Phospholipase Region of *Mycobacterium tuberculosis* Is a Preferential Locus for IS6110 Transposition

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Enzymes with phospholipase C activity in *Mycobacterium tuberculosis* have been recently described. The three genes encoding these proteins, *plcA*, *plcB*, and *plcC*, are located at position 2351 of the genomic map of *M. tuberculosis* H37Rv and are arranged in tandem. We have previously described the presence of variations in the restriction fragment length polymorphism patterns of the *plcA* and *plcB* genes in *M. tuberculosis* clinical isolates. In the present work we investigated the origin of this polymorphism by sequence analysis of the phospholipase-encoding regions of 11 polymorphic *M. tuberculosis* clinical isolates. To do so, a long-PCR assay was used to amplify a 5,131-bp fragment that contains the *plcA* and *plcB* genes and part of the *plcC* gene. In the *M. tuberculosis* strains studied the production of an amplicon ~1,400 bp larger than anticipated was observed. Sequence analysis of the PCR products indicated the presence of a foreign sequence that corresponded to an IS6110 element. We observed insertion elements in the *plcA*, *plcB*, and *plcC* genes. One site in *plcB* had the highest incidence of transposition (5 out of 11 strains). In two strains the insertion element was found in *plcA* in the same nucleotide position. In all the cases, IS6110 was transposed in the same direction. The high level of transposition in the phospholipase region can lead to the excision of fragments of genomic DNA by recombination of neighboring IS6110 elements, as demonstrated by finding the deletion, in two strains, of a 2,837-bp fragment that included *plcA* and most of *plcB*. This can explain the negative results obtained by some authors when detecting the *mtp40* sequence (*plcA*) by PCR. Given the high polymorphism in this region, the use of the *mtp40* sequence as a genetic marker for *M. tuberculosis sensu stricto* is very restricted.

The *mtp40* gene was first described as a 402-bp open reading frame (ORF) encoding a 13.8-kDa specific protein of *Mycobacterium tuberculosis* (21). This gene was cloned in a 3.1-kbp *Bam*HI fragment, and, after sequencing the whole insert, Leão et al. noted the presence of an ORF of 1,170 bp and the beginning of another (15). Johansen et al. (13) completely sequenced the second ORF, and they also demonstrated in vitro that these genes encode phospholipase C activities. They named the ORFs *mpcA* and *mpcB*. The sequence called *mtp40* actually constitutes only a part of the *mpcA* gene. After the whole *M. tuberculosis* H37Rv genome was sequenced, two more phospholipase genes were described: an ORF beside *mpcB* and another related sequence at position 1755 of the genome, located beside an IS6110 element (4). From this point on these genes were designated *plc* (phospholipase C) genes; the three ORFs arranged in tandem were called *plcA*, *plcB*, and *plcC*, and the fragment at position 1755 in *M. tuberculosis* H37Rv was called *plcD*. We use this nomenclature in the present work.

Since there have been conflicting results concerning the presence of the *mtp40* sequence in the *M. tuberculosis* complex, we previously studied its distribution within a collection of *M. tuberculosis* clinical isolates. PCR amplification of the *mtp40* region revealed that some strains were negative for this sequence (28). To rule out the presence of mutations or dele-

tions in the primer annealing sites that cause a false-negative result, we carried out Southern blot assays using the *Pvu*II enzyme and a PCR product corresponding to *mtp40* as a probe. We observed that *M. tuberculosis* H37Rv and H37Ra presented two bands: one of 0.75 kbp, which we demonstrated to correspond to *plcA*, and one of 2.1 kbp that corresponds to *plcB* and that cross-reacts with the probe for *mtp40* (which is part of *plcA*). We also found strains presenting variations of this pattern, showing extra bands or a shift in the molecular weight of the band corresponding to the *plcB* gene from 2.1 to 2.5 kbp (28). Some other clinical isolates presented changes in both bands or were negative in the Southern blot analysis.

To explain the changes in the restriction fragment length polymorphism (RFLP) patterns, in this work we studied by long PCR the phospholipase-encoding regions of selected strains followed by sequence analysis of the amplicons.

MATERIALS AND METHODS

Bacterial strains. Most of the *M. tuberculosis* strains used in this study were obtained from the National Reference Centre for Tuberculosis of the Laboratories for Health Canada (Winnipeg, Canada) and were identified by conventional methods. All the strains were maintained at –70°C in skim milk and subcultured on Lowenstein-Jensen medium when needed. DNA samples from two strains, which we named RIVM-7 and RIVM-13 were kindly donated by Kristin Kremer from the National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands (14).

