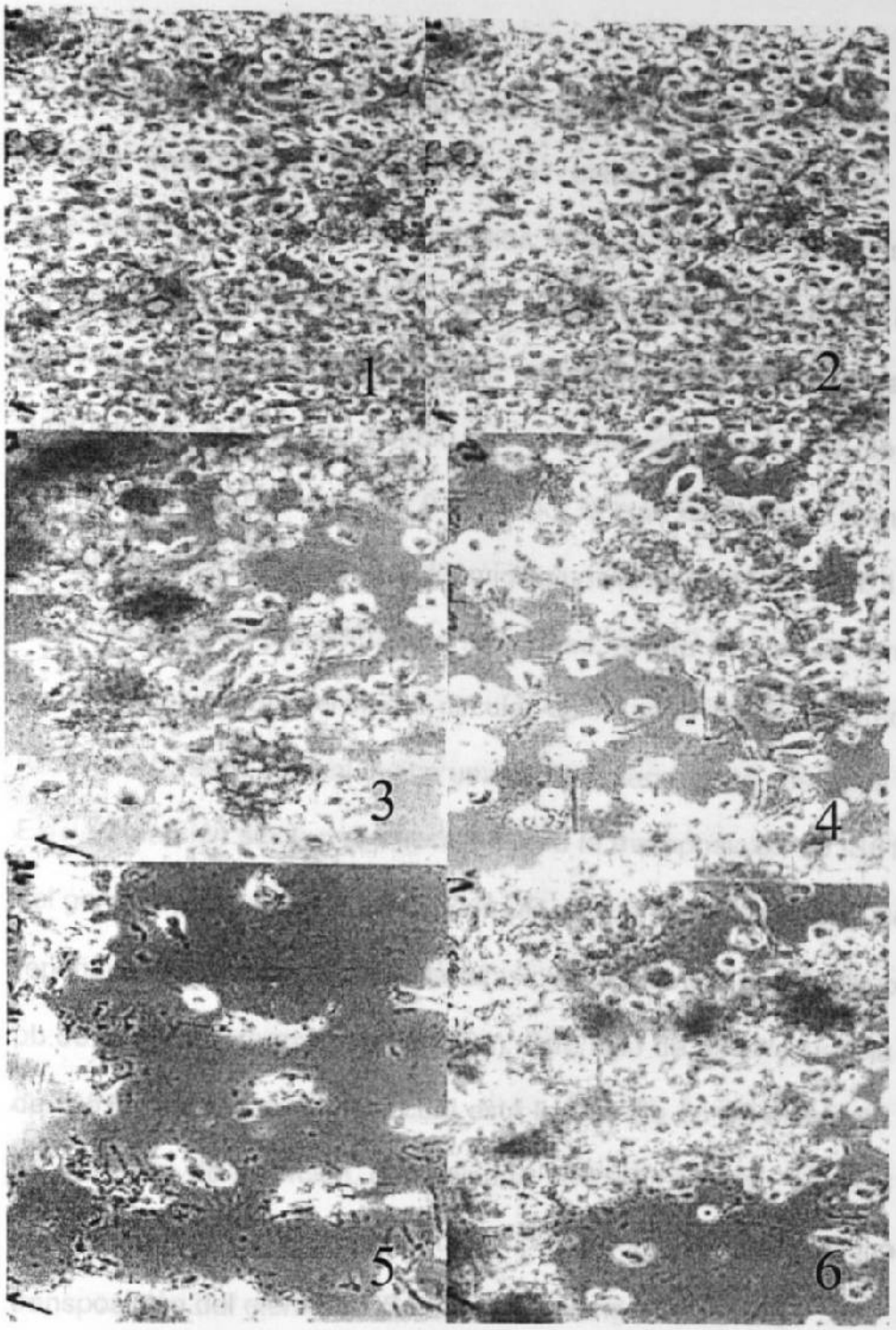

Figura 20.- Células de la línea THP-1 infectadas con *M. tuberculosis* a un multiplicidad de infección 1:1 (bacterias: células). Las fotografía 1 y 2 correponden a las células control sin infectar, las fotos 3, 4, 5 y 6 corresponden a las cepas DR-342, DR-494, H37Rv y Dr-689, respectivamente. Observadas a las 72 h de incubación por microscopía, a una ampliación de 250X.

4.- DISCUSIÓN

El elemento IS*6110* de *M. tuberculosis* pertenece al grupo de elementos móviles descritos en *Escherichia coli* como IS3, los cuales tienen como caracteristica una secuencia repetida imperfecta de 28-pb en sus extremos (Thierry *et al.*,1990; Otal *et al.*,1991; Lewis, 1996). El elemento *IS6110*, mide 1361-pb, y es la secuencia de inserción mejor caracterizada de *M. tuberculosis*. Existe un diverso número de copias en las cepas de este microorganismo que varia de 0 a 25, por lo cual es utilizada como un sistema de tipificación para estudios epidemiológicos (Van Embden *et al.*,1993).

