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Comparison of Various Repetitive DNA Elements as Genetic Markers for Strain Differentiation and Epidemiology of *Mycobacterium tuberculosis*

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Five different genetic elements have been found to be associated with genetic rearrangements in *Mycobacterium tuberculosis* complex strains. Of these elements, the insertion sequence IS*6110* is presently the most frequently used genetic marker for strain differentiation of *M. tuberculosis*. In the present study we compared five genetic elements for their potentials to differentiate a given cluster of *M. tuberculosis* strains. Because of the presence of only a single copy of IS*6110* or two IS*6110* copies at the same chromosomal locus, a large number of strains could not be differentiated by IS*6110* fingerprinting. Most strains, including the low-copy-number IS*6110* strains, could be differentiated by fingerprinting with the 36-bp direct repeat or the polymorphic GC-rich repetitive DNA element. Less discriminative power was obtained with the major polymorphic tandem repeat and the insertion element IS*1081*. One strain which did not contain IS*6110* DNA was encountered. Until now, this element has invariantly been found to be present in all *M. tuberculosis* complex strains. On the basis of classical taxonomic criteria and sequencing of the 16S rRNA gene, this strain was shown to be a genuine *M. tuberculosis* strain. Therefore, the use of this element as a target for polymerase chain reaction-facilitated detection of *M. tuberculosis* should be reconsidered.

Since the discovery of polymorphic DNA in *Mycobacterium tuberculosis*, strain differentiation has become an important tool in the epidemiology of tuberculosis. Among the various genetic elements that have been found to contribute to DNA polymorphism in *M. tuberculosis*, the insertion element IS*6110* has been studied most intensively (8, 15, 25, 32). This 1,355-bp insertion sequence (IS) element is related to the IS*3* family of ISs and has been found exclusively in all isolates of the *M. tuberculosis* complex group of mycobacteria (12, 17, 25). Because of the highly variable number of copies and the variable sites of integration in the chromosome, epidemiologically unrelated strains display an extremely high degree of polymorphism of IS*6110*-containing restriction fragments (3, 12, 14, 16). This polymorphism is presumably due to the ability of IS*6110* to transpose within the genome without much target sequence specificity (11, 12, 16, 17). Despite the high degree of IS*6110*-associated restriction fragment length polymorphism (RFLP) among *M. tuberculosis* strains, no IS*6110* transposition has been observed during prolonged in vitro culturing or in vivo growth (12, 18), suggesting that the frequency of transposition is relatively low. This is in agreement with observations which indicate that IS*6110* is an excellent genetic marker for tracing individual *M. tuberculosis* strains during microepidemics, nosocomial infections, and the clonal dissemination of multiple-drug-resistant strains (1, 2, 5–7, 9, 10, 12, 14, 17, 18, 22, 25, 29).

Although most *M. tuberculosis* complex strains carry multiple IS*6110* copies, isolates which contain only one or a few copies have occasionally been encountered. Virtually all *Mycobacterium bovis* BCG strains contain a single IS copy in a region of the chromosome in which multiple short directly repeated sequences are present. Because the majority of *M. tuberculosis* complex strains also carry one or two copies of the IS element at this chromosomal locus, this site seems to be a preferred site for integration of IS*6110* (11).

During a preliminary analysis of strains originating from India, we encountered a large number of strains with only a single IS copy. These strains could not be differentiated by IS*6110* DNA fingerprinting, because IS*6110* DNA was found to be associated with a unique restriction fragment in these strains. As expected, IS*6110* was found to be inserted in the region containing the direct repeats (DRs). Because to our knowledge these isolates originated from epidemiologically unrelated cases of tuberculosis, we wondered whether such strains might be differentiated by other polymorphic DNA markers.

In addition to IS*6110*, four other targets for RFLP analysis have been described: (i) IS*1081*, an insertion element related to the *Staphylococcus aureus* transposable element IS*254* (4, 30); (ii) a polymorphic GC-rich repetitive sequence (PGRS) present on the recombinant plasmid TBN12 (20); (iii) the major polymorphic tandem repeat (MPTR) sequence (13); and (iv) the 36-bp DR sequence of which multiple copies are present at the hot spot of IS*6110* integration (11). The cluster of DR sequences is present at a single chromosomal locus, whereas the other four elements are located at many different positions in the chromosome. Although each of these five repetitive DNA sequences has been shown to be implicated in RFLP in *M. tuberculosis*, no attempt has been made to compare the RFLPs of each of these genetic markers in *M. tuberculosis*. The study described here was undertaken to determine which of these five polymorphic genetic markers was most suitable for strain differentiation of *M. tuberculosis*, especially strains containing few IS*6110* copies. We