Genomic-DNA extraction. The mycobacteria were heat killed at 85°C for 30 min, and the DNA was extracted in accordance with a technique using cetyltrimethylammonium bromide-NaCl (31). The DNA was suspended in Tris-EDTA buffer, quantified, and stored at 4°C until use.

Southern blot assay. For Southern blotting of clinical isolates, 2 µg of genomic DNA was digested with 5 U of *Pvu*II (28) for 4 h at 37°C. The electrophoretic

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TABLE 1. Oligonucleotide primers used in this work

Primer	Sequence	Nucleotides
TB20	5' CGC AGC AAC ACC CTT ATC AAG T	19275–19296 ^a
TB21	5' GTG ATT GTC GGC GAA ATG AAG T	24406–24385 ^a
TB25	5' CTC CGG CGG GTA CCT CCT CG	90–71 ^b
TB26	5' AGG CTG CCT ACT ACG CTC AAC G	1272–1293 ^b
INS-1	5' CGT GAG GGC ATC GAG GTG GC	633–652 ^c
INS-2	5' CGT AGG CGT CGG TGA CAA A	876–858 ^c

^a Sequence MTCY98, accession no. Z83860.^b Sequence accession no. X17348.^c Reference 25.

separation of the digested fragments was done in a 20- by 25-cm 0.8% agarose gel by applying 30 V overnight. After electrophoresis, the DNA samples were transferred to a nylon membrane using the Turbo blotter system (Schleicher & Schuell, Keene, N.H.) in accordance with the manufacturer's directions. The blot was prehybridized and then probed overnight at 42°C with peroxidase-labeled amplicons prepared with the enhanced chemiluminescence kit (ECL; Amersham, Arlington Heights, Ill.). Hybridization, washing, and development of the filters were performed according to the manufacturer's instructions.

As a probe we used a PCR product with primers PT1 and PT2, which amplify a region of *plcA* (6). To determine the phylogenetic relationships among some of the studied strains that presented identical insertions, we incubated the blots with a PCR probe derived from primers INS-1 and INS-2, which amplify a fragment of 243 bp in the *IS6110* right arm.

Synthesis of oligonucleotide primers and sequence analysis of the amplicons. The oligonucleotides used in this study (Table 1) were prepared on a 392 DNA-RNA synthesizer (Applied Biosystems, Foster City, Calif.) utilizing the standard phosphoramidite method. The sequences of the PCR products were determined with the Prism Dye Terminator sequencing kit (Applied Biosystems) in an ABI 377 automated sequencer.

Long-PCR assay. To determine the genetic changes that lead to the polymorphism in the phospholipase region, we designed a pair of primers located 1,000 bp outwards of the *plcA* or *plcB* genes, which we called TB20 and TB21, respectively (Table 1). The predicted size of the amplicon was 5,131 bp. The PCR assay was carried out with 100 ng of genomic DNA in a PTC-200 thermocycler (MJ Research, Watertown, Mass.) by utilizing PCR assay kit XL (Perkin-Elmer) under the following conditions: 94°C for 2 min and 10 cycles of 94°C for 15 s, 60°C for 30 s, and 68°C for 4 min. A second round of 20 cycles was carried out at 94°C for 15 s, 60°C for 30 s, and 68°C for 4 min, adding 20 s every cycle. A final extension step at 68°C for 10 min was performed. The PCR products were applied to a 0.8% low-melting-point agarose gel, and after the electrophoresis the gel slices containing the bands were excised and purified utilizing the Gene-Clean III (BIO 101, Inc., Vista, Calif.) kit. The DNA was quantified spectrophotometrically and stored at 4°C.

