8. DISCUSIÓN

El pasaje serial *in vitro* de microorganismos ha producido en muchos casos una disminución en la virulencia o cambios en sus propiedades genéticas o fenotípicas, de tal forma que esta atenuación ha sido un factor importante para la producción de vacunas y el desarrollo de blancos terapéuticos. En este trabajo se llevó a cabo el pasaje serial de cepas de *M. tuberculosis* con deleciones en el genoma por 100 pases y se investigaron los efectos sobre su composición genética utilizando la tecnología de microarreglos y el análisis de RFLP para IS*6110*; además se estudió el efecto sobre la capacidad citotóxica en una monocapa de macrófagos THP-1.

El pasaje serial se llevó a cabo en un medio de cultivo líquido (M7H9) en dos versiones, a una de las cuales se le añadió bilis de buey; con el propósito de emular un componente del medio que utilizaron Calmette y Guerin. La bilis está compuesta de sales que actúan como detergentes emulsionando los ácidos grasos y se ha observado que causa daño en el ADN sobre *S. enterica* e induce rearreglos y mutaciones puntuales (Prieto *et al.*, 2004). La bilis puede tener efectos similares sobre el ADN de *E. coli* como se observó en un estudio donde la exposición a sales biliares indujeron genes de la cadena de estrés oxidativo y de daño al ADN (Bernstein *et al.*, 1999). Se han propuesto varios mecanismos por los cuales las sales biliares causan daño oxidativo sobre S. enterica: (a) la exposición a bilis induce transiciones CC↔ AT, b) el desoxicolato de

sodio activa la transcripción de genes que corresponden a la respuesta al daño oxidativo, los *oxyR* y *soxSR*, c) los mutantes de *S. enterica* carecen de exonucleasas que son extremadamente sensibles a la bilis (Prieto *et al.*, 2004).

De la misma manera, en este trabajo se consideró que la adición de bilis pudo ser un factor para que se dieran las mutaciones en las bacterias.

8.1. Análisis de la Región Cercana al Locus PLC.

Las fosfolipasas C son factores de virulencia de varios microorganismos incluyendo *Clostridium* spp., *Listeria monocytogenes*, *Pseudomonas aeruginosa*, etc. (Songer, 1997). Actúan destruyendo los tejidos celulares como en las infecciones por *Clostridium* (Williamson y Titball, 1993) o lisando las membranas fagosomales y liberando microorganismos intracelulares al citoplasma como en el caso de *L. monocytogenes* (Tilney y Portnoy, 1989). *M. tuberculosis* posee 4 genes que codifican enzimas fosfolipasas tipo C: *plcA, plcB, plcC* y *plcD.* Este último está ausente o interrumpido en muchos aislamientos clínicos e incluso en la cepa H37Rv. Reynaud *et al* en el 2002 demostraron la importancia de las fosfolipasas en la virulencia. Ellos inactivaron de manera individual y en conjunto los ORF´s de las fosfolipasas y analizaron la virulencia en un modelo murino. Los resultados mostraron que en los mutantes triples o cuádruples estaba disminuida su capacidad para multiplicarse en los pulmones y vaso de los animales infectados, sugiriendo su importancia para la supervivencia de *M. tuberculosis* en los tejidos. Se ha observado en humanos que a mayor presencia de deleciones genéticas en un aislamiento clínico de *M. tuberculosis* es menor la enfermedad cavitaria (Kato Maeda *et al.*, 2001). En este estudio, algunos de los genes ausentes incluyeron los genes de fosfolipasa. La deleción de fragmentos de ADN en los aislamientos clínicos de

M. tuberculosis se ha reportado previamente, con varios de ellos asociados a elementos de transposición IS*6110* (Fleischmann *et al.*, 2002; Tsolaki *et al.*,2004; Kato Maeda eta al., 2001; Ho *et al.*, 2000). En este trabajo se observó en todos los casos la inserción de IS*6110* en la región PLC en los aislamientos Δ*plcA–plcB–plcC*. Como se hipotetizó previamente (Vera-Cabrera eta al., 2001), es probable que las deleciones sean producidas por un mecanismo de recombinación homóloga ya que en ningún caso se asociaron repetidos invertidos al mecanismo de transposición. En el asilamiento DR-689, los tres genes estaban completamente ausentes y en el resto de los aislamientos se observó una copia incompleta de *plcC*. En los aislamientos RIVM 97–488 y RIVM 97–1389 se observó la presencia de secuencias de ADN similares a MT2421 y Mt2420 de *M. tuberculosis* CDC1551. MT2420 muestra homología con las proteínas de la familia ESAT-6 y MT2421 es idéntica en un 95 % a la familia de proteínas QILSS (Betts *et al.*, 200). Ambos genes están ausentes en H37Rv y su presencia varía en otros aislamientos clínicos (Fleischmann *et al.*, 2002). MT2420 y MT2421 tienen muchos genes ortólogos en *M. tuberculosis* H37Rv (Rv2436, Rv2437), *M. tuberculosis* CDC1551 (MT1066, MT1067, MT2411, MT2412, MT1841, MT1842), *M. bovis* (Mb1067, Mb1066) y *M. leprae* (Ml1181). Más que inserciones, la presencia de estas secuencias parece representar variación en arreglos genéticos entre los aislamientos de *M. tuberculosis* como se ha reportado anteriormente (Fleischmann *et al.*, 2002). Aunque se ha conjeturado que la transposición de elementos IS*6110* ocurre al azar, existen varios sitios preferenciales, entre ellos los genes de fosfolipasa (Vera-Cabrera *et al.*, 2001). Estas secuencias parecen atraer los elementos IS*6110* y cuando dos de ellos se encuentran cercanos entre sí, puede ocurrir una recombinación homóloga, como se ha visto en los aislamientos estudiados. El mecanismo de atracción es desconocido, aunque

probablemente estén implicadas secuencias repetitivas o palindrómicas. Otras secuencias repetitivas tal como las que codifican para proteínas PE y PPE, se han asociado también a deleciones en el genoma de *M. tuberculosis* (Tsolaki *et al.*, 2004), lo cual refuerza esta hipótesis. El papel de la transposición de IS*6110* en la organización del genoma de *M. tuberculosis* es todavía materia de estudio, aunque esto podría ser importante ya que parece afectar los genes adyacentes (Safi *et al.*, 2004).

Tsolaki *et al* en el 2004 reportaron el análisis por hibridación con microarreglos de 100 cepas de aislamientos de *M. tuberculosis* del área de San Francisco y observaron patrones de deleción comunes, a los que llamaron regiones de diferencia (RD). Comparando los patrones de deleción presentados por las cepas de este estudio, se observaron patrones de deleción similares. Un grupo de cepas, las cuales presentaron la mayoría de las deleciones incluyendo RD's 105, 149, 150, 152, 181 y 207, fueron muy similares en el patrón de RFLP para IS*6110* de una de las cepas analizadas en este trabajo (DR-689). La única característica en común fue que todas ellas correspondían a la familia Beijing. La familia Beijing es un grupo de cepas ampliamente diseminado que es muy dominante en Asia y otras partes del mundo (van Soolingen *et al.*, 1995). Estas cepas comparten patrones de RFLP-IS*6110* similares con múltiples bandas así como un patrón de Spoligotyping idéntico (Kremer *et al.*, 2004). DR-689 comparte un patrón de RFLP casi idéntico con los aislamientos de San Francisco teniendo todas estas deleciones, aunque éstas no están relacionadas epidemiológicamente. Es posible que estas cepas tengan un ancestro en común que ya presentaba este patrón de deleciones y que fueron adquiridas y transmitidas hace mucho tiempo. Estas cepas representan un grupo muy bien adaptado de bacterias pues son muy dominantes en ciertas áreas (hasta 85 % en el área de Beijing), y los aislamientos con todas estas deleciones pueden

representar a los más adaptados de todos los grupos filogenéticos que circulan en el mundo.