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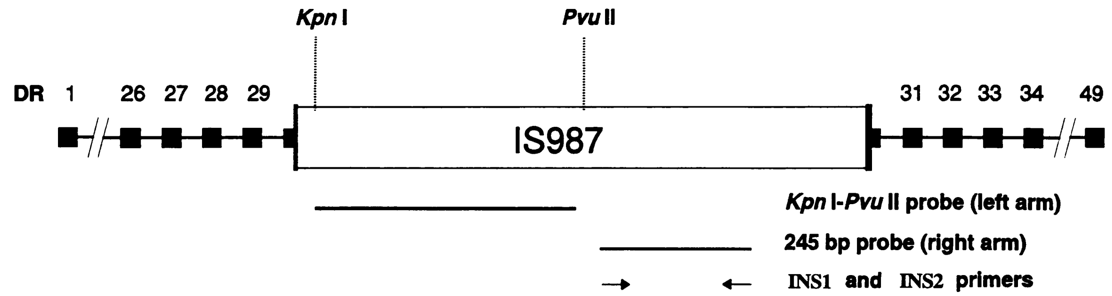

FIG. 1. Physical map of the *M. bovis* BCG chromosomal region containing the IS*6110*-like element IS*987*. The DRs are depicted as small, numbered blocks. The DRs are spaced by unique intervening sequences of 35 to 41 bp in length (11). The probes and primers used in the present study are indicated.

showed that epidemiologically nonrelated strains which cannot be differentiated with IS*6110* because of the presence of only one or a few copies can be distinguished by other genetic markers, in particular, by DR and PGRS DNAs. Furthermore, we describe a strain which is exceptional in that it lacks IS*6110* DNA and show by 16S rRNA gene sequencing and other methods that this strain is a genuine *M. tuberculosis* strain.

MATERIALS AND METHODS

Bacterial strains and bacteriological testing. Sixty-three strains originating from India were obtained in 1984 from D. W. Smith (University of Wisconsin, Madison). *M. tuberculosis* strains isolated in the People's Republic of China were supplied by Wang Guozhi and Wang Zheng (National Institute for the Control of Pharmaceutical and Biological Products, Beijing, People's Republic of China). Strains carrying a single IS*6110* copy isolated in The Netherlands were sent to us by Regional Health Laboratories. Other strains with single copies of IS*6110* were kind gifts of R. Hussain (Aga Khan University, Karachi, Pakistan), T. Aisu (Uganda Tuberculosis Investigations, Wandegeya Kampala, Uganda), P. M. Small (Howard Hughes Medical Institute, Stanford, Calif.), and J. Perera (University of Colombo, Colombo, Sri Lanka). Bacteriology based on biochemical and growth characteristics and sensitivity testing of guinea pigs for pathogenic mycobacteria were performed as described previously (23, 31). Fatty acid analysis of mycobacterial cells was done as described by Tisdall et al. (26). The reactivity of mycobacteria with the *M. tuberculosis* complex-specific monoclonal antibody F42-2 was determined as described by Schöningh et al. (21).

DNA techniques. Genomic DNA extraction and Southern blotting were performed as described previously (27–29). DNA fragments for Southern blot hybridization were labeled by using the enhanced chemiluminescence gene detection system (Amersham International plc, Amersham, United Kingdom). Oligonucleotides were labeled by using the ECL 3′ oligo labeling system (Amersham International plc).

Two different IS*6110* probes were used in the present study: a 245-bp product amplified by polymerase chain reaction (PCR) (IS*6110*R) (12) and a *Kpn*I-*Pvu*II fragment of IS*6110* (IS*6110*L) (12). The latter DNA was purified by agarose gel electrophoresis of *Kpn*I-*Pvu*II-cleaved pPH1001 plasmid DNA (11). Plasmid pTBN12 (20) was a kind gift of B. Ross (Fairfield Infectious Disease Hospital, Fairfield, Australia). This plasmid contains a cluster of short PGRSs, and the plasmid was used in its entirety for hybridization experiments. IS*1081* DNA was obtained by PCR amplification as described previously (30). The probe DR-r, which was used to detect the direct repeats in the hot spot integration region of IS*6110*, has been described previously (11). As a probe for the MPTR, we used the synthetic oligonucleotide TR2 (13).

Gen-Probe tests were performed according to the recommendations of the manufacturer (Gen-Probe Incorporated, San Diego, Calif.). The 5′ 550-bp region of the 16S rRNA gene was amplified by PCR by using the broad-host-range primers 16S1F, AGAGTTTGATC(A/C)TGG(T/C)TCAG, and 16S1R, CTTTACGCCCA(A/G)T(G/A)A(A/T)TCCG. The PCR product was sequenced on an automatic sequencer (model 370A; Applied BioSystems).

RESULTS

Determination of DNA polymorphism among *M. tuberculosis* strains by using five different genetic markers. In order to compare the ability of the five DNA sequences to detect DNA polymorphism among *M. tuberculosis* strains, these DNA sequences were used as probes in RFLP typing of 63 strains isolated in southern India. The IS*6110* fingerprints obtained with the "right arm" probe IS*6110*R (Fig. 1) of this set of strains are shown in Fig. 2. Although the majority of the *M. tuberculosis* strains differed in their IS*6110* banding patterns, 21 of 63 strains harbored a single IS*6110* copy, and in 20 of these strains, the IS element was found to be present on a 1.5-kb *Pvu*II restriction fragment. In contrast, none of the DNA fingerprints of the strains carrying multiple copies of IS*6110* were identical. Unexpectedly, the DNA of one *M. tuberculosis* strain, strain IN4, did not hybridize with the IS*6110* probe (Fig. 2B). This indicates that at least part of the IS*6110* DNA is absent from this strain.