Aunque se considera que IS*6110* se inserta al azar, es raro encontrarlo en el primer cuarto del mapa circular de la cepa de *M. tuberculosis* H37Rv (Cole *et al.*,1998), lo cual indica una cierta selección de sus sitios de inserción. Existen también sitios preferenciales para la transposición de IS*6110* a lo largo del genoma, uno de ellos es el locus DR, el cual está compuesto de secuencias repetidas directas de 36-pb separadas por segmentos no repetitivos de 36 - 41-pb de longitud (Hermans *et al.*,1991). Otro sitio de alta frecuencia de inserción de IS*6110* es el locus *ipl*, el cual está localizado en un elemento semejante a un elemento de inserción, denominado 1S*1547* (Fang y Forbes. 1997). El sitio DK1 también ha sido reportado como un sitio seleccionado para la transposición del elemento IS*6110* (Fomukong *et al.*,1997).

Los elementos IS*6110* encontrados en el presente trabajo se encuentran distribuidos en la región de los genes de PLC. Posiblemente esta distribución no es completamente al azar en este locus; al parecer existen sitios

preferenciales para la inserción de IS*6110* en la región de fosfolipasas, como lo son las posiciones 20569 en el gen *plc*B y 22124 del gen *plc*A encontradas en el presente trabajo. En las cepas de *M. tuberculosis* H37Rv existe un elemento IS*6110* interrumpiendo al gene *plc*D de fosfolipasa (Brosch,,1999). Otras cepas de *M. tuberculosis* también presentan un elemento de inserción en el gene *plc*D en la misma posición (Sampson *et al.*,1999). Estos datos parecen apoyar la idea de que existen secuencias altamente atractivas dentro de los genes *plc* para los elementos de inserción, convirtiendo esta zona en un sitio preferencial. Es necesario hacer un estudio con un gran número de cepas conteniendo elementos IS*6110* para determinar si existe una secuencia consenso involucrada en la transposición de IS*6110* en los genes *plc*.

Los elementos transponibles IS*6110* han sido propuestos como responsables en la evolución del genoma de *M. tuberculosis*, actuando como agentes de mutaciones adaptativas (Ho *et al.*,2000). Se ha observado que cambios en el medio ambiente, como la reducción en la tensión de oxígeno puede estimular la transposición de este elemento, provocando re-arreglos del genoma (Ghanekar *et al.*,1999). Una situación semejante puede darse en los granulomas, donde existe una reducción de oxígeno, cambios de pH y limitaciones de nutrientes, favoreciendo las condiciones para la transposición del elemento IS*6110* (Ghanekar *et al.*,1999; Brosch *et al.*,2000; Li *et al.*,2002). La transposición del elemento IS*6110* dentro de regiones que codifican proteinas es más común (64%) de lo que se sugería (Sampson *et al.*,1999). El impacto de IS*6110* en el fenotipo de *M. tuberculosis* depende del sitio donde se inserte y se ha observado sobre todo en genes con aparente redundancia

funcional, lo que indicaría que genes con varias copias pueden compensar las pérdidas naturales creadas por la inserción de IS*6110* en alguno de ellos. Por ejemplo, la familia de genes denominados PE y la familia de genes PPE (genes que codifican proteínas ricas en glicina y alanina que se expresan en la superficie extracelular), están dispersos en múltiples copias en todo el genoma y son una fuente importante de variación debido a que son considerados antígenos potenciales para la inmunidad del huésped (Fleischmann *et al.*,2002).

Durante la transposición del elemento IS*6110* tres o cuatro pares de bases de la secuencia de ADN en los sitios de inserción se duplican por mecanismos de reparación y llenado del espacio producido por este evento (Spielmann-Ryser *et al.*,1991) lo cual es típico de este fenómeno. En la cepa de *M. tuberculosis* RIVM7 no se observó esta repetición de nucleótidos en los extremos del elemento IS*6110*; en su lugar se encontró la duplicación de dos nucleótidos en uno de los extremos. La ausencia de secuencias repetidas a los lados de la inserción del IS*6110* es indicativa de una recombinación mediada por estos elementos (Mahillon y Chandler. 1998), lo cual produce la supresión de la región entre los dos elementos. En el caso de la cepa de *M. tuberculosis* RIVM7 no existe tal supresión, lo que indica la posible existencia de otros mecanismos de reparación ó de transposición.

Se ha reportado que la transposición de IS*6110* en sitios de alta preferencia tales como el locus *ipl* produce la pérdida de fragmentos de ADN vecinos por recombinación homóloga de dos elementos IS*6110* adyacentes y orientados en la misma dirección como lo propusieron Fang y col. (Fang *et*

al.,1998). También se encontró que la pérdida de varias regiones de *M. tuberculosis* H37Rv, tales como RvD2, RvD3 y RvD5 con tamaños que van de 0.8- a 4-kpb son debidas a la escisión de fragmentos de ADN por la transposición de IS*6110* (Brosch *et al.*,1999; Ho *et al.*,2000; Lari N *et al.*,2001; Brondi *et al.*,2002). En un estudio previo (Vera-Cabrera *et al.*,1997), observaron que algunas cepas no hibridaron con las sondas para los genes *plc*A y *plc*B y en el presente trabajo confirmamos la ausencia parcial o total de dichos genes en estas cepas. La perdida de parte de los genes de *plc* por recombinación homóloga puede explicar la ausencia de genes en algunas cepas de *M. tuberculosis* descritas por otros autores (Weil *et al.*,1996) (Figura 21).

