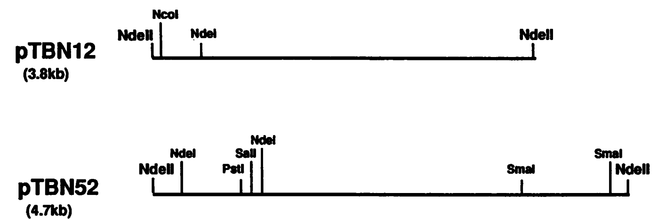

FIG. 2. Restriction map of the two DNA fragments cloned from *Nde*II-digested *M. tuberculosis* DNA containing the repeated sequence. No sites were found for *Bam*HI, *Eco*RI, *Hin*dIII, *Pvu*II, *Xba*I, or *Xho*I.

ent agar plates. *E. coli* cells harboring recombinant plasmids were identified on agar plates containing isopropyl-β-D-thiogalactopyranoside and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Plasmids containing inserts were then extracted and analyzed by agarose gel electrophoresis. Plasmids with inserts that were 2 kb or larger were labelled with digoxigenin and used as probes in Southern blotting of *Nde*II-digested *M. tuberculosis* DNA.

After plasmids containing the desired DNA fragments were identified, they were excised from pUC-8 by digestion with *Hin*dIII and *Eco*RI before subcloning into plasmid pT7T3 (Pharmacia). This enabled the synthesis of RNA probes by utilizing the T3 transcription promoter as described above. Such digoxigenin-labelled RNA probes offer the practical advantage of higher specific activity and were used for all hybridizations using the cloned DNA.

RESULTS

Cloning of *M. tuberculosis* fragments. *M. tuberculosis* DNA fragments representing the highest-molecular-weight bands after *Nde*II digestion were identified by screening recombinant plasmids for insert size. Plasmids with inserts of greater than 2 kb would represent the upper six bands of the digest (15, 16). After extraction of plasmids from individual recombinants and size estimation by agarose gel electrophoresis, two clones were identified, pTBN12 and pTBN52, with inserts of 3.8 and 4.7 kb, respectively. The inserts of these plasmids (Fig. 1B, lanes 1 and 2) migrate in agarose gels with the same apparent molecular weight as the two upper bands of *Nde*II-digested *M. tuberculosis* DNA (Fig. 1B, lane 3). When used as probes, pTBN12 and pTBN52 hybridize to multiple DNA fragments in *Nde*II-digested *M. tuberculosis* DNA (Fig. 1B, lanes 5 and 7). This indicates that these plasmids contain a repetitive sequence, and the similarity of the banding patterns suggests that they both contain the same repeated sequence. Previously, we have reported a comparison of different restriction enzymes to distinguish between strains of *M. tuberculosis* (16). Our results indicated that *Alu*I produced the largest number of bands with variable molecular weights, and it is our restriction enzyme of choice for epidemiological purposes. Interestingly, pTBN12 and pTBN52 hybridized to most of the high-molecular-weight bands from *Alu*I-digested DNA (Fig. 1B, lanes 6 and 8), indicating that these probes could be used on *Alu*I-digested DNA for epidemiologic studies.

Characterization of the repetitive sequence. The restriction maps of the inserts from pTBN12 and pTBN52 are illustrated in Fig. 2. When compared to the restriction sites present in IS*986* (10) and IS*6110* (21), there are no apparent similarities. In addition, the clones share no common restriction enzyme sites, suggesting that the repeated sequence may be located between the sites shown.

To compare the repetitive element with the previously characterized elements, a probe was synthesized by PCR using the published PCR primers to IS*6110* (10). Since IS*986* and IS*6110* differ by only three nucleotide substitutions, a probe to IS*6110* would hybridize to both elements. DNA from *M. tuberculosis* H37Rv was digested with *Nde*II and *Alu*I, followed by Southern hybridization with probes from either pTBN12 or IS*6110* (Fig. 3). This comparison clearly shows the repetitive sequence in pTBN12 to be different from the insertion sequence.