We selected the 22 *M. tuberculosis* strains used for the DNA fingerprinting shown in Fig. 2B for comparative analysis with other DNA probes. This subset contained many strains with a single, identically sized IS*6110*-containing *Pvu*II fragment, in addition to strains with highly related RFLPs. The filter containing chromosomal *Pvu*II fragments of this subset was rehybridized with the labeled *Kpn*I-*Pvu*II fragment of IS*6110* (the "left arm," IS*6110*L; Fig. 1), IS*1081*, PGRS, DR, and MPTR DNAs. The DNA fingerprints of seven *M. tuberculosis* strains showing a single 1.5-kb fragment hybridizing with the IS*6110*R probe also showed a single, unique *Pvu*II fragment when IS*6110*L was used as a probe (Fig. 3A). This strongly suggests that the IS element in these strains is inserted at a unique position in the chromosome.

The fingerprints obtained with IS*1081* were much less polymorphic compared with those obtained with IS*6110*

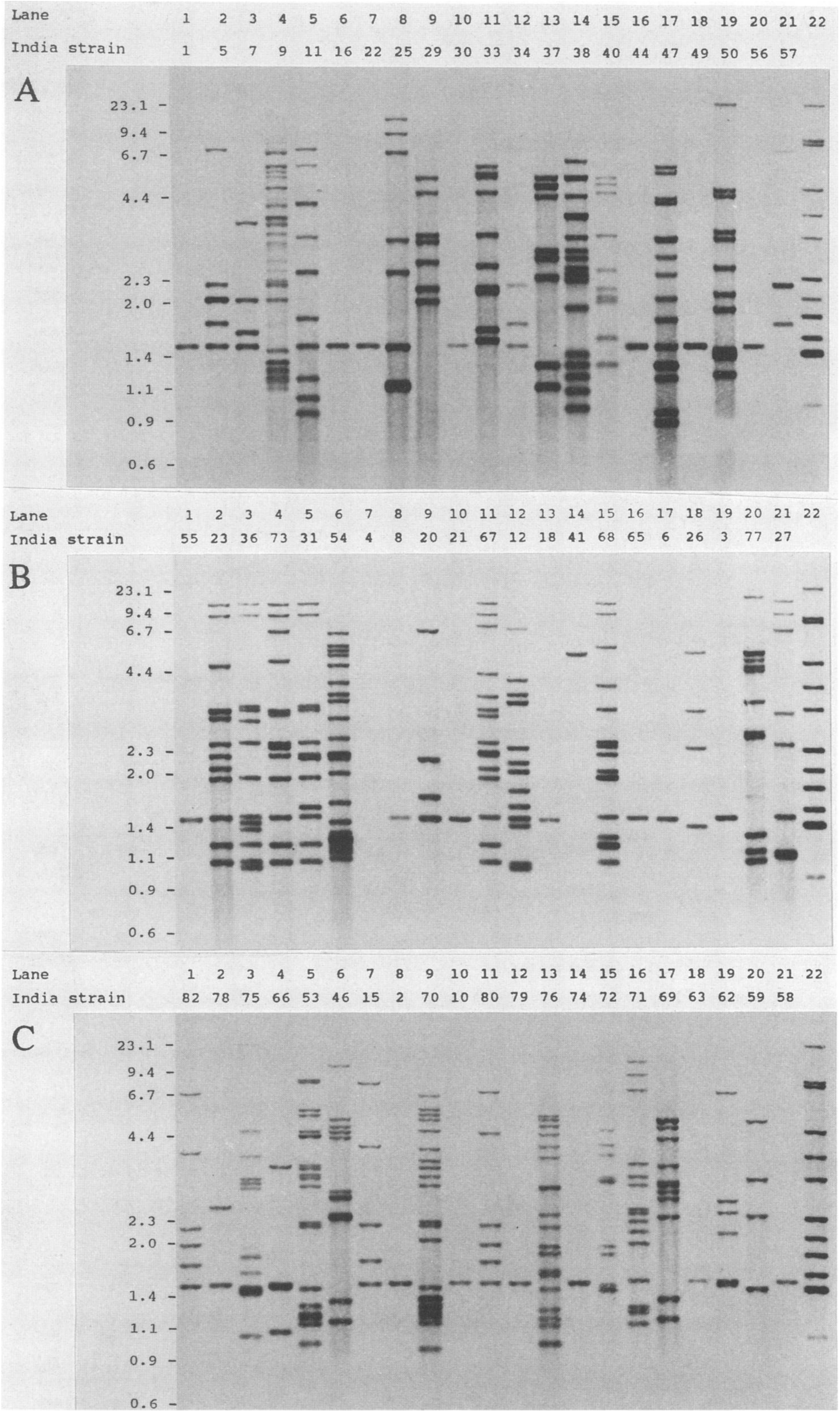

FIG. 2. DNA fingerprints of 63 *M. tuberculosis* strains originating from India. Chromosomal DNA was digested with the restriction enzyme *Pvu*II. The 245-bp PCR fragment (IS*6110*R) of IS*6110* was used as a probe to visualize *Pvu*II restriction fragments containing the right arm of IS*6110*. Numbers on the left indicate the sizes of standard DNA fragments (in kilobase pairs). Lane 22 of each panel represents DNA of *M. tuberculosis* reference strain Mt.14323.