Las secuencias nucleotídicas en los genes de fosfolipasa C parecen atraer la transposición de IS*6110*. Esto también se apoya por la presencia de otro gen para fosfolipasa C (*plcD*) en la cepa H37Rv de *M. tuberculosis* (Cole *et al.*,1998) que se encuentra interrumpido por un elemento IS*6110*. La co-existencia de dos elementos IS*6110* en dos genes de fosfolipasa C contiguos o en genes vecinos como los de la familia PE/PPE, que también atraen elementos IS*6110* (Sampson *et al.*,1999), o los genes que codifican para la cutinasa, podrían dar origen a una escisión con la consecuente pérdida de las secuencias de los genes de fosfolipasa C.

En el presente estudio observamos en dos de las cepas una escisión de un fragmento de 2.8-kpb perteneciente a la secuencia de los genes de fosfolipasa C. Este proceso pudo haberse producido por recombinación homóloga de dos elementos IS*6110* (Figura 21) dado que, como se mencionó

anteriormente, el elemento IS*6110* presente en estas cepas no tiene repetidos directos en sus extremos, y esto es una evidencia de recombinación entre elementos de inserción (Mahillon y Chandler, 1998; Ho *et al.*,2000). Es posible que la transposición de elementos IS*6110* contribuya a la movilización de estos genes produciendo cepas de *M. tuberculosis* sin genes *plc*, o cepas con solo fragmentos de los genes o bien, cepas con secuencias extras producidas por duplicación, como es el caso de las cepas pertenecientes al grupo C reportado previamente por Vera-Cabrera *et al.*, (Vera-Cabrera *et al.*,1997)

Por otro lado, los cambios en el patrón de IS*6110* ocurren cada 1 ó 2 años (Cave *et al.*,1994; Daley *et al.*,1992), en este trabajo se observó que en cepas *de M. tuberculosis* relacionadas, los cambios son mínimos en los patrones RFLP para IS*6110* pero cambios radicales o la pérdida completa de los genes de fosfolipasa también han sido observados (Vera-Cabrera *et al.*,1997).

El elemento IS*6110* podría estar involucrado también en la pérdida de la virulencia de la cepa de *M. tuberculosis* H37Ra durante su cultivo en laboratorio (Lari *et al.*,2001), ya que la transposición de IS*6110* podría ser estimulada por condiciones microaeróbicas, y los cambios son más rápidos en ciertas áreas del genoma, particularmente en aquellas donde se encuentran varios elementos IS*6110* separados por distancias pequeñas (Ghanekar *et al.*,1999).

En el presente trabajo, las cepas polimórficas con genotipos *plc*A::IS*6110*, *plc*B::IS*6110*, *plc*C::*IS6110*, *plc*A-*plc*B y *plc*A-*plc*B-*plc*C expresaron diferencias en actividad enzimática con respecto a la cepa control