RESULTS

Southern blot analysis with PT1 and PT2. The Southern blot patterns of the strains with the *plcA* probe used in this study are presented in Fig. 1. According to the restriction map of the region (not shown), the 0.75-kbp band corresponds to the *plcA* gene; the 2.1-kbp band corresponds to *plcB* and part of *plcC*. In Fig 1, we observe that strains in lanes 4 to 6 and 8 to 12 lack the 2.1-kbp band corresponding to *plcB*; instead they have bands of different sizes. Strains in lanes 2, 3, 7, and 8 lack the 0.75-kbp band that corresponds to the *plcA* gene. Strains 9 to 12 present identical RFLP patterns, with a band of 2.5 kbp instead of the normal *plcB* band of 2.1 kbp, as well as another band of about 1.0 kbp and the 0.75-kbp band. By using a probe for *plcB* we observed that the 2.5-kbp band corresponds to this gene (data not shown). The strain in lane 8 presents only a band of about 2.8 kbp. As a control (lane 1) we used the *M. tuberculosis* 14323 strain, kindly donated by J. D. A. van Embden, which is used worldwide as a control for *IS6110* studies.

FIG. 1. Southern blot analysis of the *M. tuberculosis* strains studied in this work. The blots were incubated with a PCR probe, prepared with primers PT1 and PT2 as described before (28), that amplifies a 396-bp region near the 3' end of the *plcA* ORF. Lanes: 1, *M. tuberculosis* strain 14323; 2, Dr-561; 3, RIVM-7; 4, RIVM-13; 5, Dr-351; 6, Dr-468; 7, Dr-194; 8, Dr-494; 9, Dr-116; 10, Dr-169; 11, Dr-170; 12, Dr-342.

Sequencing analysis of the TB20-TB21 amplicons. When amplifying genomic DNA from the polymorphic clinical isolates with primers TB20 and TB21, we observed the presence of amplicons bigger than those produced by control strain *M. tuberculosis* H37Rv (Fig 2). Other bands of less intensity were also observed. After sequencing analysis of some of these bands we concluded that they corresponded to less-specific annealing sites for the primers. First, we did the sequencing analysis with primers TB20 and TB21 and observed in one strain an *IS6110* element at the end of an amplicon produced with TB20 and TB21 (TB20-TB21 amplicon). To simplify the analysis, we then performed the sequencing analysis using primers TB25 and TB26, which anneal to a region close to the end of the *IS6110* sequence. These primers are directed outward in such way that, when performing the sequencing PCR, we could detect the insertion site in one run.

In Fig. 3 we describe the sequences at the junction between the insertion elements and the genomic mycobacterial DNA. We observed the duplication of three or four nucleotides at the site of the transposition. Interestingly in strain RIVM-7 there is only the duplication of two nucleotides and the duplicated nucleotides remained on one side of *IS6110*. These results were confirmed by preparing the amplicons and performing the sequencing analysis in duplicate.

FIG. 2. Long-PCR assay of mycobacterial genomic DNA from polymorphic strains for the phospholipase genes with primers TB20 and TB21. Lanes: 1 and 10, 1-kb ladder (Gibco); 2, RIVM-7; 3, Dr-169; 4, Dr-170; 5, Dr-342; 6, Dr-351; 7, Dr-561; 8, Dr-468; 9, *M. tuberculosis* H37Rv.

FIG. 3. Locations of the IS6110 elements inserted in the phospholipase regions of the *M. tuberculosis* strains studied in this work. At the right are positions of the insertion elements in the phospholipase locus. Shaded boxes, duplicated nucleotides. The nucleotide numbers are taken from GenBank sequence MCTY 98 (accession no. Z38360). For comparison purposes, sequence data from strain Dr-194, which have been published before, are shown (28). IR_R and IR_L, right and left imperfect repeats, respectively.

Instead of producing an amplicon bigger than that from *M. tuberculosis* H37Rv with TB20 and TB21, a smaller PCR fragment was obtained in strains Dr-494 and Dr-426. By sequencing analysis we observed, in both strains, that the right imperfect repeat of IS6110 was anchored on nucleotide 20569 (*plcB* gene) and that the left repeat was anchored on nucleotide 23406 (*plcA*), with the loss of a 2,837-bp fragment (Fig. 3). Interestingly no direct repeats were found at the ends of the IS6110 elements. These sequence analysis findings were corroborated by digestion of the TB20-TB21 amplicon with *Pvu*II (data not shown), as mentioned below.

To confirm that the changes in the Southern blot patterns are only due to the insertion of the IS6110 element, we gel purified the amplicons and digested them with *Pvu*II. In Fig. 4 we show the map with the predicted changes as well as the electrophoretic separation of the digested amplicons. In all the cases there was concordance between the expected and the obtained fragments.