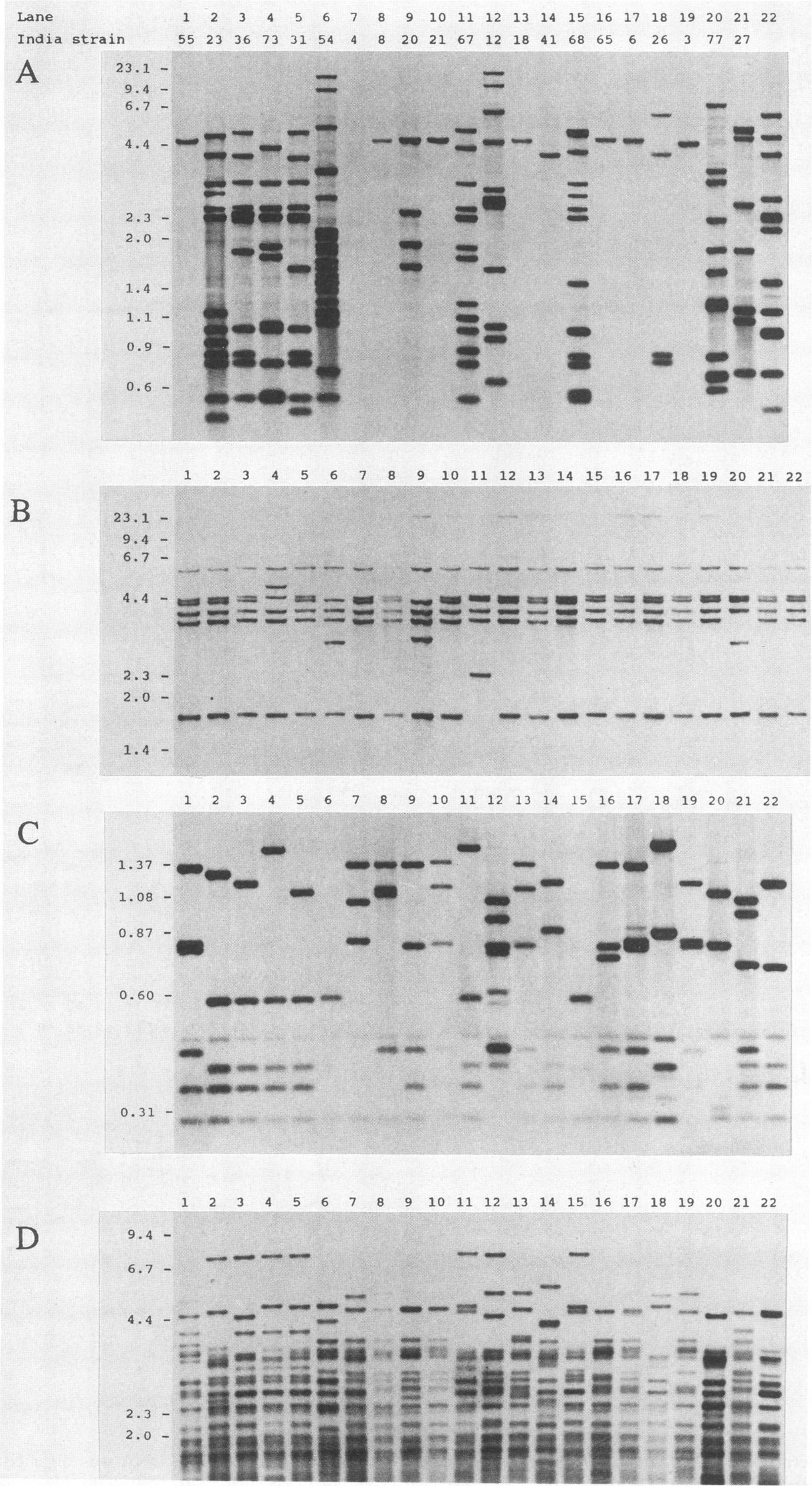

FIG. 3. RFLP of *M. tuberculosis* DNA by using the left arm of IS*6110* (A), IS*1081* (B), DR (C), and PGRS DNA (D) as probes. The strains analyzed are identical to those in Fig. 2B. (A and B) Fingerprints from *Pvu*II-digested DNAs; (C and D) fingerprints from *Alu*I-digested DNAs. Numbers on the left indicate sizes of standard DNA fragments (in kilobase pairs). In all panels, lane 22 was loaded with the DNA of *M. tuberculosis* reference strain Mt.14323.