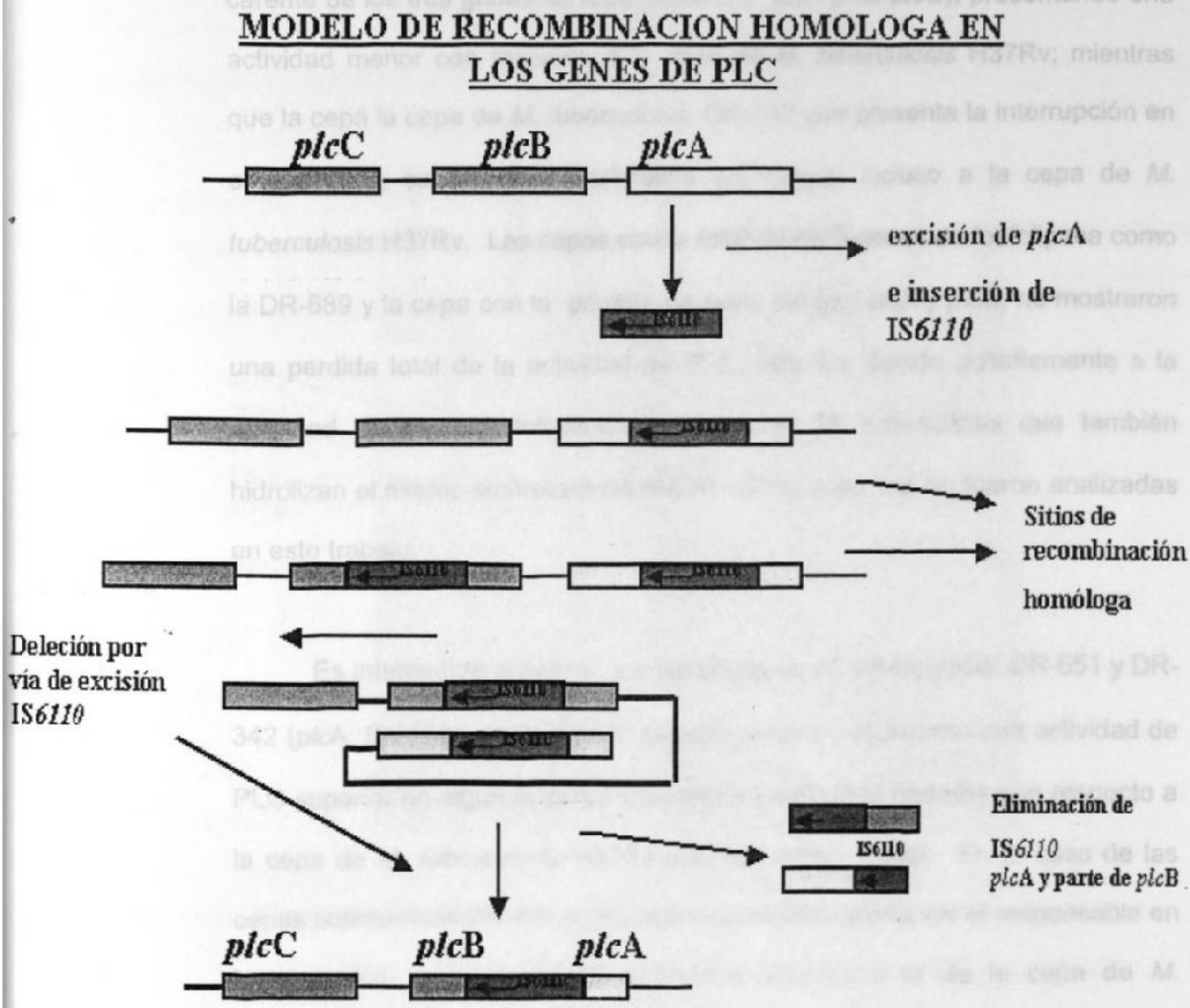

Figura 21.- Modelo de recombinación homóloga para la pérdida de un segmento de la región de *plc*A y *plc*B de *M. tuberculosis*

de *M. tuberculosis* H37Rv. En la cepa de *M. tuberculosis* con genotipo *plc*A-*plc*B se observa un comportamiento similar a la cepa de *M. tuberculosis* carente de los tres genes de fosfolipasa C (*plc*A-*plc*B-*plc*C), presentando una actividad menor con respecto a la cepa de *M. tuberculosis* H37Rv; mientras que la cepa la cepa de *M. tuberculosis* DR-342 que presenta la interrupción en el gen *plc*B, su actividad enzimática fue mayor, incluso a la cepa de *M. tuberculosis* H37Rv. Las cepas con la falta de los 3 genes de fosfolipasa como la DR-689 y la cepa con la pérdida de parte del gen *plc*A y *plc*B, no mostraron una perdida total de la actividad de PLC, esto fue debido posiblemente a la actividad de otras fosfolipasas presentes en *M. tuberculosis* que también hidrolizan el mismo sustrato (fosforil-[^{3}H]-colina) pero que no fueron analizadas en este trabajo.

Es interesante observar que las cepas de *M. tuberculosis* DR-651 y DR-342 (*plc*A::*IS6110* y *plc*B::*IS6110* respectivamente) mostraron una actividad de PLC superior en algunas de las concentraciones de la proteína con respecto a la cepa de *M. tuberculosis* H37Rv utilizada como control. En el caso de las cepas polimórficas DR-651 y DR-342 el gene *plc*D podria ser el responsable en la expresión de la actividad enzimática superior a la de la cepa de *M. tuberculosis* H37Rv, ya que estas cepas poseen este gene de PLC, mientras que la H37Rv no lo tiene.

El principal componente de la membrana celular son los fosfolípidos, los cuales son hidrolizados por las fosfolipasas ocasionando una serie de efectos en la célula. Los efectos resultantes pudieran contribuir en el proceso de