Since one explanation of the selection of the same insertion site could be that the strains actually belong to the same clone, we analyzed the RFLPs for IS6110 in those strains showing identical insertion sites. In Fig. 5A we show the Southern blot analysis of two of the most closely related strains. Both are isolates from British Columbia, Canada, and present nine similar bands. It is possible they have the same ancestor. On the other hand, although strain RIVM-7 from Mongolia and strain Dr-561 from Alberta, Canada (Fig. 5B), have the IS6110 element inserted in the same nucleotide position in *plcA*, they seem to be unrelated.

In all the strains studied in this work we found the IS6110 elements transposed in the same direction.

DISCUSSION

IS6110 has a 1,361-bp sequence with a 28-bp imperfect repeat at the ends (25, 26). This insertion element belongs to the IS3 family of mobile elements and is widely distributed in the *M. tuberculosis* complex members. IS6110 polymorphism is currently used as a genetic target to differentiate individual

clones of *M. tuberculosis*, because they can contain from 0 to 25 copies distributed in the entire genome (20, 27). Although it is considered that IS6110 transposes randomly, it is rarely found in the first quarter of the *M. tuberculosis* H37Rv circular map (4), which indicates a certain form of selection of the insertion sites. This is demonstrated also by the observation of "hot spots" for IS6110 transposition in several sites. The first is the direct repeat locus, which is composed of directly repeated sequences of 36 bp separated by nonrepetitive segments of 36 to 41 bp (12). Other high-frequency locations of IS6110 are the *ipl* locus, which is itself located in an insertion element-like element, IS1547 (7), and the DK1 site (10).

In this work we found IS6110 elements distributed along the phospholipase region; however this distribution was not random. It seems likely that even in the phospholipase genes there are preferential sites for the IS6110 insertion, such as nucleotides 20569 and 22124. In *M. tuberculosis* H37Rv and H37Ra there is an IS6110 interruption of the *plcD* gene (2), and other *M. tuberculosis* strains demonstrate similar insertion elements at the identical position (22). These data support the idea that there are hot spots, which exist in the *M. tuberculosis* genome and within the phospholipase genes specifically, that attract IS6110 insertion elements. Only by doing a study involving a great number of strains bearing IS6110 elements can we determine if there are consensus sequences within the phospholipase genes that stimulate IS6110 transposition.

During transposition, 3 or 4 bp of the DNA sequence at the insertion site is duplicated by the repair and filling mechanisms of the nick produced during this event (24). In the amplicon derived from RIVM-7 DNA, we did not observe direct repeats at the ends of the IS6110 element; instead we observed the duplication of two nucleotides in one of the sides. The absence of flanking direct repeats can be an indication of recombination mediated by insertion elements (16), which usually produces the deletion of the region between the elements. For RIVM-7 there was not such a deletion, which indicates the possible existence of other reparation or transposition mechanisms.

IS6110 transposition in high-preference sites such as the *ipl* locus has been found to produce the excision of neighboring DNA fragments (8, 9), possibly by homologous recombination between two adjacent IS6110 elements oriented in the same direction, as proposed by Fang et al. (9). Several regions of *M. tuberculosis* H37Rv (RvD2, RvD3, and RvD5 regions), ranging in size from 0.8 to 4 kbp (2), have also been attributed to DNA excised during IS6110 transposition. In a previous study (28) we observed that some strains did not hybridize with the probes for *plcA* and *plcB*. The excision of part of the phospholipase genes by IS6110 recombination can explain the lack of these genes in some *M. tuberculosis* strains described by us and others (29). Although initially the *mtp40* sequence inside *plcA* was considered to exist only in *M. tuberculosis*, and thus was used to identify *M. tuberculosis* sensu stricto, it appears that these genes are very mobile and unstable, and this may restrict their clinical use as genetic markers.

Our data suggest that the phospholipase genes seem to attract IS6110 transposition. Thus the presence of an IS6110 element in two phospholipase genes at a time or in nearby genes (such as the neighboring cutinase or PE or PPE gene, all of which have also been found to attract IS6110 elements) (22)

M. tuberculosis strains

FIG. 4. Internal *PvuII* restriction sites of the TB20-TB21 amplicon. (Top) Map of the expected fragments obtained from amplicons derived from *M. tuberculosis* H37Rv strain and some of the polymorphic strains according to the *PvuII* restriction sites and the position of the inserted *IS6110* element in each strain. (Bottom) A 1.5% agarose gel with the digested amplicons. Lanes: 1, *M. tuberculosis* H37Rv; 2, Dr-468; 3, Dr-170; 4, Dr-342; 5, RIVM-7; 6, Dr-169; 7, Dr-561; 8, Dr-351; lane 9, 100-bp ladder (Gibco). Molecular sizes of the fragments obtained from *M. tuberculosis* H37Rv are at the top.