8.2. Ensayo de Microarreglos.

El género *Mycobacterium* es uno de los que se han obtenido más secuencias completas de los genomas de sus especies (Cole *et al.*, 1998). La comparación de estos genomas ha resultado un medio muy útil y un inicio para elucidar los mecanismos involucrados en la virulencia y realizar el análisis de la evolución de *M. tuberculosis* y otras especies del género(Behr *et al.*, 1999; Brosch *et al.*, 2000; Fleischmann *et al.*, 2002; Kato Maeda *et al.*, 2001; Mahairas *et al.*, 1996; Brenan *et al.*, 1996; Vera-Cabrera *et al.*, 2007). Una de las tecnologías genómicas recientemente desarrollada son los microarreglos (Lashkari *et al.*, 1997). El microarreglo constituye un arreglo de secuencias de fragmentos de ADN inmovilizadas y bien ordenadas que están adheridas a una superficie sólida generalmente de cristal. Cada secuencia corresponde a un gene diferente. Los microarreglos han sido usados para el análisis de la expresión génica al comparar el ARNm producido para múltiples genes por un organismo en diferentes condiciones, así como para comparar la presencia o ausencia de genes entre dos diferentes cepas de un organismo. En este último caso, generalmente se tiene la secuencia completa de una de las cepas. (Butcher, 2004). La tecnología de los microarreglos es altamente confiable y existen varios formatos para el genoma de *M. tuberculosis* incluyendo conjuntos de oligonucleótidos comerciales para que el cliente realice sus propios microarreglos (Operon/Qiagen) y varios microarreglos caseros (Butcher, 2004). Salamon *et al* en el 2000 fueron los primeros en presentar una nueva

aplicación de un microarreglo (Affymetrix GeneChip) para efectuar una búsqueda extensa de polimorfismos de deleción en el genoma de cepas de *M. tuberculosis*.

En este trabajo se llevó a cabo una comparación de genomas de cepas de *M. tuberculosis* sometidas a pasaje serial y su contraparte parental, utilizando un sistema de microarreglos. El análisis de microarreglos reveló algunas diferencias en la presencia o ausencia de genes entre las cepas parentales y aquellas sometidas a pasaje serial que suponían grandes deleciones generadas por efecto del subcultivo. Dado que el análisis de las cepas parentales fue realizado en un laboratorio distinto a donde se realizó el análisis de las cepas sometidas a subcultivo, fue necesario comprobar mediante reacciones de PCR la presencia o ausencia de los genes discordantes utilizando iniciadores específicos para cada gen. Esta discordancia pudo ser debida a las distintas condiciones de hibridación utilizadas en ambos laboratorios. En el ensayo de PCR se encontró que no había diferencia entre las cepas parentales y aquellas sometidas a pasaje serial, es decir que el subcultivo aparentemente no generó ninguna deleción genética importante que involucrara los genes de *M. tuberculosis* H37Rv. La reacción de hibridación en los microarreglos que se utilizaron en este trabajo, se llevó a cabo entrè el ADN de la muestra y una molécula de oligonucleótidos inmovilizado sobre la superficie del microarreglo. Estos arreglos contienen 25-80 oligonucleótidos y esto constituye una desventaja porque puede existir una deleción en otra parte del gen que no está representada en el microarreglo, por lo que en nuestro estudio no fue posible determinar la presencia de otras deleciones distintas a las que estaban incluidas en el microarreglo.

8.3. RFLP y Sspoligotyping

Dentro del genoma de *M. tuberculosis* se han descrito varios elementos genéticos móviles o genes "saltarines" conocidos como secuencias de inserción (Gordon *et al.*, 1999). Estos elementos son capaces de moverse de un lado a otro en el cromosoma por un proceso llamado transposición y su dinámica natural ha sido implicada en las características fenotípicas de varias bacterias patógenas (Parkhill *et al.*, 2003; Brugger *et al.*, 2004). Debido a la cantidad y polimorfismo de posición dentro del genoma de *M. tuberculosis*, las secuencias de inserción (IS) *6110* han sido utilizadas ampliamente como marcadores genotípicos en estudios epidemiológicos (McEvoy *et al.*, 2007). Sin embargo, además de su invaluable papel en la epidemiología molecular no solo es un elemento genético pasivo y debido a su movilidad, es capaz de insertarse en la secuencia de uno o varios genes y alterar o evitar su expresión y de ese modo contribuir a una diversidad fenotípica entre distintas cepas de *M. tuberculosis* (McEvoy *et al.*, 2007). En base a esta última hipótesis, en este trabajo se decidió analizar el efecto del pasaje serial de cepas de *M. tuberculosis* con deleciones en el locus PLC sobre el patrón de RFLP para IS*6110*. El análisis de RFLP para IS*6110* de las cepas parentàles y las cepas sometidas a pasaje en el medio M7H9 con y sin bilis reveló que 5 de las cepas estudiadas permanecieron sin cambio en sus patrones de bandas para IS*6110*. Sin embargo, en la cepa DR-689 (Beijing) subcultivada en el medio con bilis, se produjo un cambio que involucró el elemento transponible IS*6110*. El cambio generado en esta cepa fue el cambió de posición en el patrón de hibridación de IS*6110* de ~1,400 pb a ~1,600 pb.

Previamente se había asumido que la tasa de transposición era constante entre cepas (McEvoy *et al.*, 2007). Un análisis para determinar si el pasaje serial *in vitro* de cepas de

M. tuberculosis producía cambios en el patrón de RFLP mostró que no se produjeron cambios después de aproximadamente 25 pases (van Soolingen *et al.*, 1991). Diferentes cepas de *M. tuberculosis* han mostrado variación en las tasas de transposición (de Boer *et al.*, 1991; Niemann *et al.*, 1999; Yeh *et al.*, 1998; Warren *et al.*, 2002a,b) cuando se analizan aislamientos seriados de las cepas obtenidas de pacientes por lo que éste es el primer reporte de un evento de transposición generado *in vitro*. Una característica a resaltar en el aislamiento en el cual se generó el cambio, es el elevado número de copias de la secuencia de inserción. Los estudios en torno a la tasa de transposición dependiendo del número de copias de IS*6110* en un aislamiento, sugieren que las cepas con un número de copias más alto tienen una tasa de evolución más alta y una ventaja potencialmente selectiva que las cepas con un bajo número de copias (Wall *et al.*, 1999). El resultado obtenido en este estudio para la cepa DR-689 avala esta hipótesis; coincidentemente esta es una cepa Beijing, esta cepa es dominante en el este y sureste de Asia así como en Euroasia norte y se ha diseminado rápidamente a otras partes del mundo donde también ha incrementado su incidencia (Bifani *et al.*, 2002). De esta forma el gran éxito en la adaptación de estas cepas con alto número de copias puede estar directamente relacionado al número de copias de IS*6110*. Esto sin embargo pasa por alto el hecho de que cepas con un bajo número de copias pueden causar brotes y desarrollo de algunos rasgos como drogoresistencia (Valway *et al.*, 1998; Victor *et al.*, 2007) y que otros miembros del complejo, como *M. bovis*, que generalmente contiene solo un elemento IS*6110*, todavía causa infección y se transmite de manera eficiente. Estos hallazgos son sugestivos de distintos mecanismos de evolución adaptativa exitosa (McEvoy *et al.*, 2007).

La variación en el número de copias parece ser debido en gran parte a la naturaleza de la región genómica donde el elemento se inserta (Wall *et al.*, 1999). Cuando reside en una región genética con trascripción silenciosa, IS*6110* es inactivo y raramente sufre transposición. Sin embargo, cuando se inserta en una región del genoma transcripcionalmente activa, la tasa de transposición se aumenta considerablemente, debido muy probablemente al incremento en la expresión de la transposasa, enzima codificada en la secuencia del elemento IS*6110* (Wall *et al.*, 1999). Este hallazgo sugiere que un simple evento de transposición (como el que observamos en nuestra investigación) puede generar un repentino estallido de actividad transposicional si el elemento es incorporado a un sitio activo de transcripción (Wall *et al.*, 1999). La localización exacta de la banda de IS*6110* que se movió en la cepa DR689 no fue determinada y por lo tanto no conocemos el sitio de inserción. Las consecuencias de este evento de transposición pueden ser muy variadas. Dependiendo de la posición de la integración, un evento transposicional puede resultar en amplio rango de alteraciones fenotípicas sobre el hospedero que varían de la letalidad a la neutralidad o posibles efectos benéficos ocasionales (McEvoy *et al.*, 2007).

El hecho de que el movimiento de la bande IS*6110* se haya dado en el medio conteniendo bilis y en la cepa con un mayor número de secuencias de inserción sugiere que la bilis puede ser una fuente de polimorfismo genético para este marcador.

Por otro lado también analizamos el efecto que podría tener el pasaje serial de cepas de *M. tuberculosis* en el polimorfismo de ADN presente en un locus cromosomal particular, la región del "Repetido Directo" (DR). El estudio de esta región también se ha hecho con fines epidemiológicos (Hermans *et al.*, 1992). El método para analizar el locus DR se conoce como Spoligotyping. El análisis de las cepas parentales y de sus

similares sometidas a subcultivo en M7H9 con y sin bilis no mostró algún cambio en el patrón de Spoligotyping, demostrando así la estabilidad de la región DR del cromosoma de las cepas analizadas aquí.