Distribution of the repeated sequence. Genomic DNA samples from various mycobacterial species were digested with *Pvu*II, Southern blotted, and probed with pTBN12 to determine the distribution of this sequence among mycobacteria (Fig. 4). The repeated sequence is present in all members of the *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis* BCG, *M. africanum*, *M. microti*, and *M. bovis* (Fig. 4). It is also present in *M. gastri*, *M. gordonae*, *M. kansasii*, and *M. szulgai*. Additionally, it may be present in smaller numbers in *M. malmoense* and *M. phlei*. The single band present in certain species is due to regions flanking the repeated element, as evidenced by probing an identical blot with pTBN52, which highlighted a band with a different molecular weight (data not shown). This distribution of the repeated sequence differs from that of IS*986*, which is not present in *M. gordonae* or in *M. kansasii*, and from that of IS*6110*, which is present only in the *M. tuberculosis* complex. By

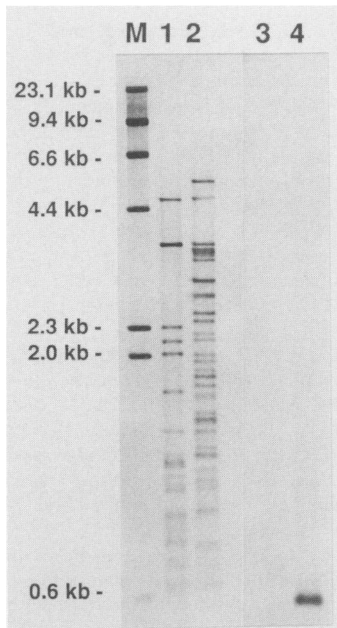

FIG. 3. Southern blot of *M. tuberculosis* DNA from the type strain H37Rv digested with *Nde*II (lanes 1 and 3) and *Alu*I (lanes 2 and 4). After Southern transfer, the filter was divided into two pieces before being probed with pTBN12 (lanes 1 and 2) and IS*6110* (lanes 3 and 4). The molecular weights of DNA markers in lane M are indicated.

comparison with the Southern blots of IS*6110* (9, 10), our cloned repeated sequence appears to be present in a larger number of copies per genome. By counting the bands present in Fig. 4, we estimate that at least 30 copies per genome may be present in *M. tuberculosis* strains, and the repeat has been detected in all of approximately 200 isolates tested to date.

To examine the potential use of this cloned repeated sequence in epidemiologic investigations, we analyzed the

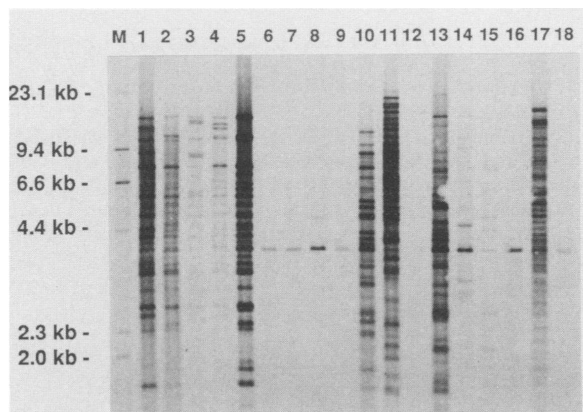

FIG. 4. Southern blot of DNA from various mycobacterial species digested with *Pvu*II by using pTBN12 as the probe. Lane 1, *M. tuberculosis* H37Rv; lane 2, *M. bovis* BCG; lane 3, *M. africanum*; lane 4, *M. microti*; lane 5, *M. bovis*; lane 6, *M. asiaticum*; lane 7, *M. avium*; lane 8, *M. chelonae*; lane 9, *M. flavescens*; lane 10, *M. gastri*; lane 11, *M. gordonae*; lane 12, *M. intracellulare*; lane 13, *M. kansasii*; lane 14, *M. malmoense*; lane 15, *M. phlei*; lane 16, *M. simiae*; lane 17, *M. szulgai*; lane 18, *M. fortuitum*. The molecular weights of DNA markers in lane M are indicated.