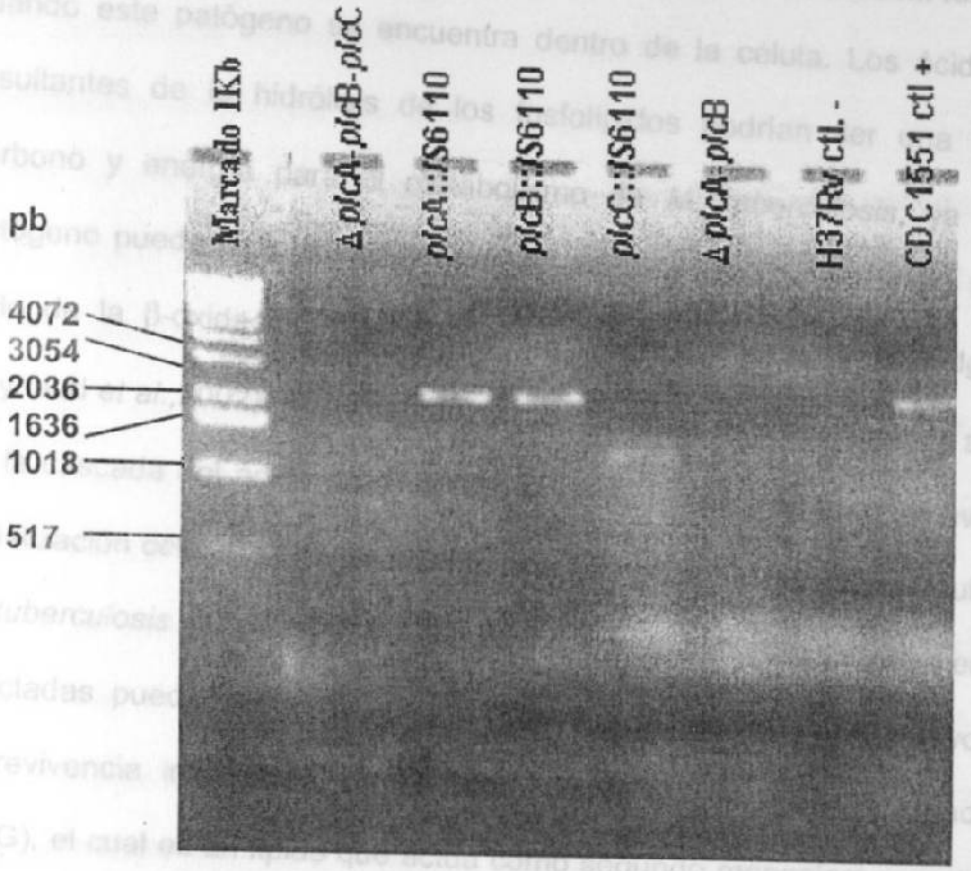

Figura 17.- Amplicones de la región del gene *plc*D de cepas de *M. tuberculosis* polimórficas.

infección por *M. tuberculosis* ya que la membrana celular es la primera barreara contra su invasión y al ser hidrolizados estos fosfolípidos, facilitaría la entrada del patógeno a la célula o proveer a la bacteria de nutrientes.

No está bien determinado el papel exacto de las PLC de *M. tuberculosis*, cuando este patógeno se encuentra dentro de la célula. Los ácidos grasos resultantes de la hidrólisis de los fosfolipidos podrían ser una fuente de carbono y energía para el metabolismo de *M. tuberculosis*, ya que este patógeno puede usar los ácidos grasos como fuente de carbono a través del ciclo de la β-oxidación y la vía del glyoxylato (Wheeler y Ratledge, 1991; Raynaud *et al.*,2002). Por otro lado, las fosfolipasas intervienen en la activación de la cascada del ácido araquidónico e inositol para intervenir en eventos de señalización celular, como es la inhibición de apoptósis por cepas virulentas de *M. tuberculosis* (Miao, 1997). La presencia de fosfolipasa exógena en células infectadas puede desbalancear los procesos de señalización y favorecer la sobrevivencia intracelular de *M. tuberculosis* al generarse 1,2,diacilglicerol (DAG), el cual es un lípido que actúa como segundo mensajero para transmitir señales intracelulares para TNF y IL-1 (Schutze, 1994).

En nuestros ensayos de infección de monocapa de macrófagos THP-1 con la cepa de *M. tuberculosis* DR-689 (con genotipo *plc*A-*plc*B-*plc*C), la cual carece de los genes de PLC en forma natural, no se observó efecto citotóxico a las 72 h después de infectar a los macrófagos a una multiplicidad de infección de 0.1, esto indica que estos genes son importantes para la citotoxicidad de *M. tuberculosis* en macrófagos humanos.

En las cepas con elemento IS*6110* insertado en los genes *plc*A, *plc*B ó *plc*C también se observa una reducción del efecto citotóxico a las 72 h de infección al compararse con la cepa *M. tuberculosis* H37Rv.

Los hallazgos de este estudio indican que los genes de *plc* pueden tener importancia como factor de virulencia para *M. tuberculosis*, y la transposición dentro de un ORF de *plc* por el elemento móvil IS*6110* representa una forma de perder la funcionalidad del gene, o la pérdida del gene en sí al ocurrir una recombinación. La pérdida en las secuencias nucleotídicas, como se observó en la cepa DR-426 repercutió en la reducción de la actividad enzimática. Estos cambios pueden ser experimentados por *M. tuberculosis* modificando su fenotipo y por tanto la eficiencia para entrar a los macrófagos (Li *et al.*,2002).

Los resultados apoyan fuertemente la importancia de PLC en la patogénesis de *M. tuberculosis*, sin embargo, con cepas como la DR-689 carente del locus completo de *plc* (*plc*A-*plc*B-*plc*C) que fue aislada de un caso de tuberculosis en un paciente con enfermedad clínica, sugiere un papel no primordial de estas enzimas como factores de virulencia y además apoya que la virulencia de *M. tuberculosis* depende de varios factores.

