Insertion Element IS986 from Mycobacterium tuberculosis: a Useful Tool for Diagnosis and Epidemiology of Tuberculosis

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IS986 of Mycobacterium tuberculosis belongs to the IS3-like family of insertion sequences, and it has previously been shown to be present in multiple copies in the chromosome of M. tuberculosis. In this study we investigated the value of a IS986-based DNA probe in the diagnosis and epidemiology of tuberculosis. IS986 was found only in species belonging to the M. tuberculosis complex. Independent isolates of M. tuberculosis complex strains showed a very high degree of polymorphism of restriction fragments which contained IS986 DNA. In contrast, Mycobacterium bovis BCG vaccine strains as well as clinical isolates of M. bovis BCG contained only one copy of IS986, which was present at the same location in the chromosome. Different M. tuberculosis isolates from a recent M. tuberculosis outbreak showed an identical banding pattern. We concluded that IS986 is an extremely suitable tool for the diagnosis and epidemiology of tuberculosis.

Tuberculosis is a highly contagious disease that is mainly transmitted from person to person. Because of the long incubation time needed for definitive laboratory diagnosis of this disease, the tracing of individuals who might have been in contact with infected people is a major strategy for limiting the dissemination of Mycobacterium tuberculosis. The typing of strains from infected individuals could play an important role in tracking the sources of infection. Phage typing (4) can be used for differentiating several groups of M. tuberculosis strains but is of limited value in the epidemiology of tuberculosis because only a few different phage types can be recognized (7). In addition, phage typing is a rather time-consuming and cumbersome technique which can be carried out by only a very specialized laboratory. Serotyping, on the other hand, cannot differentiate strains within the M. tuberculosis complex (10). Therefore, there is a great need for an improved method to subtype M. tuberculosis strains by a simple and rapid method. Restriction fragment length polymorphism (RFLP) in genomic DNA is commonly exploited to detect genetic variability (9). Several investigators have identified M. tuberculosis DNA element which are present in multiple copies per genome and which show a polymorphic banding pattern among the few isolates that have been investigated (6, 15, 24). One of these elements, IS986, was recently sequenced and was found to show considerable homology with insertion sequences of the IS3 family of enterobacteria (R. A. McAdam, P. W. M. Hermans, D. van Soolingen, Z. F. Zainuddin, D. Catty, J. D. A. van Embden, and J. W. Dale, submitted for publication). The DNA sequence was virtually identical to the recently described M. tuberculosis insertion element IS6110 (20). Although the transposition of these elements has not yet been described in M. tuberculosis, the sequence data indicate that the insertion element is a functional transposable element. The 1,358-kilobase-pair (kb) element is flanked by inverted repeats of 30 base pairs (bp), and it contains a large open reading frame which shares homology with the transposase of the enterobacterial IS3 family. In this study, we evaluated the usefulness of IS986 as a probe for RFLP analysis. For this purpose, we analyzed a large number of isolates of M. tuberculosis complex strains from both epidemiologically nonrelated and related sources.

MATERIALS AND METHODS

Bacterial strains, genomic DNA, and plasmids. The bacterial strains and plasmids used in this study and their origins are listed in Table 1. Media, reagents, and enzymes were used as described previously (21). Culturing of the mycobacterial strains and isolation of genomic DNA was performed as reported by Hermans et al. (12a).

Labeling of DNA probes. The mycobacterial DNA fragments of pRP5000 were labeled with [α-32P]dCTP by using the multiprime DNA labeling kit (Amersham International plc, Amersham, United Kingdom) or with horseradish peroxidase by using the enhanced chemiluminescence gene detection system (Amersham International plc). The detection of horseradish peroxidase-labeled probes was carried out by the peroxidase-catalyzed oxidation of luminol and subsequent enhanced chemiluminescence. The emitted light was detected on X-ray film (16, 23).