can produce excision and may lead to the loss of phospholipase sequences and, ultimately, function. This is also supported by the presence in *M. tuberculosis* H37Rv of a phospholipase gene (*plcD*) (4) that is interrupted by an *IS6110* element. In the present study, two strains of *M. tuberculosis* were observed to produce 2.8-kbp excision fragments of the phospholipase genes. This could have been due to recombination of two *IS6110* elements since, as mentioned above, *IS6110* did not present direct repeats, and this is evidence of recombination between IS elements (16). It is possible that transposition of *IS6110* elements mediates the mobilization or duplication of these genes, producing strains with no phospholipase genes,

fragments of the genes, or extra bands produced by duplication, such as those strains belonging to group C (28). We are currently working on the characterization of the *M. tuberculosis* strains lacking the entire phospholipase locus; data from this work can help us to explain the mechanisms of the loss of DNA in this region.

The change in sequence divergence has been found useful in establishing and calibrating molecular clocks. Changes in the *IS6110* pattern have been observed to occur over 1 or 2 years (3, 5). We observed in related *M. tuberculosis* strains minimal changes in the *IS6110* patterns but radical changes in or the complete loss of the phospholipase genes (28). It is possible that environmental (11) or culture conditions may rapidly induce these changes in certain genomic areas, particularly in those where there are several *IS6110* elements separated by small distances.

It has been claimed that phospholipases play an important

FIG. 5. Southern blot analysis of some of the *M. tuberculosis* polymorphic strains showing identical insertion sites probed with an IS6110 right-arm PCR probe. (A) Lane 1, Dr-169; lane 2, Dr-170. (B) Lane 1, Dr-561; lane 2, RIVM-7.

role in virulence, either by producing tissue damage, as for the alfa toxin of *Clostridium perfringens* (30) or *Pseudomonas aeruginosa* phospholipase (19), or by allowing the escape of the microorganism from the phagolysosome to live freely in the macrophage cytoplasm (23). Recently, phospholipase C and D and sphingomyelinase activities have been detected in *M. tuberculosis* (13). *M. tuberculosis* phospholipase proteins resemble those encoded by *P. aeruginosa* *plcH* and *plcS* genes, which contribute to the virulence of this opportunistic lung pathogen. The phospholipase role in intracellular survival and as a virulence factor has been probed in vitro and in vivo by studying the effect of deletions of *Listeria monocytogenes* phospholipases encoded by *plcA* and *plcB*. Indeed a Δ *plcA-plcB* double mutant lost its ability to escape from the phagocyte and to spread from cell to cell (23). The production of hemolytic plaques of this microorganism was reduced to nearly 70% of that for the wild type and the mutant was 500-fold less virulent than wild-type bacteria in mice, which suggests a role for phospholipases as a virulence factor. It is possible that the *M. tuberculosis* cluster comprising *plcA*, *plcB*, and *plcC* can have a similar role in pathogenesis. It is important to note that *M. bovis* lacks this cluster of genes (1). The clinical diseases produced by *M. tuberculosis* and *Mycobacterium bovis* are indistinguishable. However, it has been observed that *M. bovis* has a decreased ability to reactivate and spread from person to person (18). Since phospholipase activity in other microorganisms has an important role in their virulence, it is possible that this activity confers to *M. tuberculosis* the ability to survive intracellularly in macrophages and therefore to grow and spread to other cells or tissues.

Recently, Miller and Shinnick (17) reported that *Mycobacterium smegmatis* cells complemented with PCR-generated

plcA and *plcB* genes from *M. tuberculosis* did not show an increased rate of intracellular survival in THP-1 macrophages in comparison with wild-type bacteria. However, these results do not rule out a possible involvement of *plc* genes in the whole mechanism of pathogenesis of tuberculosis, as these genes may be involved in processes interacting with other factors present in *M. tuberculosis* but not in *M. smegmatis*, which is a limitation of that assay.

M. tuberculosis strains with naturally knocked out genes, such as those described in this paper, as well as strains lacking the complete cluster of phospholipase genes, can constitute a good model to study the role of these enzymes in *M. tuberculosis* pathogenesis.

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