Se puede plantear que la cepa carente de los genes de *plc* pudiera haber sufrido la pérdida de la secuencia de la región de PLC por la transposición de IS*6110* al multiplicarse dentro de las células infectadas del paciente, ya que las condiciones microaeróbicas pueden estimular y producir estos cambios, en particular en aquellas zonas donde se encuentran varios elementos IS*6110* separados por distancias pequeñas (Ghanekar *et al.*,1999),

y al parecer la región de PLC es una zona de atracción para el IS*6110*.

Se ha publicado que cepas de *M. tuberculosis* con un mayor número de deleciones en su genoma muestran reducción en la virulencia, ya que las cavitaciones pulmonares en los pacientes infectados con estas cepas son menores que en aquellos pacientes infectados con cepas donde el genoma esta integro o con pocos genes eliminados (Kato-Maeda *et al.*,2001).

La falta de daño celular en la monocapa de macrófagos THP-1 a las 72 h después de la infección por la cepa *M. tuberculosis* carente de los genes de *plc*, podría ser debido a que en esta cepa, además de los genes de PLC pudieran faltar otras secuencias nucleotídicas que contribuyen como factores importantes junto a los genes *plc* para causar citotoxicidad como la observada en la infección con la cepa H37Rv de *M. tuberculosis*.

La cepa de *M. tuberculosis* carente del locus de *plc*, como la descrita en este trabajo, constituye un excelente modelo para estudiar la contribución de estas enzimas en la patogenia de *M. tuberculosis*. Mediante la utilización de técnicas modernas de comparación genómica, será posible identificar otros genes de *M. tuberculosis* que contribuyan en el desarrollo de la enfermedad clínica.

5.- CONCLUSIONES

1. El polimorfismo observado en la región de fosfolipasa C en *M. tuberculosis* esta dado por la transposición del elemento móvil IS*6110*

2. El IS*6110* se encuentra insertado en la misma dirección en cualquiera de los tres genes de fosfolipasa C, y puede estar implicado en la pérdida de secuencias debido a una recombinación homologa entre dos elementos cercanos

3. La región de PLC es una zona de alta frecuencia (hot spot) para la transposición de elementos de inserción IS*6110*

4. La interrupción de los genes PLC, por la secuencia de inserción IS*6110*, se manifiesta en cambios de la actividad enzimática de fosfolipasa C

5. Los genes que codifican a la enzima fosfolipasa C en *M. tuberculosis* son importante para producir daño citológico en macrófagos *in vitro*

6.-PERPECTIVAS DE INVESTIGACIÓN

Existen cepas que carecen de los genes *plc* o que se encuentran suprimidos de manera natural y que han sido analizadas en este trabajo de investigación mostrando un efecto citotóxico disminuido sobre macrófagos de la línea THP-1 comparadas con la cepa H37Rv, sugiriendo que posiblemente en estas cepas existen otros genes suprimidos que contribuyen a la patogenicidad de *M. tuberculosis* o que existen otros genes asociados con la actividad de las fosfolipasas. Por lo tanto una perspectiva de este trabajo es investigar el efecto *in vivo* e *in vitro* de la virulencia en cepas de *M. tuberculosis* que carecen de los genes de fosfolipasa C.

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Phospholipase Region of *Mycobacterium tuberculosis* Is a Preferential Locus for IS*6110* 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 IS*6110* 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, IS*6110* 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 IS*6110* 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 decribed: an ORF beside *mpcB* and another related sequence at position 1755 of the genome, located beside an IS*6110* 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 deletions 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 cetyltrimethyamonium 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 Turboblotter 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 IS*6110* 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 GeneClean 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 IS*6110* studies.

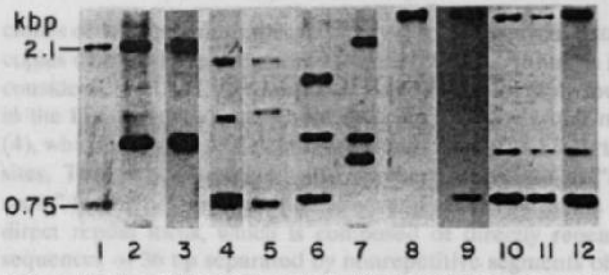

FIG. 1. Southern blot analysis of the *M. tuberculosis* strains studied in this work. The blots were incubated with a PCR probe, prepared with primers PT1 and PT2 as described before (28), that amplifies a 396-bp region near the 3′ end of the *plcA* ORF. Lanes: 1, *M. tuberculosis* strain 14323; 2, Dr-561; 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 IS*6110* 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 IS*6110* 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 IS*6110*. These results were confirmed by preparing the amplicons and performing the sequencing analysis in duplicate.