(Fig. 3B). All 22 strains carried either six or seven IS*1081* copies, and only six different patterns were observed. This limited polymorphism is consistent with our previous observations on the IS*1081* RFLP among other *M. tuberculosis* strains.

A poor differentiation between fingerprints was obtained with the remaining three DNA probes because the MPTR and the PGRS probes hybridized to many *Pvu*II restriction fragments, resulting in almost identical overcrowded banding patterns and because DR DNA hybridized to only a few fragments that differed only slightly in size (data not shown). Therefore, chromosomal DNAs of this subset were cleaved with another restriction enzyme, *Alu*I, which cleaves more frequently than *Pvu*II. Again, little strain differentiation was obtained with the MPTR probe (data not shown); however, the patterns obtained with PGRS and DR probes were more polymorphic compared with the patterns obtained with the *Pvu*II fragments. Figure 3D shows the fingerprints obtained with PGRS DNA as a probe. A vast number of *Alu*I PGRS-hybridizing fragments are visible in Fig. 3D, and the RFLP was most conspicuously visible among fragments larger than 3 kb. The banding patterns of all 22 strains were different, including those from strains with a single IS*6110* element.

The DR fingerprints are shown in Fig. 3C. Twenty different *Alu*I patterns could be distinguished among the 22 *M. tuberculosis* strains. All except two of the seven strains with a single IS*6110* copy were differentiated by the DR probe (Fig. 2B and 3C, lanes 10 and 13). The other strain pair with an identical DR fingerprint was from a group of strains that displayed closely related IS*6110* fingerprints (Fig. 2B, 3A, and 3C, lanes 4 and 11).

It should be noted that the 1.5-kb *Pvu*II IS*6110*-containing fragment in the strains with a single copy of IS*6110* cohybridized with DR DNA, indicating that the IS*6110* element is inserted in the DR region of the chromosome. We conclude that the DR and the PGRS probes allow differentiation of strains which are indistinguishable by the IS*6110* probe because of the IS*6110* insertion into a unique position in the chromosome.

DNA polymorphism in the DR cluster of epidemiologically nonrelated strains with identical IS*6110* fingerprints. The results described above indicate that *M. tuberculosis* strains with an identical IS*6110* DNA fingerprint are not necessarily identical, because the strains with a single IS*6110* copy located on a 1.5-kb *Pvu*II fragment generally show a different fingerprint when probed with PGRS or DR DNA. Previously, we have observed the occurrence of *M. tuberculosis* strains containing a single IS*6110* element (13, 28a, 29, 30). In the majority of these strains, the IS element was found to be present at a 1.5-kb *Pvu*II fragment, whereas most of these strains originated from epidemiologically unrelated cases of tuberculosis.

To investigate more systematically the DNA polymorphism of such strains by using other genetic markers, we subjected all 21 single-IS*6110*-copy strains from the set of 63 strains from India described above and 16 single-IS*6110*-copy strains from six other countries to RFLP analysis using the DR probe. In 29 of these 37 strains, the IS*6110*R probe hybridized with a *Pvu*II fragment of 1.5 kb, and in the remaining strains the IS*6110*R probe hybridized with either a 1.3- or a 5.0-kb fragment.

The results of the DR DNA hybridization experiment are shown in Fig. 4. A high degree of *Alu*I RFLP was also observed among the majority of these strains.

Thirty-four different DR-containing fragment patterns were distinguished among the 37 strains investigated. This indicates that the majority of these strains are different, which is consistent with the fact that these strains were supposed to be epidemiologically unrelated.

In a previous report (29), we have described an outbreak of tuberculosis among homeless people in Amsterdam, and the vast majority of the strains from that outbreak showed an IS*6110* fingerprint consisting of two *Pvu*II bands with sizes of 2.9 and 2.1 kb. Strains with this characteristic banding pattern have been encountered repeatedly since then, although no apparent epidemiological relationship with the outbreak was suspected. To investigate whether the latter strains were identical to the ones involved in the outbreak in Amsterdam, we analyzed by DR typing 11 outbreak strains and seven strains isolated elsewhere in The Netherlands 1 to 2 years after the outbreak in Amsterdam. All 11 outbreak strains and 3 other strains were identical by DR fingerprinting, whereas the remaining 4 strains clearly differed from the outbreak strains. Of the latter four strains, two showed identical DR fingerprints, whereas the other two had different patterns (data not shown). Among the same 18 strains, each of which carried only two copies of IS*6110*, DNA fingerprints were also produced by using the PGRS probe. Strains sharing identical DR DNA fingerprints also appeared to have the same PGRS patterns, whereas the two strains that were epidemiologically unrelated but that shared the same DR DNA fingerprint also had identical PGRS patterns (data not shown). We conclude that the DR and the PGRS probes are equally suitable for differentiating *M. tuberculosis* strains, which are difficult to distinguish with the IS*6110*R DNA probe because of the presence of a single IS*6110* element in the DR region.