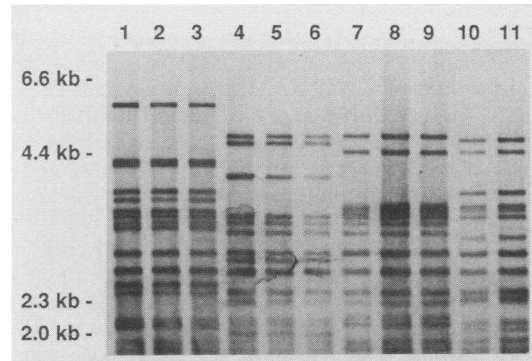

FIG. 5. Southern blot of *Alu*I-digested DNA from epidemiologically related isolates of *M. tuberculosis*. Lanes 1 to 3, school outbreak strains C1 to C3; lanes 4 to 6, group of fellow sewer workers' strains C4 to C6; lanes 7 to 9, family outbreak strains C7 to C9; lanes 10 and 11, family outbreak strains C10 and C11.

banding patterns of *M. tuberculosis* DNA from four small clusters of infection occurring in Melbourne, Australia. Within each of the four clusters, the banding patterns are identical, although there are a large number of band differences between the clusters (Fig. 5), showing the discriminatory nature of the repetitive element as a probe in RFLP analyses.

DISCUSSION

A number of repetitive DNA elements have been described previously in *M. tuberculosis* (9, 10, 14). Of these, only the insertion elements IS*6110* and IS*986*, which differ by only three nucleotide substitutions, have been shown to distinguish between *M. tuberculosis* isolates when used as probes on restriction enzyme-digested genomic DNA (9, 10). In the present study, we have cloned another repetitive DNA element which is distinct from IS*6110* and IS*986* in a number of ways. By restriction map comparison, pTBN12 and pTBN52 (Fig. 2) do not contain sites for *Pvu*II, *Bam*HI, or *Xho*I that are present in the previously described element (10). The distribution of the repeated sequence in other mycobacteria suggests that, apart from the species of the tuberculosis complex, it is present in *M. gastri*, *M. kansasii*, and *M. szulgai* (Fig. 3), whereas IS*6110* and IS*986* are confined to the tuberculosis complex (9, 10). The number of copies in *M. tuberculosis* appears to be at least 30, and *M. bovis* which is different from IS*6110* and IS*986*, which are present in 1 to 19 copies in *M. tuberculosis*, 1 to 5 copies in *M. bovis*, and 1 copy in *M. bovis* BCG (22). Finally, by using a probe for IS*6110* in comparison with the use of plasmid pTBN12, the two probes identified different DNA fragments (Fig. 3). However, of the previously reported repetitive elements one is considerably similar to our element (14). Reddi et al. probed restriction enzyme-digested mycobacterial DNA with a gel-purified 5.6-kb fragment from *Alu*I-digested *M. tuberculosis* DNA. They found that the 5.6-kb probe hybridized to a large number of bands, indicating the presence of a repeated sequence within the fragment. In addition, they used this probe to identify cloned fragments containing this repeat in a genomic clone library in λgt11. However, this group of investigators provided no restriction enzyme mapping, only limited data on mycobacterial species cross-hybridization, and no comparison among *M. tuberculosis* isolates by Southern blotting. Thus, in the absence of any subsequent characterization of their repeated element,

comparison is difficult, although it seems likely that the repeated element is the same as the one reported here.

We have shown the cloned repeated element to be useful in small epidemiologic studies. Strains within the small clusters of infection studied in this report appear identical, although there is a large number of DNA fragment differences between clusters (Fig. 5). Clearly, this method will be useful in larger epidemiologic studies and provides a number of variable DNA bands for comparison between isolates. One difficulty that may be encountered, especially when a strain is endemic to a particular geographical location, is the attempt to ascribe meaning to a single band difference between strains. The variation seen between isolates has resulted from mutations in the genomic DNA with time, and an estimate of how many serial passages of a strain needed to cause a mutation resulting in a DNA band variation is important to such considerations. This will become clearer as we examine multiple isolates from patients and strains from larger clusters of infection.

The reasons for the variable banding patterns among strains will require further study. After nucleotide sequencing, the nature of the element will be determined more readily, and this sequencing is currently under way. However, the relatively narrow distribution of the element among other mycobacteria would suggest that it is not widely shared among them and therefore may not be an essential functional component of mycobacteria.

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