8.4. Ensayo de Citotoxicidad en Macrófagos.

Los macrófagos son la primera línea de defensa del hospedero contra los microbios. Los macrófagos son críticos tanto para la supervivencia de *M. tuberculosis* como para vincular la inmunidad innata y adaptativa del hospedero (Flynn y Chan, 2003). Ellos promueven la activación y reclutamiento de células T que son cruciales para contener a la micobacteria dentro de los granulomas en el pulmón. Las micobacterias virulentas pueden replicarse dentro del ambiente hostil de los macrófagos, secuestrados en los fagosomas pobremente acidificados que fracasan en la fusión con los lisosomas (Brown *et al.*, 1969; Mwandumba *et al.*, 2004; Russel, 2001). *M. tuberculosis* puede evadir la función bactericida inhibiendo la señalización mediada por IFN-γ (Flynn, 2004). Se ha reportado que *M. tuberculosis* interfiere con la presentación de antígenos, múltiples vías de señalización y respuesta transcripcional dentro del macrófago (Flynn y Chan, 2003).

La virulencia de cepas de tuberculosis ha sido tradicionalmente evaluada en términos de la capacidad de los organismos de replicarse dentro de órganos específicos de ratones y cobayos siguiendo la infección con aerosoles. También los modelos de monocitos y macrófagos humanos han sido utilizados para evaluar la virulencia de aislamientos clínicos de *M. tuberculosis* (Laochumroonvorapong y Kaplan, 1996; Zhang *et al.*, 1998, 1999; Riendeau y Kornfel, 2003; Theus *et al.*, 2004, 2006). Para determinar si el pasaje serial tenía un efecto sobre la capacidad citotóxica producida por *M. tuberculosis*, en este trabajo se llevaron a cabo ensayos de citotoxicidad en macrófagos derivados de la

línea celular de monocitos THP-1 infectados con las cepas parentales y las sometidas a subcultivo en el medio de cultivo con y sin bilis. En tres de las cepas estudiadas sometidas a subcultivo se observó una disminución en la citotoxicidad sobre la línea celular TH-P 1 con respecto a sus contrapartes parentales que siguen conservando un efecto citotóxico mayor a 85 % al día 7 post-infección. Estos resultados corroboran que las cepas utilizadas tienen la capacidad de producir efecto citotóxico al día 7 post-infección aunque tienen una deleción en el locus PLC y que siguen conservando los genes necesarios para su supervivencia en los macrófagos. Los resultados de la disminución en la capacidad citotóxica de las cepas sometidas a pasaje serial no representan necesariamente una atenuación de su virulencia. Probablemente este efecto fue debido a la adaptación de las bacterias a un medio diferente al hospedero en donde las bacterias dejan de expresar los genes necesarios para su crecimiento en macrófagos, aunque siguen conservando los genes constitutivos necesario para su supervivencia *in vitro*. Una estrategia para comprobar lo anterior es re-introducir la bacteria en un modelo de infección *in vivo* o a macrófagos tal como lo realizado por McDonough *et al* en el 2000. Ellos realizaron un pasaje intracelular de la cepa *M. tuberculosis* H37Rv dentro de macrófagos y esto afectó el tránsito del bacilo virulento en la re-infección de otros macrófagos de una manera suero-dependiente. Es posible que la disminución en la virulencia de esta cepa sobre la monocapa de macrófagos haya sido derivada del efecto que tuvo el cambio de la secuencia de inserción aunque las demás cepas estudiadas mostraron el mismo efecto.

Una mayor caracterización de estas cepas es necesaria para determinar si la disminución en la capacidad de citotoxicidad sobre la monocapa de macrófagos no fue

solamente debida a un cambio en la expresión de ciertos factores producido por la adaptación de las micobacterias al cultivo *in vitro*.

La atenuación de microorganismo por pasajes *in vitro* ha sido reportada por varios autores. La mayoría de las vacunas virales utilizadas en animales y humanos han sido derivadas de esta forma (Jennings y Potter, 1999). El pasaje de *Entamoeba histolytica* en cultivos axenicos a largo plazo disminuyó algunas actividades relacionadas a la virulencia (González-Garza *et al.*, 2000). El cultivo prolongado *in vitro* de *Trypanosoma cruzi* produjo sub-líneas con una baja infectividad para mamíferos (Basombrio *et al.*, 2000). En bacterias *Staphylococcus aureus*, donde el pasaje serial provocó cambios en la fisiología, producción de factores de virulencias y mutaciones dentro del operón agr (Somerville *et al.*, 2000). En *Borrelia anserina* (Sakharoff) que fue pasada 39 veces en un medio de cultivo líquido perdió su capacidad de infección en un modelo de pollos (Levine *et al.*, 1990). Pero el caso más representativo de la atenuación por pasaje serial es la obtención de la vacuna que se utiliza contra la tuberculosis: la cepa *M. bovis* BCG a partir de *M. bovis*. Sin embargo, debido al requerimiento para pasaje continuo de cepas derivadas, para cuando los stock liofilizados fueron preparados en 1960, diferentes cepa hijas habían sufrido hasta 1000 pases adicionales. El más reciente estudio sobre las diferentes vacunas circulando en el mundo concluye que las vacunas iniciales han sido las más efectivas para la protección contra la TB. Debido a la variable eficacia protectora de BCG contra la tuberculosis en adultos, que va de 0 a 90 % (Clemens *et al.*, 1983), se han hecho numerosos esfuerzos para desarrollar nueva vacunas con mejor efecto protector. Ya que una variedad de genes son necesarios para la virulencia *in vitro*, las cepas que carezcan de estos genes pueden ser posibles candidatos como vacunas. Por lo anterior nosotros hipotetizamos que las cepas de *M. tuberculosis* analizadas en este

trabajo y que disminuyeron su virulencia después de ser sometidas a pasaje serial pueden en un futuro ser utilizadas como vacuna, ya que a diferencia de *M. bovis* BCG pueden seguir conservando los antígenos de *M. tuberculosis* los cuales serían más especie-específicos y pueden proporcionar un mejor respuesta inmune protectora que BCG. Aunque también se considera que la obtención de una cepa atenuada de *M. tuberculosis* debe ser un proceso racional. Se requerirá que las cepas estén lo suficientemente atenuadas para que no induzcan efectos patológicos y que la pérdida de la virulencia sea irreversible.

9. CONCLUSIONES

1 En la cepa DR-689 (Beijing) subcultivada en el medio con bilis se produjeron cambios genéticos que involucraron el elemento transponible IS*6110*.

2 El análisis de microarreglos reveló que a parte de las deleciones ya reportadas para estas cepas, no se produjeron nuevas deleciones importantes que involucraran los genes de *M. tuberculosis* H37Rv.

3 El subcultivo continuo produjo cambios en la citotoxicidad de *M. tuberculosis* sobre la monocapa de macrófagos THP-1. La adaptación de *M. tuberculosis* al crecimiento en medios artificiales induce al microorganismo a modificar la expresión de genes que no son esenciales para su supervivencia *in vitro*, cambiando algunas de sus propiedades biológicas y su virulencia.

10. PERSPECTIVAS

- El pasaje serial de las cepas de *M. tuberculosis* tiene que continuarse para observar posteriores cambios.

- Para determinar si el subcultivo produjo cambios pequeños como mutuaciones puntuales, SNP's, duplicaciones, etc, la alterativa es un análisis del transcriptoma o la secuenciación completa de los genomas.

- La relevancia de la evolución de *M. tuberculosis in vitro* no está clara, por lo que la continuación del pasaje serial y posteriores estudios de expresión de genes y estudios de virulencia serán de importancia para el entendimiento de la biología de este microorganismo.