Comparison of IS*6110* and PGRS DNA typing of *M. tuberculosis* strains carrying multiple IS*6110* copies. Previous studies have shown that outbreak strains are identical by IS*6110* typing (12, 29). To determine whether this is also true for PGRS typing, we analyzed 12 isolates from a previously described outbreak (12) and 2 strains with the same IS*6110* type isolated 2 years after the main microepidemic. All 14 strains exhibited identical *Alu*I PGRS banding patterns (data not shown).

Furthermore, seven strains from a recent microepidemic which had identical IS*6110* fingerprints were also found to have the same PGRS fingerprints (data not shown). These data indicate that outbreak strains can be well recognized by the PGRS DNA probe and that PGRS DNA restriction fragment patterns have enough stability to be used as effective tools in the epidemiology of tuberculosis.

Previous studies suggested that IS*6110* fingerprints from epidemiologically unrelated isolates in countries with a high rate of tuberculosis transmission are more similar than are such strains from countries with a low transmission rate (29). To compare the discriminative power of IS*6110* with PGRS DNA for strains from areas of high endemicity, we analyzed a set of 18 *M. tuberculosis* strains originating in the People's Republic of China. As shown in Fig. 5A, the IS*6110* banding patterns showed much relatedness, but each pattern was distinct. The *Alu*I PGRS banding patterns also showed much relatedness, and only 13 different patterns were observed among the 18 strains. Identical fingerprints were found among four strains (Fig. 5B, lanes 4, 11, 12, and 15) and two pairs of strains (Fig. 5B, lanes 5 and 16 and lanes 7 and 9).

Finally, to investigate the changes in the IS*6110* and PGRS DNA banding patterns of isogenic *M. tuberculosis* strains which have been cultured separately for many years, the laboratory strains H37Rv and its avirulent derivative H37Ra