Southern blot hybridization. Digests of chromosomal DNAs were electrophoretically separated on 0.8% agarose gels containing ethidium bromide (500 ng/ml). After denaturation and transfer to Gene Screen Plus membranes (Dupont, NEN Research Products, Boston, Mass.) by vacuum blotting (14, 18) (Milliblot V-system; Millipore Corp., Bedford, Mass.), the DNA was hybridized with [32P]-labeled probes, as described by Noordhoek et al. (13). Hybridization and washing were done at 65°C. Membranes were exposed to X-Omat film (Eastman Kodak Co., Rochester, N.Y.) at −70°C for various lengths of time. The experimental proce-
TABLE 1. Bacterial strains and plasmids used in this study

<table>
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<th>Strain or plasmid</th>
<th>Species</th>
<th>Property or origin</th>
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<td></td>
<td>Ampicillin resistance; pBR322 derivative</td>
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<td>pAT153 derivative carrying a 4.6-kb insertion element-containing M. tuberculosis DNA fragment</td>
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<td>pEX2 recombinant containing a 2.4-kb DNA fragment of M. tuberculosis</td>
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<sup>c</sup> Organon Teknika N.V., Turnhout, Belgium.
<sup>d</sup> Institut Armand Frappier, Laval, Quebec, Canada.
<sup>e</sup> Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium.
<sup>f</sup> American Type Culture Collection, Rockville, Md.

Synthetic oligonucleotides. Based on the sequence of IS986 (McAdam et al., submitted), two oligonucleotides were synthesized by using a DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). The oligonucleotides INS-1 (5'-GTGTAGGGGATCGAGGTTGGG) and INS-2 (5'-CGG TAGGGCGTGGTGACAAA) correspond to bp 631 to 650 and 856 to 875 of the IS986 sequence, respectively. Oligonucleotides INS-1 and INS-2 were complementary to opposite strands and positioned 205 bp apart. Two homologous regions within the 16S rRNA genes of M. bovis BCG (19) and the 16S rRNA genes from various other procaryotic species described by Dams et al. (5) were selected and used for synthesizing the oligonucleotides R (5'-GTGCCAGCGACCGCGTAA) and S (5'-GTGCCAGGCAGAGTCCTACATTC). These oligonucleotides correspond to residues 504 to 523 and 910 to 929 on the opposite strands of the M. bovis BCG gene, respectively.

Polymerase chain reaction. The polymerase chain reaction (PCR) (17) was performed with thermoresistant DNA polymerase from *Thermus aquaticus* (Taq polymerase; The Perkin-Elmer Corp., Norwalk, Conn.) as recommended by the manufacturer. One unit of *Taq* polymerase was added to 100 μl of 50 mM NaCl–2 mM MgCl<sub>2</sub>–10 mM Tris hydrochloride–0.01% (wt/vol) gelatin–0.2 mM of each of the deoxynucleotides dGTP, dATP, dTTP, and dCTP (Boehringer GmbH, Mannheim, Federal Republic of Germany) (pH 9.6),

![Figure 1](http://jcm.asm.org/) FIG. 1. Physical map of the 4.6-kb *EcoRI* insert of pRP5000 containing IS986 flanked by inverted repeats (IR). Also, the large 1,037-bp open reading frame (ORF) is depicted.
FIG. 2. Southern blot analysis of PvuII-digested chromosomal DNA of various mycobacterial species hybridized with the 32P-labeled 4.6-kb EcoRI fragment of pRP5000 (A) and the 2.4-kb EcoRI fragment of pH7301 (B). Lanes 1, M. leprae 90; lanes 2, M. tuberculosis 13; lanes 3, M. tuberculosis 14; lanes 4, M. tuberculosis 15; lanes 5, M. tuberculosis 19; lanes 6, M. tuberculosis 23; lanes 7, M. tuberculosis 21; lanes 8, M. tuberculosis 22; lanes 9, M. tuberculosis 32; lanes 10, M. tuberculosis 10; lanes 11, M. bovis 40; lanes 12, M. bovis 41; lanes 13, M. bovis BCG 45; lanes 14, M. africanum 38; lanes 15, M. microti 46; lanes 16, M. avium 50; lanes 17, M. fortuitum 60; lanes 18, M. gordonae 71; lanes 19, M. intracellulare 56; lanes 20, M. kansasii 53. Numbers on the left indicate sizes of standard DNA fragments in kilobase pairs.
containing 100 ng of target DNA and 500 ng of each primer. DNA amplification was performed in a PCR processor (Bio-med GmbH, Theres, Federal Republic of Germany) with 35 temperature cycles: 1 min, 94°C; 1 min, 65°C; and 2 min, 72°C. The amplified DNA was analyzed by agarose gel electrophoresis.