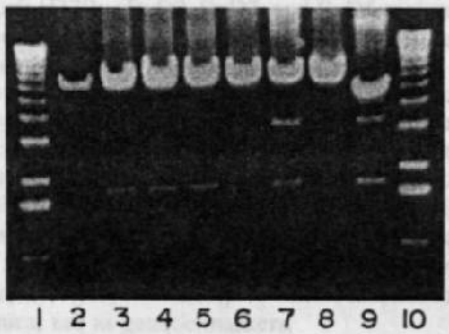

FIG. 2. Long-PCR assay of mycobacterial genomic DNA from polymorphic strains for the phospholipase genes with primers TB20 and TB21. Lanes: 1 and 10, 1-kb ladder (Gibco); 2, RIVM-7; 3, Dr-169; 4, Dr-170; 5, Dr-342; 6, Dr-351; 7, Dr-561; 8, Dr-468; 9, *M. tuberculosis* H37Rv.

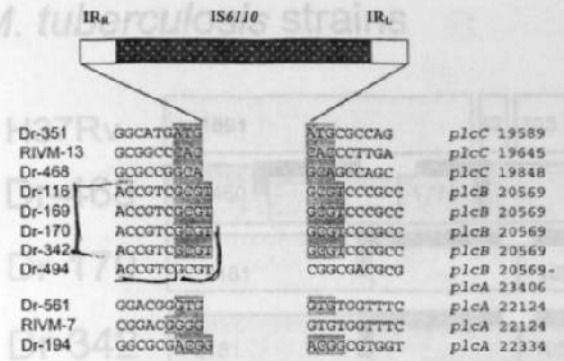

FIG. 3. Locations of the IS*6110* elements inserted in the phospholipase regions of the *M. tuberculosis* strains studied in this work. At the right are positions of the insertion elements in the phospholipase locus. Shaded boxes, duplicated nucleotides. The nucleotide numbers are taken from GenBank sequence MCTY 98 (accession no. Z83860). 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 IS*6110* 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 IS*6110* 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 IS*6110* 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 IS*6110* 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 IS*6110* 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 IS*6110* elements transposed in the same direction.

DISCUSSION

IS*6110* 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. IS*6110* 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 IS*6110* 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 IS*6110* 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 IS*6110* are the *ipl* locus, which is itself located in an insertion element-like element, IS*1547* (7), and the DK1 site (10).

In this work we found IS*6110* 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 IS*6110* insertion, such as nucleotides 20569 and 22124. In *M. tuberculosis* H37Rv and H37Ra there is an IS*6110* 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 IS*6110* insertion elements. Only by doing a study involving a great number of strains bearing IS*6110* elements can we determine if there are consensus sequences within the phospholipase genes that stimulate IS*6110* 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 IS*6110* 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.

IS*6110* 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 IS*6110* 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 IS*6110* 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 IS*6110* 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 IS*6110* transposition. Thus the presence of an IS*6110* 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 IS*6110* elements) (22)