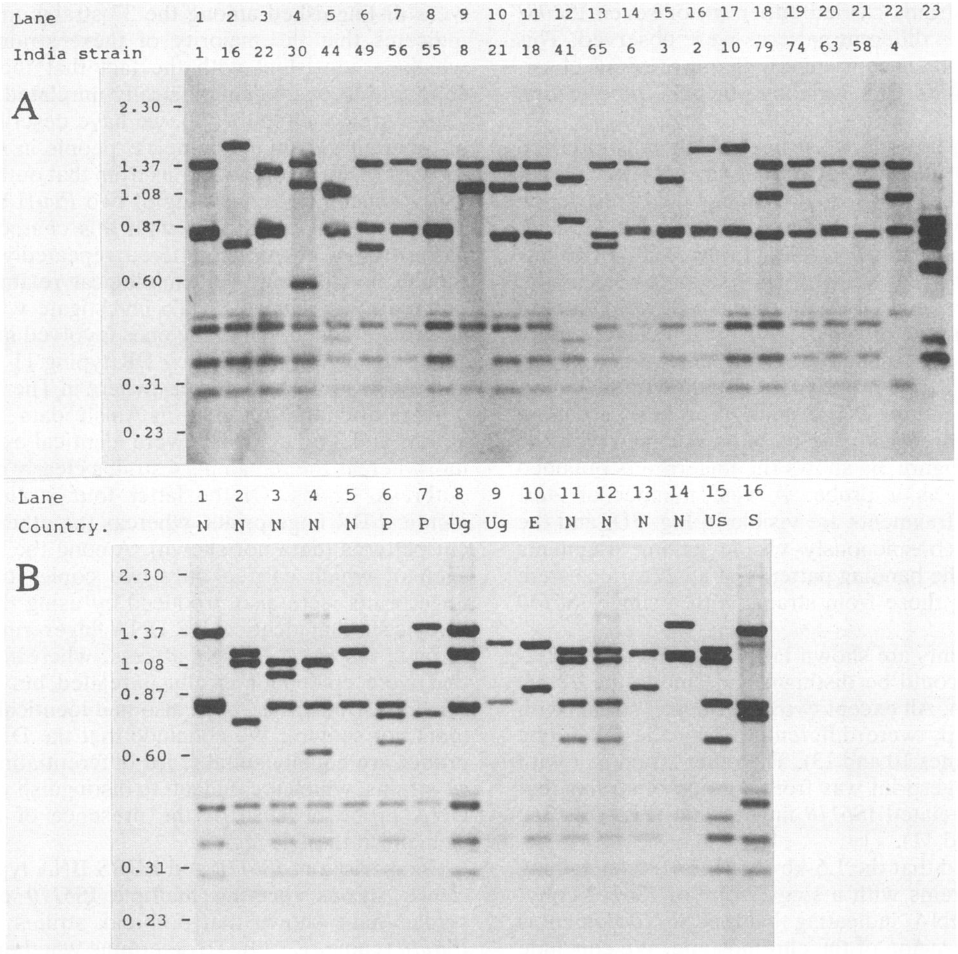

FIG. 4. DR RFLP of *M. tuberculosis* strains carrying a single IS*6110* element. All DNAs were digested with the restriction enzyme *Alu*I. The strains shown in panel A are from India, and the strains in panel B originated from The Netherlands (N), Pakistan (P), Uganda (Ug), Ethiopia (E), the United States (Us), and Sri Lanka (S). Numbers on the left indicate the sizes of standard DNA fragments (in kilobase pairs). Lane 22 of panel A contains DNA of *M. tuberculosis* reference strain Mt.14323. In these strains, IS*6110*R DNA is present on a *Pvu*II fragment of either 1.5 kb (panel A, lanes 1 to 13 and lanes 15 to 21; panel B, lanes 1 to 8 and 16), 1.3 kb (panel B, lanes 11, 12, and 15), or 5.0 kb (panel A, lane 14; panel B, lanes 9, 10, 13, and 14) (data not shown).

were compared. The IS*6110* fingerprints of these strains differed in 5 of 15 bands, whereas the only difference between the PGRS patterns was one additional band in the lane of H37Rv (data not shown).

Characterization of an *M. tuberculosis* strain without IS*6110* DNA. As described above, DNA extracted from one of the *M. tuberculosis* strains originating from India did not hybridize with IS*6110* DNA. Also, by PCR with the INS1 and INS2 primers (Fig. 1), we found no amplifiable IS*6110* DNA in strain IN4. Strain IN4 is exceptional among *M. tuberculosis* strains because this was the first strain without detectable IS*6110* DNA among at least 700 *M. tuberculosis* complex strains tested so far. Hybridization experiments with other genetic markers revealed the presence of DR, PGRS, and IS*1081* DNAs in the genome of this exceptional strain (Fig. 3B, C, and D). Because this strain might also differ from *M. tuberculosis* in other characteristics, strain IN4 was subjected to a thorough bacteriological identification procedure.

The Gen-Probe test for *M. tuberculosis* complex strains was positive with strain IN4. Because this test is based on the hybridization of only a 20-bp stretch of the 16S rRNA sequence, we sequenced the 5′ 550 bp of the 16S rRNA gene in strain IN4. The sequence obtained was identical to the ones previously published for *M. tuberculosis* and *M. bovis* BCG (19, 24) (EMBL data base accession numbers X52917 and M20940, respectively). Strain IN4 was also subjected to classical bacteriological determination, gas chromatography of cell wall-associated lipids, and drug susceptibility testing with an extended spectrum of antibiotics. Again, the results were characteristic for *M. tuberculosis*. Furthermore, strain IN4 was reactive with the monoclonal antibody F24-2, which has been shown to be specific for bacteria of the *M. tuberculosis* complex (21). Finally, guinea pigs showed the characteristic tuberculous lesions 2 months after subcutaneous inoculation with strain IN4.

DISCUSSION

Although five different genetic repetitive DNA elements have been shown to be implicated in chromosomal DNA polymorphism of *M. tuberculosis*, only IS*6110* and PGRS DNA have previously been tested with a large number of

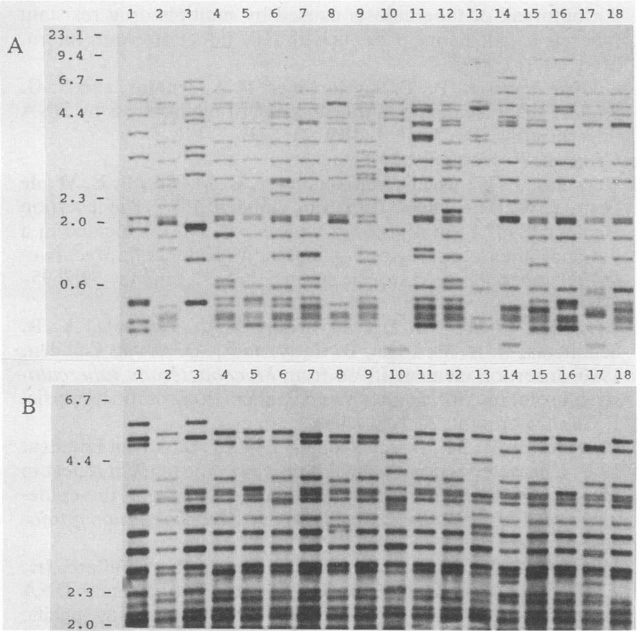

FIG. 5. IS*6110* (A) and PGRS DNA (B) containing restriction fragment patterns of *M. tuberculosis* strains originating from the People's Republic of China. An identical set of strains was analyzed in both panels. The DNAs in panel A were digested with *Pvu*II, and the DNAs in panel B were digested with *Alu*I. Numbers on the left indicate the sizes of standard DNA fragments (in kilobase pairs).

isolates. The results of the present study confirm the conclusions of previous studies that both IS*6110* and PGRS DNA are excellent markers for use in differentiating *M. tuberculosis* strains. Furthermore, the DR region was also found to be very polymorphic, allowing the differentiation of virtually all 63 Indian *M. tuberculosis* strains investigated in the present study. As observed previously (30), the IS*1081* element was found to be less useful for strain differentiation because most of the five to seven copies of IS*1081* are present at identical positions in the *M. tuberculosis* chromosome. Similarly, the MPTR sequence allowed only limited strain differentiation.