**Animal experiments.** TRL guinea pigs (weight, about 250 g) were subcutaneously and intramuscularly inoculated in one leg with 0.5 ml of a *M. tuberculosis* suspension of 10^2 bacilli per 1 ml of phosphate-buffered saline. After 8 weeks, the animals were sacrificed, *M. tuberculosis* was isolated from the spleens, and the bacteria were further cultured in vitro.

**RESULTS**

**Occurrence of IS986 among the various mycobacterial species.** In order to determine the occurrence of IS986 in mycobacteria, plasmid pRP5000 was used for the isolation of the insertion element. pRP5000 is a derivative of a lambda recombinant that was selected from a *M. tuberculosis* gene library as a result of its hybridization to the *M. fortuitum* plasmid pUS300 (24). The physical map of the mycobacterial insert of pRP5000 is shown in Fig. 1. The 4.6-kb EcoRI insert was isolated and hybridized with *PvuII*-digested genomic DNA from different mycobacterial species. Multiple hybridizing bands were observed in DNA from *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis* BCG, and *M. microti*, whereas the insert did not hybridize with strains of *M. leprae*, *M. avium*, *M. fortuitum*, *M. gordonae*, *M. intracellulare*, *M. kansasi* (Fig. 2A), *M. flavescens*, *M. malmoense*, *M. phlei*, *M. scrofulaceum*, *M. paratuberculosis*, *M. chelonae*, *M. smegmatis*, or *M. perigrinum* (data not shown). These results indicate that the occurrence of the IS986-like elements is restricted to strains belonging to the *M. tuberculosis* complex group.

**Polymorphism of restriction fragments containing IS986.** All 14 *M. tuberculosis* complex strains shown in Fig. 2A contained multiple DNA fragments which hybridized with the 4.6-kb insert of pRP5000, which is consistent with the previous findings of Zainuddin and Dale (24). Furthermore, each strain displayed a different, unique banding pattern. To demonstrate that this RFLP is a special property of the fragments containing the IS986 element, we rehybridized the same blot with the 2.4-kb EcoRI fragment of pPH7301. This plasmid contains another repeated sequence that is present in the *M. tuberculosis* complex species *M. gordonae* and *M. kansasi* (12a). In contrast to the hybridization patterns obtained with the 4.6-kb EcoRI fragment of pRP5000, virtually identical banding patterns were observed among the different *M. tuberculosis* complex strains (Fig. 2B). Sequence data showed that IS986 is located between two *BspMII* sites (McAdam et al., 24) which are unique sites in the inverted repeats of this insertion sequence element (Fig. 1). To confirm that the RFLP is due to restriction fragments that contain IS986, we used the 386-bp *BamHI*-XhoI fragment located within the insertion element of pRP5000 as a probe for the hybridization of *PvuII*-digested chromosomal DNA of the same mycobacterial
strains used in Fig. 2. As expected, this small fragment did not hybridize with species other than those of the M. tuberculosis complex. The banding patterns obtained with the M. tuberculosis complex strains were similar but not identical to those obtained with the 4.6-kb EcoRI probe (Fig. 3). This is consistent with the idea that the RFLP is due to the presence of IS986 at different sites in the genome of the various strains that we analyzed.

**IS986 in M. bovis BCG strains.** As shown in Fig. 3, lane 12, only one fragment in M. bovis BCG hybridized with the 386-bp IS986-specific DNA probe. This indicates that IS986 is present as a single copy in this clinical isolate. We examined six additional M. bovis BCG strains, both vaccine strains as well as clinical isolates. All M. bovis BCG strains tested were found to contain a unique 1.7-kb PvuII fragment that hybridized with the 386-bp fragment IS986 (Fig. 4). We conclude that IS986 is present as a single copy in all M. bovis BCG strains and that it is inserted at the same location in the genome.