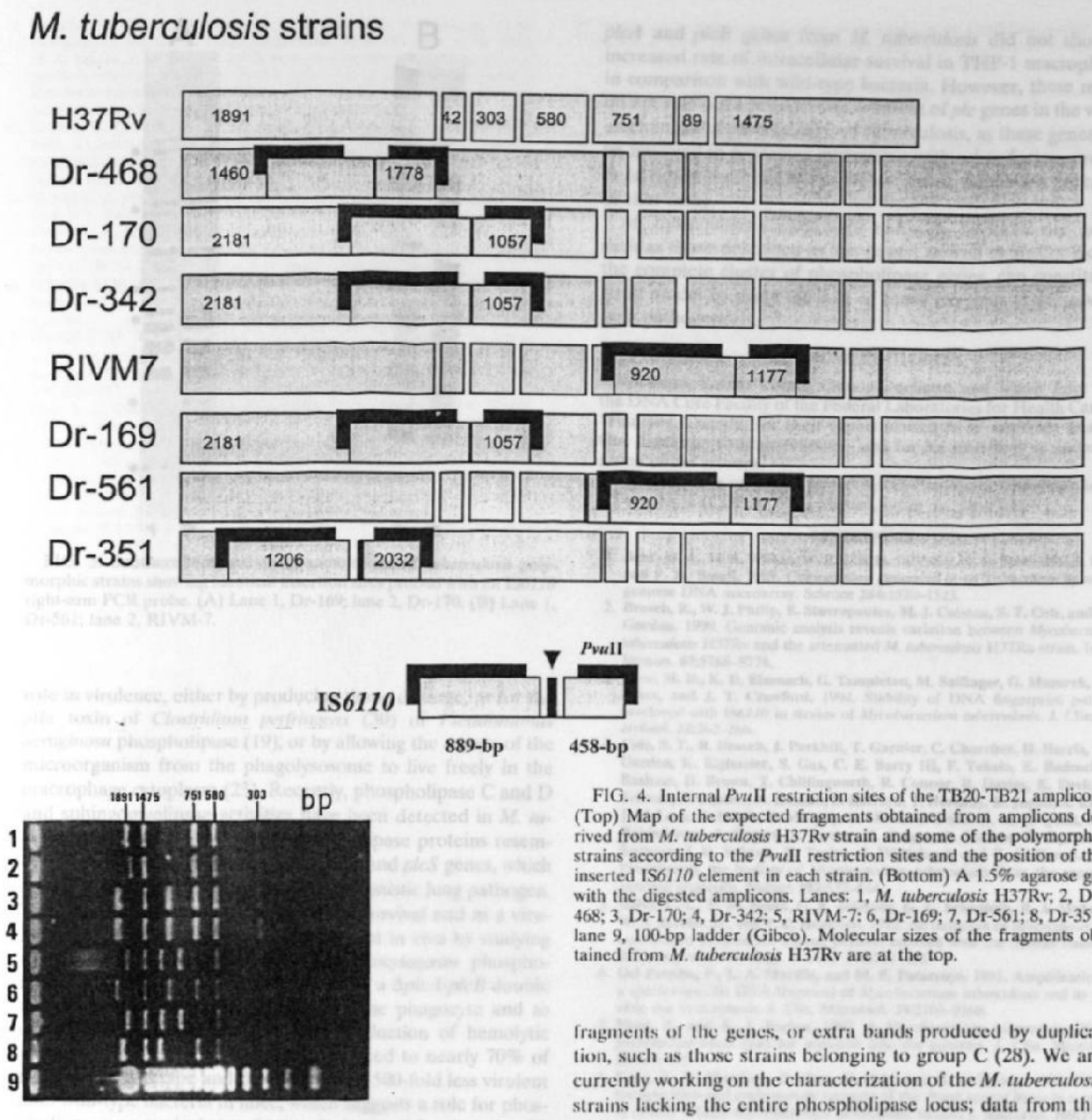

FIG. 4. Internal *Pvu*II restriction sites of the TB20-TB21 amplicon. (Top) Map of the expected fragments obtained from amplicons derived from *M. tuberculosis* H37Rv strain and some of the polymorphic strains according to the *Pvu*II restriction sites and the position of the inserted IS*6110* element 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 IS*6110* 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 IS*6110* elements since, as mentioned above, IS*6110* did not present direct repeats, and this is evidence of recombination between IS elements (16). It is possible that transposition of IS*6110* 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 IS*6110* pattern have been observed to occur over 1 or 2 years (3, 5). We observed in related *M. tuberculosis* strains minimal changes in the IS*6110* 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 IS*6110* elements separated by small distances.

It has been claimed that phospholipases play an important

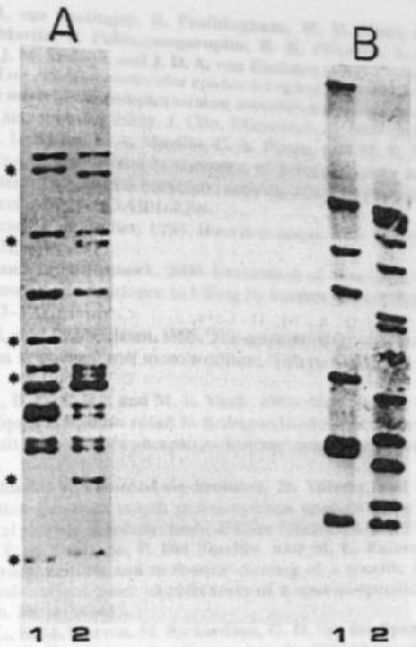

FIG. 5. Southern blot analysis of some of the *M. tuberculosis* polymorphic strains showing identical insertion sites probed with an IS*6110* right-arm PCR probe. (A) Lane 1, Dr-169; lane 2, Dr-170. (B) Lane 1, Dr-561; lane 2, RIVM-7.

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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Phospholipase C Genes are Essential for *Mycobacterium tuberculosis* Cytotoxicity to Human Macrophages.

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Running title: Phospholipase C is important for MTB macrophage cytotoxicity

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ABSTRACT

Mycobacterium tuberculosis phospholipase C genes are encoded by three genes arranged in tandem, starting at position 2351 of the *M. tuberculosis* strain H37Rv genome and named *plcA*, *plcB* and *plcC* respectively. In a previous publication (24), we described that preferential insertion of IS*6110* elements in any of these three genes produces polymorphism at this region. We found three isolates with deletions in this region produced by homologous recombination of two nearby elements; two strains had an identical deletion of *plcA* and most of *plcB* whereas another showed the loss of the complete phospholipase C (PLC) locus. In the present work we studied the effect of the disruption or loss of any of the *plc* genes on enzyme activity and the ability of these *M. tuberculosis* strains to elicit cytotoxicity using human macrophage monolayers. We observed that isolates having the inactivated *plc* genes did not show any significant decrease in enzyme activity; whereas the *plcA-plcB* and *plcA-plcB-plcC* strains demonstrated a lower PLC activity. The cytotoxicity for human macrophages (THP-1) was also decreased, and, remarkably, the *M. tuberculosis* Δ*plcA-plcB-plcC* strain produced a limited toxicity to macrophage monolayers. These results confirm the importance of PLC for *M. tuberculosis* virulence.