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RESUMEN CURRICULAR

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Candidato para el Grado de

DOCTOR EN CIENCIAS con Especialidad en Microbiología

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Genetic characterization of *Mycobacterium tuberculosis* clinical isolates with deletions in the *plcA–plcB–plcC* locus

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KEYWORDS
Mycobacterium tuberculosis;
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Summary
Setting: The basis for *Mycobacterium tuberculosis* virulence is not completely understood. Analysis of the genomic structure of clinical isolates will give information that can be related to biological activities involved in virulence.
Objective: To determine the extension of the deletion in the *plcA–plcB–plcC* locus of selected *M. tuberculosis* isolates, as well as other changes in the chromosome.
Design: In the present work we characterized a group of *M. tuberculosis* isolates devoid of the *plcA–plcB–plcC* locus by PCR, sequencing and microarrays.
Results: PCR amplification of this region demonstrated a complete lack of *plcA* and *plcB* ORF's in all of the isolates. The *plcC* gene was completely deleted in one of the strains (DR-689) and the other three isolates still conserved part of this ORF. The loss of lateral DNA sequences ranged from 3723 to 7646 bp. An IS*6110* element was present in all tested strains cases, and some isolates presented the insertion of ORF's coding for proteins homologous to the ESAT-6 and QILSS families. Genomic DNA of all the strains was extracted and analyzed with an in-house microarray system to observe loss of other genes possibly implicated in attenuated virulence. Two of the strains presented novel deletions; the rest of the isolates showed deletions already reported for other *M. tuberculosis* strains. DR-689, a Beijing type *M. tuberculosis*

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strain isolated in Canada, showed an IS*6110* RFLP and a genomic deletion pattern similar to a San Francisco family of strains, although completely unrelated epidemiologically.
Conclusion: Genomic changes in *M. tuberculosis* seem to occur in a controlled manner and they are possibly related to changes in its pathogenic properties.
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Introduction

Tuberculosis (TB) is an infectious, primary pulmonary disease, caused by *Mycobacterium tuberculosis* that remains an important public health problem worldwide with approximately eight million new cases and three million deaths per year.[1] TB is considered the most important disease caused by a single infectious agent and its control has been difficult due to both the lack of an effective vaccine and the progressive development of resistance to anti-tuberculous drugs. Although it was considered that changes in the *M. tuberculosis* genome occur very rarely,[2] the recent determination of the complete genomic sequence and the development of microhybridization assays have made possible the comparative analysis of the whole genome of many *M. tuberculosis* clinical isolates[3,4] showing that large sequences of DNA can be deleted more frequently than expected. Loss of DNA fragments ranging from 189 to 10,982 bp have been reported,[5] most of them in intergenic regions, although the interruption of non-vital genes has also been reported.[6] *M. tuberculosis* clinical isolates vary in their ability to spread and produce clinical infection (e.g. highly transmissible isolates 210, CDC1551).[7,8] Additionally, it has been observed that the greater the deletions in their genome, the lesser the production of cavitary disease.[9] Comparative studies of virulent properties of these clinical isolates can shed information on the bases of pathogenicity of *M. tuberculosis*.

In the present work we determined the extension of the deletion in the PLC region of five *M. tuberculosis* clinical isolates as well as the presence of other deletions in their entire genomes.

Methods

Mycobacterial strains, culture and growth conditions

Five clinical isolates of *M. tuberculosis* with deletion of the *plcA–plcB–plcC* region were utilized in this study. *M. tuberculosis* DR-689 was previously described[10]; *M. tuberculosis* 97–448, 97–1389, 97–1177 and 97–1289 which are also ΔPLC, were kindly donated by Kristin Kremer from the Diagnostic Laboratory for Infectious Diseases and Perinatal Screening, National Institute of Public Health and the Environment, Bilthoven, The Netherlands. These strains have been partially characterized by Viana-Nero et al.[11]

Stock cultures of mycobacterial strains were prepared in Middlebrook 7H9 OADC at mid-logarithmic-phase growth and stored at −70 °C until needed. Colony Forming Units (cfu) for each stock culture was determined in triplicate using Middlebrook 7H10 agar.

Determination of the extension of the PLC locus deletions

In order to determine if there were other ORFs deleted than the PLC genes, we utilized a long-PCR assay to map this region based on the reported genes of *M. tuberculosis* H37Rv and *M. tuberculosis* CDC1551.[3,4] Oligonucleotides were designed using the DNA Star program (Madison, WI) and included LGB-L (5′–CCA GCC CGC GCC CGA GAT GTT-3′) located at Rv2347, LGB-U (5′–GGG GTC GGG GCC GTT GTT TAT G-3′) located at Rv2352, LGB-U3 (5′–GGG GGT GCT GGG GCG GGT AT–3′) located at Rv2352, and LGB-UP6 (5′–ATC ACT CCG GGC AAC TTC ATC–3′) located at Rv2357. Primers IS25 and IS26, located at the extremes of the IS*6110*[10], were used for internal sequencing analysis as it is known these strains possess an IS*6110* element interrupting the PLC genes (Fig. 1).

Long PCR assay

The PCR assay was carried out with 100 ng of genomic DNA in a PTC-200 thermocycler (MJ Research, Watertown, MA) by utilizing PCR assay kit XL (Perkin-Elmer) under the following conditions: 94 °C for 2 min and 15 cycles of 94 °C for 15 s, 65 °C for 30 s, and 68 C for 4.5 min. A second round of 14 cycles was carried out at 94 °C for 15 s, 65 °C for 30 s, and 68 °C for 4.5 min, adding 15 s every cycle. A final extension step at 72 °C for 10 min was

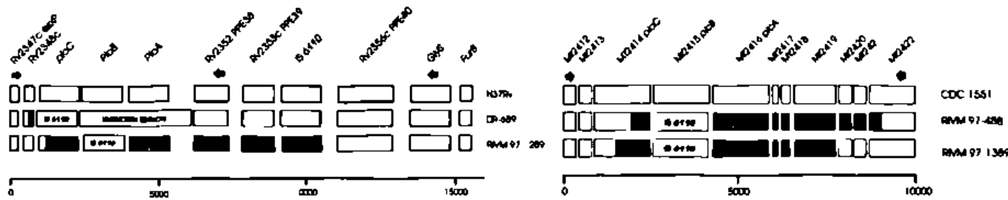

Figure 1 PLC region of *M. tuberculosis* strains H37Rv and CDC1551 and genetic changes in ΔPLC strains used in this study. Deleted regions are represented in black boxes; in gray boxes we show the presence of unexpected DNA sequences. In the left panel we present the physical map of the changes in strains DR-689 and RIVM 97-1289 compared to H37Rv, and in the right panel we present the changes in isolates RIVM 97-488 and RIVM 97-1389, which are more similar to CDC1551.

performed. The PCR products were applied to a 0.8% low-melting-point agarose gel, and after electrophoresis the gel slices containing the bands were excised and purified utilizing the GeneClean III (BIO 101, Inc., Vista, CA) kit. The DNA was quantified spectrophotometrically and stored at 4 °C. The products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide.

Microarray preparation

DNA microarrays were constructed with the *M. tuberculosis* ORFmer™ PCR Primer Pairs set (Sigma Genosys, The Woodlands, TX) which is based on the whole genome of *M. tuberculosis* strain H37Rv. The primer set was designed to amplify the most unique segment (⩽500 bp) of 3876/3918 open reading frames (ORFs) known to H37Rv.[12] The remaining 42 ORFs have extensive homology, making the primers redundant. PCR reactions were prepared in 50-μl reactions in 96-well plates as per manufacturer instructions and subsequent amplicons were evaluated by agarose gel electrophoresis to ensure they were the expected size. Only 29 (0.7%) ORFs were unable to be amplified, and were discarded from the experiment. Unpure products from the 96-well plates were transferred to 384-well plates, dried down and resuspended in a 1.5 M betaine/3X SSC solution as per Diehl et al.[13] Amplicons were spotted in duplicate sets onto GAPS II slides (Gamma Amino Propyl Silane II, Corning Life Sciences, Fisher Scientific Canada) using VersArray ChipWriter robotic arrayer (Bio-Rad Laboratories, Inc., Hercules, CA).

Sample preparation

DNA isolation was carried out by the method of van Embden et al.[14] The concentration and quality of DNA was measured with a Nanodrop (Nanodrop technologies, IL) and 2 μg were used for labeling. Whole genomic DNA from H37Rv (control) and the test strain were used as templates for direct incorporation of fluorescent nucleotide analogs (Cy3 and Cy5 dCTP, Amersham Biosciences) by a randomly primed polymerization reaction. Labeling reactions were carried out with Bioprime kits (Invitrogen, ON) as per manufacturer's instructions and contained 2 μg of template DNA, 20 μL of 2.5 × buffer/random primer mix, 5 μL dNTPs, 2 μl of concentrated Klenow (40 U/μL) and 3 μL Cy dye. Yeast tRNAs (100 μg) were added to the labeled DNAs and the samples were purified and concentrated with Micron YM30 filters (Millipore, ON.). The control and test strain were combined to a volume of 5 μL to which 60 μl DIG-Easy hyb buffer (Roche, Canada) was added. The mixture was heated at 95 °C for 5 min, cooled to 65 °C and applied to the microarray slide.