**Specific DNA amplification of IS986 by PCR.** Based on the sequence of IS986 (McAdam et al., submitted), two oligonucleotides, INS-1 and INS-2, were synthesized. These oligonucleotides were used as primers for amplification by PCR of chromosomal DNA corresponding to residues 641 to 885 of IS986. As expected, after amplification of either M. tuberculosis DNA or pRP5000, a fragment of approximately 245 bp was visible on agarose gels (Fig. 5A). Chromosomal DNAs of M. africanum, M. bovis, M. bovis BCG, and M. microti were also found to contain the 245-bp amplifiable fragment. Furthermore, chromosomal DNAs from 22 M. tuberculosis strains, 6 M. bovis BCG strains, and 1 M. bovis strain were tested by PCR and all were shown to contain the 245-bp amplifiable fragment (data not shown). In contrast, no such fragment was found in M. avium, M. kansasii, M. fortuitum, M. scrofulaceum, M. smegmatis, or M. leprae.

**As a positive control for successful DNA amplification in vitro, the two oligonucleotide primers R and S, which are homologous to the conserved regions of the procaryotic 16S rRNA genes (5, 19), were used to test the genomic DNA isolates.** This resulted in the amplification of a 426-bp fragment from all mycobacterial species mentioned above (Fig. 5B).

**Genetic stability of IS986 during animal passage.** The high degree of RFLP in IS986-containing sequences of M. tuberculosis complex strains suggests that this insertion element could transpose with a high frequency. To investigate whether genetic rearrangement of IS986 occurs after passage in animals susceptible to M. tuberculosis, we analyzed the restriction fragment patterns of four strains before and after passage during 2 months in guinea pigs. The results are shown in Fig. 6. No changes in the banding pattern were observed.
Twenty-three different banding fragments in that legend to Fig. 3. Numbers on the left indicate sizes of standard DNA fragments in kilobase pairs.

observed, indicating that IS986 is stable during a limited number of generations in vivo.

**Use of IS986 for epidemiological purposes.** The observed RFLP caused by IS986 suggests the possibility that IS986 can be used for precise epidemiological investigations. Therefore, we tested an additional 15 clinical isolates of *M. tuberculosis*. These isolates were obtained from various regional hospitals and laboratories in The Netherlands. A total of 6 of the 15 strains were not known to originate from epidemiologically related cases of tuberculosis, and these strains showed different PvuI banding patterns when the 386-bp IS986 probe was used (data not shown). This is in marked contrast to the remaining nine isolates, which exhibited a mutually identical restriction fragment pattern (Fig. 7, lanes 1 to 9). These nine isolates originated from an outbreak of tuberculosis among individuals who were all treated by the same physician, who specialized in the treatment of patients with arthritis. The identities of the banding patterns from these isolates from a known outbreak indicate the potential value of IS986 as a probe for such studies. Further confirmation of the usefulness of this probe was provided by a study of 26 *M. tuberculosis* isolates which were obtained from a single regional health laboratory in January 1990; at that time, these isolates were not known to be related. Twenty-three different banding patterns were found among these isolates (Fig. 8). The banding patterns of two strains (Fig. 8, lanes 1 and 14) were identical to those shown in Fig. 7. Investigation of the patients' histories revealed that they could be traced to the same outbreak. An additional three strains (Fig. 8, lanes 15, 24, and 25) also showed characteristic banding patterns which were not related to the patterns of the strains involved in the outbreak. Case histories of these patients revealed that they were suspected of being contacts of a common source of infection. The application of the probe in this case was therefore successful in revealing a previously unsuspected cluster of cases of tuberculosis.