Because IS*6110* is the most frequently used genetic element for differentiating *M. tuberculosis* strains, it is of critical importance to know whether strains with identical IS*6110* banding patterns belong to a single group of outbreak strains that were recently disseminated in a clonal way. The present study showed that when only one or a few IS*6110* copies are present in *M. tuberculosis* strains, such isolates may appear to be polymorphic when other genetic markers are used.

Hermans et al. (11) have shown that about 85% of the *M. tuberculosis* isolates that they investigated contained one or two copies of IS*6110* in the DR-containing region of the chromosome, which seems to be a hot spot for integration of IS*6110*.

We previously found that in many strains, IS*6110* DNA is present on a 1.5-kb *Pvu*II fragment (29) and that this fragment usually contains DR DNA (11). Therefore, the finding that 32 of the 63 Indian strains investigated in the present study carried IS*6110* DNA on the 1.5-kb *Pvu*II fragment was not unexpected. Twenty of these strains contained a single IS*6110* element, and consequently, these isolates could not be differentiated with either the left arm or the right arm of IS*6110* as the DNA probe. In all of these strains, IS*6110* was found to be integrated in the DR region of the chromosome. However, most of these strains were easily differentiated by the PGRS and DR DNA probes.

Although no thorough studies on the stability of PGRS and DR DNAs in *M. tuberculosis* have been done, these results strongly suggest that the 20 strains do not belong to a recently disseminated clone, which is consistent with the unlikeliness that all of these strains are epidemiologically related. Moreover, we found that 16 strains originating from six different countries differed in their PGRS and DR restriction fragment banding patterns, although a majority of these strains contained a single copy of IS*6110* at a 1.5-kb *Pvu*II restriction fragment.

It is interesting that the DR-containing *Alu*I fragment patterns among the Indian strains appeared to be less polymorphic compared with those among the strains from other countries. This would suggest that the Indian strains belong to an evolutionarily related group of strains which is distinct from strains isolated on other continents.

It should be noted that *M. tuberculosis* strains with a single IS*6110* copy have rarely been observed (1–3, 5–7, 10, 11, 12, 14, 16–18, 22, 25, 29, 30). However, in none of those studies were fingerprints of Asian strains investigated. Results of the present study suggest that a significant proportion of the *M. tuberculosis* strains from the Far East contain a single IS*6110* element at the unique hot spot for integration of this mobile element. Recent studies have also shown the occurrence of such strains in Vietnam (21a).

We also investigated a few epidemiologically unrelated *M. tuberculosis* strains carrying two IS*6110* elements at identically sized *Pvu*II fragments. Different types could be distinguished by DR and PGRS fingerprinting, indicating that these were different strains and that perhaps a second preferred site of integration for IS*6110* exists in the mycobacterial chromosome.

Although we encountered many strains with a single IS*6110* element, the general experience is that *M. tuberculosis* isolates contain multiple IS*6110* elements and that strains with the same IS*6110* fingerprints are epidemiologically related.

The results described above indicate that certain repetitive DNA elements of *M. tuberculosis* are more unstable than other ones. This opens the interesting possibility that these elements could be used as differential molecular clocks to determine the evolutionary distances between *M. tuberculosis* strains.

It is clear that the pace of the IS*1081* clock is slower than that of IS*6110*, DRs, and PGRS DNA. However, very little is known about the quantitive differences between the pace of genetic rearrangements mediated by the remaining repetitive DNA elements identified thus far in *M. tuberculosis*.

A complicating factor in using IS elements for such purposes is undoubtedly the strong locus-dependent frequency of transposition, as suggested by the results of the present study.

The observation that the power of the PGRS probe to discriminate strains with a high multiplicity of IS*6110* elements is less than that of IS*6110* may indicate that IS*6110*-associated rearrangements occur more frequently than PGRS-associated ones do. However, the outbreak strains generally were found to be identical either by IS*6110* typing or by using PGRS DNA.

Repetitive DNA elements like the DR, MPTR, and PGRS probes might be more useful than IS elements for measuring quantitative evolutionary relationships, because the DNA rearrangements caused by these elements are likely to be more random in nature. This is because many of the mutations caused by transposition of IS elements are expected to be far from neutral, in contrast to what one might expect from variations in short repetitive DNA sequences. Recent sequence data of the DR region of various *M. tuberculosis* strains have shown that part of the DNA polymorphism in this region can be explained by homologous recombination between the 36-bp DR sequences, which are spaced by unique sequences 35 to 41 bp in length (10a).

Unexpectedly, we encountered for the first time a strain which lacked demonstrable IS*6110* DNA sequences. This strain originated from India, and by all criteria, it was found to be a genuine *M. tuberculosis* strain. If such strains would occur specifically in certain geographic areas, one should be reluctant to use IS*6110* DNA as a target for amplification by PCR for detection of *M. tuberculosis* in clinical specimens.

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