Microhybridization assays

Slides were prehybridized in DIG Easy Hyb buffer (Roche, Canada) with the addition of 0.1 mg/ml BSA for 1 h at 42 °C. The slides were washed in sterile distilled water, spun dry and used immediately for hybridization. The probe solution (65 μL) was applied and covered with a plastic cover slip (Hybri-slips, Sigma-Aldrich, ON) cut to grid size. The slide was then placed in a waterproof Genetix hybridization chamber (Genetix USA Inc.) for hybridization overnight in a 42 °C water bath. Post-hybridization washes proceeded as follows: slides were washed briefly in 2 × SSC+0.1% SDS that was pre-warmed to 42 C to remove the cover slips, transferred to a fresh container with the same wash buffer, incubated with ~95 rpm agitation for 5 min and subsequently washed with a second wash buffer (0.1 × SSC, 0.1% SDS) at room temperature

with ~95 rpm agitation for 10 min. Then the second wash buffer was replaced with wash buffer consisting of 0.1 × SSC, incubated at room temperature for 1 min, and transferred to a 0.01 × SSC wash buffer at room temperature for 15 s. Slides were then spun dry and taken for scanning.

Hybridization signal acquisition and data analysis

Microarray slides were scanned using the VersArray ChipReader laser scanner (Bio-Rad Laboratories, Inc., Hercules, CA) with independent excitation of the Cy3 and Cy5 fluorophores. The mean signal and background hybridization intensities were calculated for each DNA spot using the Array-Pro Analyzer analysis software (Media Cybernetics, Carlsbad, CA) and the net signal intensity for each spot was calculated as the difference between the mean signal intensity and the mean background intensity. These net signal intensities were normalized using loess normalization by subgrid. All hybridizations were repeated four times per experimental strain with half of these experimental replicates carried out as dye-reversal experiments.

To classify genes from the clinical strains as present or putatively divergent/absent, GACK analysis, which calculates a dynamic cutoff for deleted genes based on the shape of the signal-ratio distribution for each hybridization,[15] was performed using 0, 50 and 100% Estimated Probability of Presence (EPP) parameters. Output was binary, from which a consensus (⩾6/8 hybridizations per strain) was determined from the GACK output. Absent and present genes were visualized and clustered by strain using GeneMaths XT (Applied Maths Inc, Austin, TX,) software.

Results

Genetic characterization of the PLC locus deletion: The prefix Rv or Mt for ORFs described in the following text refers to the *M. tuberculosis* H37Rv or *M. tuberculosis* CDC1551 designation, respectively. In order to determine the extent of the deletions at the PLC locus in the studied *M. tuberculosis* strains, we amplified the nearby region using primers LGB-L, and LGB-U as shown in Fig. 1. Strain DR-689 presented an IS*6110* element with the right inverted repeat inserted in nucleotide 201993 corresponding to Rv2348c, and the complete loss of the *plcA–plcB–plcC* region. Attempts to amplify the other end of the IS*6110* were unsuccessful, although the neighboring ORFs Rv2352, Rv2353 and *glyS* genes were identified by PCR (see Fig. 1). *M. tuberculosis* RIVM 97-1289 presented the right inverted repeat inserted in 212117 which corresponds to an intergenic region close to PPE40 (Rv2356c). In none of the cases an inverted repeat produced by the transposition process of IS*6110* was observed.

The location of the IS*6110* insertions in the *plcC* gene of the rest of the strains from Kristin Kremer labs has already been published[11] and confirmed within our laboratory in this work; we also report the DNA sequence changes present in the other end (Fig. 1). Strain RIVM 97-488 has an IS*6110* element inserted in nucleotide 2630531 of the genome sequence of *M. tuberculosis* CDC1551 of an ORF denominated MT2421. The rest of the sequence is almost identical to the gene encoding for MT2422, with exception of two stretches of 64-bp. MT2422 gene is identical in this section to Rv2352 (PPE38) of H37Rv.

The right inverted repeat of the IS*6110* element in RIVM 97-1389 was located in nucleotide 2630123 (CDC1551 sequence) which corresponds to an intergenic region, followed by the complete ORFs of MT2420, MT2421 and MT2422.

Microhybridization analysis: Table 1 presents the list of putative ORFs deleted from the analyzed strains, with the exception of the *plcA–plcB–plcC* region that was described above. Most of them encode for hypothetical proteins although some genes implicated in metabolism were also deleted, e.g. *moeY*, *helZ*, etc. Upon analyzing the deleted ORFs, it was observed that many of these deleted ORFs have been associated with the insertion of IS*6110* elements. Interestingly, Rv2816c, deleted from DR-689, is the insertion site for the unique IS*6110* element in BCG Pasteur.[16] *M. tuberculosis* CDC1551 also shares two insertion sites with DR-689, Rv1758 and Rv2816c.

Some proteins with immunological or pathogenic importance were deleted, among them ESAT-6 like proteins, *esxQ* and *esxR*, which have been observed to be highly immunogenic. *M. tuberculosis* DR-689 presented the deletion of the fragment Rv2816c-2820c, which includes Rv2819c. The latter gene encodes for a protein that is downregulated in *M. tuberculosis* H37Ra, and it was the only protein associated to possible changes in virulence in that study.[17]

Comparing the deletions already published by other authors with those observed in the strains studied here we found a similar pattern to those reported in 100 strains in San Francisco.[5] However when comparing the IS*6110*-RFLP pattern for IS*6110* of our strains with those with similar deletions

Table 1 Deleted sequences, other than in the PLC region, found in the *M. tuberculosis* isolates.

M. tuberculosis ISOLAT	Rv no.	Gene designation	Possible function
RIVM 97-0488			
	Rv1354c	Hypothetical protein	Cellular processes: signal transduction mechanisms
	Rv1355c	*moeY*	Metabolism: coenzyme metabolism
	Rv1356c	Hypothetical protein	—
	Rv2101	*helZ*	Transcription, probable helicase
	Rv2271	Hypothetical protein	—
	Rv2272	Hypothetical protein	Probable transmembrane protein
	Rv2273	Hypothetical protein	Probable transmembrane protein
	Rv2274c	Hypothetical protein	—
	Rv2275	*Hypothetical protein*	—
	Rv2276	*cyp121*	Cytochrome P450 121 CYP121
	Rv2277c	Hypothetical protein	Metabolism: energy production and conversion
	Rv3017c	***esxQ***	**ESAT-6 like protein**
	Rv3021c	**PPE47**	—
	Rv3135	PPE50	—
	Rv3324c	*moaC3*	Metabolism: coenzyme metabolism
RIVM 97-1289			
	Rv0552	Hypothetical protein	—
	Rv1519	Hypothetical protein	—
	Rv1520	Hypothetical protein	Cellular processes: cell envelope biogenesis, outer membrane
	Rv2226	Hypothetical protein	—
	Rv2283	Hypothetical protein	—
	Rv2284	*lipM*	Probable esterase
	Rv2285	*Hypothetical protein*	—
	Rv2286c	Hypothetical protein	—
	Rv2353c	PPE39	—
	Rv3017c	***esxQ***	**ESAT-6 like protein**
	Rv3019c	**esxR**	**Secreted ESAT-6 like protein**
	Rv3021c	**PPE47**	—
	Rv3022c	PPE48	—
	Rv3142c	Hypothetical protein	—
	Rv3516	*echA19*	Metabolism: lipid metabolism
	Rv3517	Hypothetical protein	—
	Rv3737a (PPE)		
RIVM 97-1389			
	Rv1524	Hypothetical protein	Metabolism: carbohydrate transport and metabolism
	Rv1525	*wbbl2*	Probable rhamnosyl transferase
	Rv1526c	Hypothetical protein	Metabolism: carbohydrate transport and metabolism
	Rv1731	*gabD1*	Metabolism: energy production and conversion
	Rv2645	Hypothetical protein	—
	Rv2646	Hypothetical protein	DNA replication, recombination and repair
	Rv2652c	Hypothetical protein	—
	Rv2653c	Hypothetical protein	—
	Rv2655c	Hypothetical protein	—
	Rv2656c	Hypothetical protein	—
	Rv2657c	Hypothetical protein	—
	Rv2658c	Hypothetical protein	—
	Rv2659c	Hypothetical protein	DNA replication, recombination and repair

Table 1 (*continued*)