**DISCUSSION**

As repetitive segments of DNA are found in virtually all procaryotic and eucaryotic organisms (8), it is not surprising that these elements have recently also been identified in mycobacteria. Clark-Curtiss and colleagues (2, 3, 12) found a 2.2-kb sequence that was present in at least 19 copies per *M. leprae* genome but that was not present in other mycobacterial species. Another species-specific repetitive DNA element in *M. paratuberculosis* was identified by Green et al. (11). This repeated sequence has significant homology with IS110, an insertion element of *Streptomyces coelicolor*. Various strains of *M. leprae* and *M. paratuberculosis* have been analyzed for RFLP by using the repetitive DNA as a probe, and the nonvariability of the banding patterns indicated that these elements were inserted at a specific position in the *Mycobacterium* chromosome. In *M. tuberculosis*, repetitive DNA elements have been found that are inserted at a specific location in the genome (12a), whereas others were found to differ in genomic position from strain to strain (6, 15, 24). In this study, we exploited the latter property of
The putative insertion sequence IS986. This element is 1,386 bp in size and is flanked by inverted repeats that are homologous to those of IS3 and IS3411. Furthermore, IS986 contains an open reading frame, which encodes a putative transposase, which is significantly homologous in its amino acid sequence to the transposases of the IS3 family of enterobacterial insertion elements (McAdam et al., submitted). A similar but not identical element was recently sequenced by Thierry et al. (20).

In this study, we showed that IS986-like elements are present in 1 to 20 copies per genome and that these elements are specific for the species of the M. tuberculosis complex. We extend the preliminary observations of Zainuddin and Dale (24) that these elements differ in their chromosomal location from strain to strain. The patterns of the restriction fragments containing IS986 in all 32 independent isolates of M. tuberculosis strains were different. Such an extreme degree of polymorphism reinforces the idea that IS986 is a functional insertion element that can insert to new sites on the same replicon and that can initiate other types of genetic rearrangements such as cointegration of replicons, inversion, and deletion of sequences adjacent to the insertion sequence element (8). The latter type of genetic event has recently been suggested by Hermans et al. (12a) to be a consequence of the activity of another repetitive DNA element in M. tuberculosis. This study shows that the high degree of polymorphism of IS986-containing fragments is very useful as a tool in determining the epidemiology of tuberculosis. All strains from an epidemiologically well-defined outbreak of tuberculosis were of the same characteristic RFLP type. Furthermore, in a collection of 26 strains obtained from a peripheral laboratory in the southern part of The Netherlands, we could identify two strains with this RFLP pattern. Later, it appeared that these strains originated from patients involved in the same outbreak mentioned above. Also, three strains with another characteristic RFLP pattern were recognized, and these strains were suspected of being contacts with individuals involved in a single tuberculosis outbreak. We are in the process of determining the RFLP types of all M. tuberculosis strains that are sent to our reference laboratory. By computer analysis of a library of previously established RFLP patterns, one might be able to trace sources of M. tuberculosis from the distant past.

An interesting observation was the nonpolymorphic pattern of IS986-containing restriction fragments of M. bovis BCG. This derivative was obtained in 1921 from a pathogenic Mycobacterium strain that lost its virulence gradually during passages in liquid medium (1). Since then, many substrains of M. bovis BCG have been used to prepare vaccines around the world, and these substrains are heterogeneous in many of their characteristics. As all strains investigated in this study were of the same RFLP type and contained a single copy of IS986 in their genomes, all M. bovis BCG isolates are likely to contain a single IS986 copy at one particular location in the chromosome. This suggests that IS986 lost its transposition capability before or during the 230 passages of the parental strain of M. bovis BCG and that all substrains are identical with respect to the site of insertion of IS986 in the chromosome. This unique location in all M. bovis BCG strains will be useful in distinguishing M. bovis BCG from other mycobacteria. We will investigate whether there is a correlation between the loss of virulence of M. bovis BCG and the location of IS986 in the chromosome. The high polymorphism of fragments containing IS986 among clinical isolates of M. tuberculosis complex strains suggests either that IS986 changes frequently in its position in the chromosome or that IS986 is relatively stable but that...
the clinical isolates examined in this study have a long history of separate lineage. Our observations that no polymorphism was observed among strains from single outbreaks and that serial passage in guinea pigs does not lead to substrains with IS986 at a different chromosomal location point toward the latter possibility and suggest that IS986 is a relatively stable element which transposes with a low frequency.

Because IS986 is found only in \textit{M. tuberculosis} complex strains, this element is a useful target for in vitro amplification by PCR for the rapid and specific detection of such bacteria in clinical specimens, without the need for culturing these slowly growing mycobacteria.

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