M. tuberculosis ISOLAT	Rv no.	Gene designation	Possible function
	Rv3017c	***esxQ***	**ESAT-6 like protein**
	Rv3019c	***esxR***	**Secreted ESAT-6 like protein**
	Rv3021c	PPE47	—
	Rv3135	PPE50	—
	Rv3651	Hypothetical protein	—
DR-689			
	Rv0065	Hypothetical protein	Possible maturase
	Rv0071	Hypothetical protein	Probable glutamine transport transmembrane ABC transporter
	Rv0072	Hypothetical protein	Probable glutamine transport ATP binding protein ABC transporter
	Rv0073	Hypothetical protein	Metabolism: Nucleotide transport and metabolism
	Rv1573	Hypothetical protein	
	Rv1574-Rv1584c	Hypothetical protein	This section is RD 3 -the phRV1 phage (Rv1573–1586c)
	Rv1585c	Hypothetical protein	DNA replication, recombination and repair
	Rv1586c	Hypothetical protein	Metabolism: carbohydrate transport and metabolism
	Rv1672c	Hypothetical protein	Metabolism: energy production and conversion
	Rv1758	*cut1*	—
	Rv1760	Hypothetical protein	—
	Rv1761c	Hypothetical protein	—
	Rv1762c	Hypothetical protein	—
	Rv1765c	Hypothetical protein	
	Rv2434c	Hypothetical protein	Poorly characterized: function unknown
	Rv2544	*lppB*	Possible conserved lipoprotein
	Rv2816c	Hypothetical protein	Poorly characterized: function unknown
	Rv2817c	Hypothetical protein	Poorly characterized: function unknown
	Rv2818c	Hypothetical protein	—
	Rv2819c	Hypothetical protein	Poorly characterized: function unknown
	Rv2820c	Hypothetical protein	Poorly characterized: function unknown

Those in bold show common deletions shared by at least two of the clinical strains.

reported by Tsolaki et al. there were no relationship among them, with the exception of DR-689, which possesses a deletion pattern similar to strains 47, 55, 67, 69, 95 and 97 (Fig. 2). All these strains have deletions of RD 105, 149, 150, 152, 181, and 207. All of them are Beijing strains although no epidemiological association was known. In order to corroborate these results, we checked these deletions in series of Beijing strains from the Dutch database,[18] including NLA 009501317, NLA009600299, NLA009800636, NLA000017383, NLA 009500592, NLA000017914, NLA000016362, NLA 000017583, and NLA009801500. The only strain sharing all the same chromosomal deletions was NLA009500592, which has an identical RFLP pattern to DR-689.

Discussion

Phospholipases C are well-recognized virulence factors of several microorganisms, including *Clostridium spp.*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, etc. They act by either destroying cellular tissues, as in *Clostridium* infections,[19] or by lysing the phagolysosomal membranes and releasing intracellular microorganisms in the cytoplasm, as in the case of *L. monocytogenes.*[20] In *M. tuberculosis,* there are several genes encoding phospholipase C-type enzymes: *plcA*, *plcB*, *plcC* and *plcD.*[3] They are very similar, except for *plcD*. The latter is missing or interrupted in many *M. tuberculosis* isolates including H37Rv. Reynaud et al. demonstrated the importance of

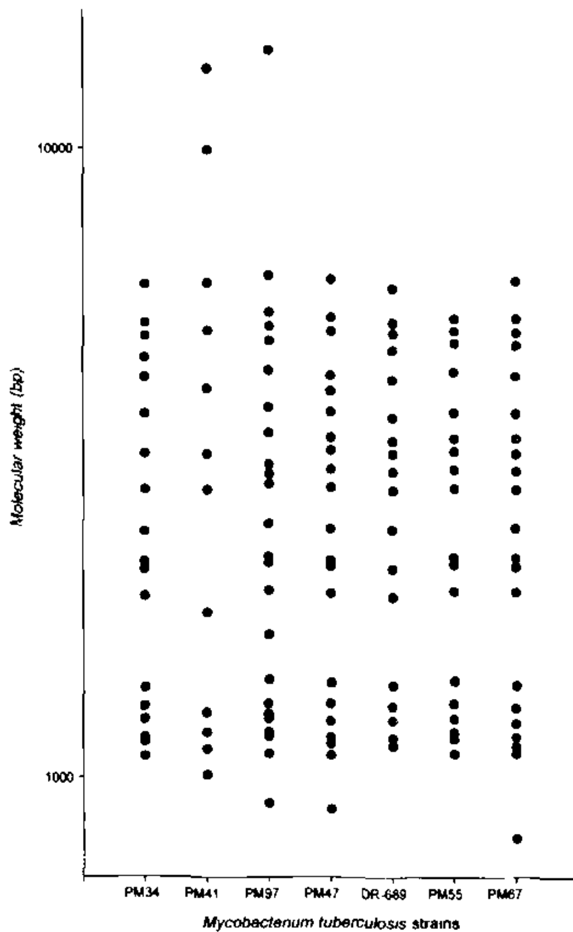

Figure 2 Comparison of the RFLP patterns for the IS*6110* element of *M. tuberculosis* strains with similar deletion patterns. Strains PM34 and PM41 possess the deletions RD105, RD149, RD152, RD181 and RD207 reported by Tsolaki et al.[5] The rest of the strains have all these deletions plus the loss of RD 150. The RFLP analysis of DR-689 was made in our lab. The RFLP data of the other strains were kindly donated by Dr. Peter Small and Dr. Midouri Kato Maeda.

phospholipases in virulence by inactivating either each individual ORF or all of them concurrently and testing their virulence to mice.[21] They observed that triple or quadruple inactivated mutants were impaired in their ability to multiply in the lungs and spleen of infected animals, suggesting their importance for the survival of *M. tuberculosis* in tissues. It has been observed that the more genomic deletions in a clinical isolate of *M. tuberculosis* the less cavitary disease is observed in humans[9]; in that study some of the deleted genes included the PLC genes. However, an in vitro test of the virulence of these strains was not performed.

The deletion of DNA fragments in *M. tuberculosis* clinical isolates has been reported previously with many of them being associated to IS*6110* elements transposition.[4,5,9,22] In this study, in all the cases, an IS*6110* inserted in the plc region was observed in the *ΔplcA plcB–plcC isolates*. As *hypothesized* previously,[10] it appears that deletions are produced by a homologous recombination mechanism since no inverted repeats associated to the transposition mechanism were observed in any case. In isolate DR-689, the three genes were completely absent, and in the rest of the isolates an incomplete copy of *plcC* was observed. In isolates RIVM 97–488 and RIVM 97 1389, we observed the presence of DNA sequences similar to MT2421 and MT2420 sequences of *M. tuberculosis* CDC1551. MT2420 shows homology to proteins of the ESAT-6 family and MT2421 is 95% identical to proteins of the QILSS family.[23] Both of them are deleted from H37Rv, and its presence varies in other *M. tuberculosis* clinical isolates.[4] MT2420 and MT2421 have many ortholog genes in *M. tuberculosis* H37Rv (Rv2346, Rv2347), *M. tuberculosis* CDC1551 (MT1066, MT1067, MT2411, MT2412, MT1841, MT1842), *M. bovis* (Mb1067, Mb1066) and *M. leprae* (Ml1181). More than insertions, the presence of these sequences seem to represent variation in gene arrangements among the *M. tuberculosis* isolates as reported before by *Fleischmann et al.*[4]

Although the transposition of IS*6110* elements is supposed to occur randomly, there exist several preferential sites, among them the phospholipase genes.[10] These sequences seem to attract the IS*6110* elements and when two of them are close enough, a homologous recombination may occur as seen in the studied isolates. The mechanism of attraction is not known, although repetitive or palindromic sequences are probably implicated. Other repetitive sequences such as those encoding for PE and PPE proteins, have also observed to be associated to deletions in the *M. tuberculosis* genome,[5] which strengthens this hypothesis. The role of IS*6110* transposition in the modeling of the *M. tuberculosis* genome is still a matter of study, although it could be important since it seems to affect the expression of neighboring genes.[24]

Tsolaki et al.[5] reported the analysis by microarray hybridization of 100 strains of *M. tuberculosis* isolated from the San Francisco area, and observed common deletion patterns that they called regions of difference (RD). Comparing the deletion patterns presented by our strains to those, similar deletion patterns were observed. A group of strains, which presented the most regions deleted including RD's 105, 149, 150, 152, 181 and 207,

were very similar in the IS*6110*-RFLP pattern to one of our strains (DR-689). The only commonality was that all of them belong to the Beijing family. The Beijing family is a widespread group of *M. tuberculosis* which is quite dominant in Asia and other parts of the world.[25] They share a similar multibanded IS*6110* RFLP patterns as well as an identical spoligotypye pattern.[18] DR-689 shares an almost identical RFLP pattern with the San Francisco isolates having all these deletions, although they are not epidemiologically related. It is possible that these strains have a common ancestor that already presented this deletions pattern and that they were acquired and transmitted long time ago. These strains represent a very well adapted group of bacteria since they are very dominant in certain areas (up to 85% in the Beijing area), and isolates with all these deletions can represent the most adapted of all them.

Free-living actinomycetes, a closely phylogenetically related group of bacteria to the *Mycobacterium* genus, possess giant chromosomes of about 6–8 mega bases, as in the case of *Streptomyces coelicolor.*[26] In contrast, *M. tuberculosis* has a chromosome of 4.1 million bases. It appears that when adapting to their hosts, the best way for the microorganisms to survive is to loss DNA material of non-essential genes, or even essential genes whose products can be provided by the host. This has been observed in *Mycobacterium leprae*[27] with a chromosome that has shrunk to about 3.1 mega bases, 1 mega base smaller than the *M. tuberculosis.*[3,4] *M. leprae* has even lost part of the genes encoding for enzymes of the Krebs cycle, which explains in part its inability to grow in vitro. It is possible that adaptation of the most successful *M. tuberculosis* strains to their human host includes the loss of part of their genome even at the expense of a decrease in their virulence, although this theory deserves future studies.

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Use of a colorimetric assay to measure differences in cytotoxicity of *Mycobacterium tuberculosis* strains

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Several techniques have been used to quantify the cytotoxicity produced by *Mycobacterium tuberculosis* bacilli on cell monolayers; however, they are semi-quantitative or time consuming. Herein, a method based on crystal violet (CV) uptake by THP-1 cell monolayers is described. This colorimetric method quantifies the cytotoxic effect as a function of the number of remaining cells after the infection with *M. tuberculosis*. Since this micro-organism is not stained by the dye, it does not produce a background that affects absorbance readings. As determined by CV assay (CVA), *M. tuberculosis* strain H37Rv destroyed 10.5 % of THP-1 cell monolayers at 24 h and 50.52 % at 72 h, while *M. tuberculosis* strains lacking the complete phospholipase C locus produced a reduced cytotoxic effect. The damage estimated by microscopy corresponded to the effect quantified by CVA. The results show that the use of CVA is a rapid, sensitive and reliable quantitative assay to measure the cytotoxicity of different *M. tuberculosis* strains.

INTRODUCTION

Mycobacterium tuberculosis is a bacterial pathogen that produces a detrimental effect on mammalian cell cultures. This effect can be due to the induction of cell apoptosis or necrosis leading to cell death. The phenotype is reported as a cytotoxic or cytopathic effect (Castro-Garza *et al.*, 2002; Danelishvili *et al.*, 2003; Dobos *et al.*, 2000; McDonough & Kress, 1995) and it can be roughly quantified by analysing the altered cell morphology (cell rounding and loss of monolayer integrity) (Daniel *et al.*, 2004; Fischer *et al.*, 1996), setting up a scale to estimate the percentage of rounded or detached cells (Read *et al.*, 1974) or counting the amount of degraded cells by electron microscopy (McDonough & Kress, 1995). A more precise quantitative assay, such as measuring lactate dehydrogenase (LDH) release by using a colorimetric kit, has also been reported (Danelishvili *et al.*, 2003; Dobos *et al.*, 2000). However, most of the above experimental procedures are only semi-quantitative or are time-consuming.

Abbreviations: CV, crystal violet; CVA, crystal violet assay; PLC, phospholipase C; WST-1, 4-[3-4(iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate.

There are other methods to quantify cytotoxicity; however, they are not practical for use with *M. tuberculosis* infection systems: exclusion or inclusion of vital dyes requires direct handling of samples, the release of radiolabelled substances increases the biosafety level, and the reduction of coloured compounds such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 4-[3-4(iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) and other tetrazolium salts by bacteria (Franzblau *et al.*, 1998; Gomez-Flores *et al.*, 1995) as well as macrophages (Ferrari *et al.*, 1990) in *in vitro* infection models would produce a high background and the results obtained would not be accurate.

Crystal violet (CV) is a triphenylmethane dye also known as gentian violet. The most commonly used application for this dye is as the primary stain in the Gram-staining procedure. Gillies *et al.* (1986) used CV to quantify the cell number in monolayer cultures as a function of the absorbance of the dye taken up by the cells. This method has been used with modifications for a wide number of applications, most of them to determine cytotoxicity or cell death produced by chemicals, drugs, or toxins from pathogens (Harhaji *et al.*, 2004; Shaik *et al.*, 2004;

Rothman, 1986), and to determine cell viability (Thomas *et al.*, 2004) or cell proliferation (Zivadinovic *et al.*, 2005) under different conditions.

A unique characteristic of *M. tuberculosis* bacilli is their outer lipid bilayer, which is the thickest biological membrane known. Because of its hydrophobic nature, the bacterial wall is not accessible to hydrophilic substances such as CV, and *M. tuberculosis* cannot be stained with the regular Gram-staining reagents, resulting in a remarkable Gram-neutral (neither positive nor negative) or Gram-ghost appearance of mycobacteria (Trifiro *et al.*, 1990). Therefore, the CV assay (CVA) becomes a very attractive method to quantify mycobacterial cytotoxicity, because CV will not react or be absorbed by the mycobacteria and any change in absorbance reading is due solely to the difference in the number of mammalian cells in a culture.

METHODS

Mycobacterial strains, culture and growth conditions. *M. tuberculosis* clinical isolates DR-689, DR-448 and DR-1289 lacking the *plcA-plcB-plcC* phospholipase C (PLC) locus were used for the present study. The extension of the deletions and the description of the changes in the locus are described in a previous paper (Vera-Cabrera *et al.*, 2007). *M. tuberculosis* H37Rv was included as a positive control for cytotoxicity. Stock cultures of mycobacterial strains were prepared in Middlebrook 7H9 broth supplemented with oleic acid/albumin/dextrose/catalase (OADC), grown to mid-logarithmic phase, and stored at −70 °C until needed. Cell density for each stock culture was determined in triplicate as c.f.u. using Middlebrook 7H10 agar.

Cell cultures. Human monocyte cell line THP-1 was maintained in RPMI 1640 medium (Gibco-BRL) supplemented with 10 % fetal bovine serum (FBS; Gibco-BRL) and 1 mM sodium pyruvate (Sigma). In order to transform the cells into macrophages, the cells were subcultured four times without sodium pyruvate and then seeded into 24-well microplates (Costar Corning) at a concentration of 5×10^5 cells per well in complete RPMI 1640 supplemented with phorbol-12-miristate-13-acetate (PMA; Calbiochem Biosciences) at a concentration of 6.25 ng ml^{-1}. Cell cultures were washed twice with RPMI 1640 every 48 h for no longer than 4 days. Before infecting the cell cultures, the number of viable cells per well was determined using the WST-1 (Roche Molecular Biochemicals) reduction assay (Ishiyama *et al.*, 1993), plotting the A_{545} obtained in a standard curve of cell density. WST-1 is a tetrazolium salt that is cleaved by viable cells into a coloured product that can be measured spectrophotometrically. A_{545} values have a linear relationship with the number of viable cells. The average cell density of three wells per plate determined by WST-1 was used as the number of cells per well for each plate.

CVA. Control and infected cell cultures were fixed at the time points indicated for each experiment in 10 % buffered formalin for 24 h at 4 °C. In all cases, the volume added to each well of the different solutions was 500 µl. Fixative solution was discarded and a 0.1 % aqueous CV solution was added to each well. The samples were incubated at room temperature for 30 min with gentle shaking. The plates were washed by submersion in flowing tap water for 15 min. Microplates were allowed to air dry and 0.2 % Triton X-100 in water was added to each well and incubated for 30 min at room temperature with gentle shaking to dissolve the dye. Then, 100 µl from each well was transferred into a fresh 96-well microplate and the A_{600} read in a microplate reader (EIA Microwell Reader II, Sigma).

Mycobacterial cytotoxicity assay. Before the beginning of the experiment, bacteria were thawed at 37 °C, mixed vigorously using a vortex, and diluted in tissue-culture medium to obtain the desired density to infect the cells with an m.o.i. of 1 : 10 (bacilli : cells). Bacterial c.f.u. from infected cultures was determined at the beginning and end of each experiment in 7H10 medium supplemented with OADC.

Macrophage (THP-1 cell line) monolayers were infected in triplicate with the *M. tuberculosis* strains tested. Infected cultures were incubated at 37 °C and 5 % CO_2 for up to 72 h. Every 24 h, the culture media from three wells from each strain and controls was discarded and the cells fixed in 10 % buffered formalin for at least 24 h at 4 °C. A different microplate was used for each time point, to avoid the effect of formaldehyde vapour on the rest of the cell culture. The cytotoxic phenotype was observed qualitatively as disruption of the confluent monolayers over time using a phase-contrast light inverted microscope Axiovert 25 (Carl Zeiss).

The quantitative analysis of cytotoxicity on the stained cultures infected with *M. tuberculosis* was based on the percentage of dead cells, employing the following equation:

Percentage dead cells = ((control A_{600} − sample A_{600})/control A_{600}) × 100

All experiments were repeated three times in triplicate.

RESULTS AND DISCUSSION

A_{600} readings were proportional to the number of cells per well. Regression and correlation analysis of data (1.5×10^4–5×10^5 cells per well) showed a slope value of 0.2019 with an r^2 value of 0.9465 (Fig. 1). This result confirms the utility of CVA to determine viability or cell culture density, as reported previously (Gillies et al., 1986).

In order to test a cytotoxic agent, we used Triton X-100, which is an anionic detergent that dissolves the cell membrane leading to cell death, and the effect depends on the concentration used. Triton X-100 produces a clear

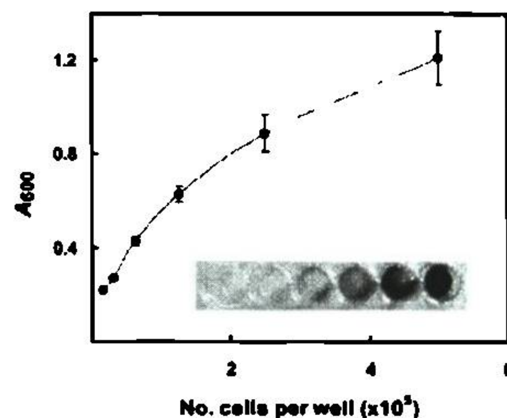

Fig. 1. CVA in the THP-1 cell line. Bars show mean ± SD of three independent experiments done in triplicate. A row of an experimental microtitre plate is shown in which the wells correspond to the numbers of cells.

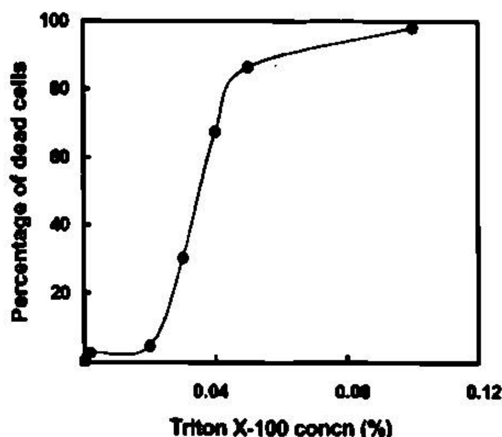

Fig. 2. Cytotoxic effect of Triton X-100 on the THP-1 cell line.

and increasing cytotoxic effect starting at a concentration of 0.05 % and reaching a maximum at 0.1 %, as measured with the CVA (Fig. 2). Regression and correlation analysis of data (three experiments each in triplicate) between 0.02 and 0.1 % Triton X-100 gave a slope value of 2821.5 and an r^2 value of 0.9865.

To test the ability of the method to quantify the cytotoxicity produced by *M. tuberculosis*, it was necessary that no background was caused by staining of the bacteria. As mentioned above, *M. tuberculosis* is not stained by CV and we found this feature very useful for this model. THP-1 cell monolayers were infected with a variable number of mycobacteria with an m.o.i. up to 10 : 1 (bacteria : cell), incubated for 6 h to allow the internalization of bacteria into the cells, and then immediately subjected to the CVA before any effect was produced by *M. tuberculosis* on the monolayers. Absorbance readings for all the different bacterial densities were very similar and no significant differences were found by an ANOVA one-way analysis ($P<0.05$). This result supports the use of the CVA to quantify mycobacterial cytotoxicity, since no background was produced by the mycobacteria. Accordingly, any change in absorbance readings in infected monolayers would be produced exclusively by *M. tuberculosis* cytotoxicity.

In order to compare the cytotoxicity of different *M. tuberculosis* strains using the CVA, THP-1 cell monolayers were infected with *M. tuberculosis* H37Rv strain and *M. tuberculosis* clinical isolates DR-689, DR-448 and DR-1289 (all of them lacking the PLC locus). *M. tuberculosis* H37Rv produced a cytotoxic effect on THP-1 cell monolayers that was observed microscopically 24 h post-infection. After 72 h, it could be visually estimated that around 50 % of the cell monolayer was destroyed (Fig. 3). On the other hand, *M. tuberculosis* DR-689 and DR-1289 did not disrupt the cell monolayer 72 h post-infection. Strain DR-448 produced cytotoxicity at 72 h as determined by CVA. The CVA values obtained from these experiments are shown in Table 1. All the strains infected the cell cultures, as shown by the c.f.u. values for each strain determined at the end of the experiment, which were: DR-689, 3.5×10^4; DR-448, 2.3×10^4; DR-1289, 2.2×10^4; and H37Rv, 3.7×10^4. The strain DR-689, with a yield of 3.5×10^4 c.f.u., produced no effect on cell cultures, while H37Rv, with a similar yield, had the highest cytotoxic effect. This result shows that the cytotoxicity is not only due to the intracellular growth but also the strain producing the activity. We have previously reported similar results with *M. tuberculosis* strains Erdman and CDC-1551, and *Mycobacterium bovis* BCG (Castro-Garza *et al.*, 1997). Although the clinical isolates used in this work lacked the PLC locus, they still had residual PLC activity (data not shown). There is a fourth phospholipase gene (*plcD*) in a different region of the genome. In H37Rv, the *plcD* gene is truncated and interrupted by a copy of the IS*6110* insertion sequence. Raynaud *et al.* (2002) have demonstrated the importance of phospholipases in virulence by inactivating either each individual ORF or all of them concurrently and testing for virulence to mice; however, phospholipases are not the only mycobacterial product able to produce cytotoxicity.

Recently, Takii *et al.* (2005) have reported the use of CV to test the pyrazinamide susceptibility of *M. tuberculosis* in a fibroblast-based assay, in which the host-cell viability is reflected by the state of the bacilli inside the host cells. In this study, we demonstrated that the CVA can quantify the cytotoxicity produced by *M. tuberculosis* in a THP-1 cell monolayer. The ability of the CVA to measure this effect with other mycobacterial species remains to be tested, although a biofilm produced by *Mycobacterium avium* can be stained with CV (Carter *et al.*, 2003). Mycobacteria other than *M. tuberculosis*, such as *Mycobacterium ulcerans* and *Mycobacterium marinum*, are natural pathogens of poikilothermic organisms (Trucksis *et al.*, 2005), but produce only localized skin infections in humans (Ranger *et al.*, 2006), while *Mycobacterium abscessus* is commonly associated with contaminated traumatic skin wounds and

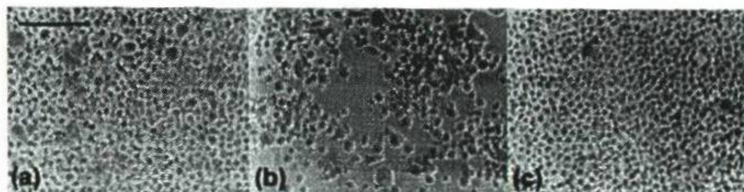

Fig. 3. Cytotoxicity of *M. tuberculosis* towards THP-1 cells. Cell monolayers 72 h post-infection are shown (×250). (a) Uninfected cells, (b) *M. tuberculosis* H37Rv infected cells, (c) *M. tuberculosis* DR-689 infected cells. Bar, 200 μm.

Table 1. Cytotoxicity of *M. tuberculosis* strains

Values show the precentage of dead cells, and represent the mean ± SD of three independent experiments done in triplicate.

Incubation time	*M. tuberculosis* strains			
	DR-689	DR-448	DR-1289	H37Rv
24 h	0	0	0	10.50 ± 1.05
72 h	0	20.51 ± 1.53	0	50.52 ± 6.32

with post-surgical soft-tissue infections (Petrini, 2006). These mycobacterial species exhibit cytotoxicity towards cell cultures or have a necrotic effect in their pathologies (Torrado *et al.*, 2007; Ranger *et al.*, 2006). It would be very interesting to apply the CVA to measure and compare their activities, either between species or among mutant strains of a single species, such as the *M. ulcerans* mutant lacking the mycolactone toxin, which has only been analysed by altered cell morphology (Daniel *et al.*, 2004).

The results of this work support the use of the CVA as a rapid, sensitive and reliable quantitative assay to measure the cytotoxicity of *M. tuberculosis* and to compare differences in activity between strains that could potentially be related to virulence.

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