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# Comparison of DNA Fragment Patterns between the Phenolic Glycolipid-Tb Producers and Non-Producers of *Mycobacterium tuberculosis*

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*Differences in ability to produce the specific phenolic glycolipid-Tb (PGL-Tb) antigen among* Mycobacterium tuberculosis *strains have been reported. One of the explanations would be the genotypic variation between the strains. In this study, we compared the DNA fragment patterns after digestion of DNA with various restriction enzymes between the PGL-Tb producing and non-producing strains of* M. tuberculosis. *Three clinical isolates of* M. tuberculosis *producing the PGL-Tb antigen detectable by thin-layer chromatography, and* M. tuberculosis *H37Rv and* M. bovis *BCG not producing the antigen were grown in Sauton medium. The chromosomal DNA was digested with the restriction endonucleases,* Eco *RI,* Sau3A *I,* BamH *I,* Xho *I,* Sma *I,* Pst *I,* Hinc *II, and* Bst *EII. Most of the restriction enzymes used gave no clear DNA bands or no DNA fragment common just to the PGL-Tb producing strains. When DNAs were digested with Bst EII, however, there was a 13 kb DNA fragment common to the PGL-Tb producing isolates of* M. tuberculosis *and not present in the H37Rv strain and* M. bovis *BCG. This study thus suggests that there might be differences in DNA fragment patterns between the PLG-Tb producing and non-producing strains of* M. tuberculosis.

**Key Words:** *Mycobacterium tuberculosis,* tuberculosis, phenolic glycolipid-Tb, antigen, endonuclease, DNA

The recent isolation and chemical characterization of specific phenolic glycolipid of *Mycobacterium tuberculosis* (PGL-Tb) has brought an unprecedented opportunity to elucidate the pathogenesis and to develop a specific serodiagnostic tool of tuberculosis (Daffe 1989; Daffe *et al.* 1978). The PGL-Tb antigen did not seem cross-reactive with antisera against other phenolic glycolipid antigens from *M. leprae, M. Kansasii, M. bovis, and M. marinum,* thus indicating the high specificity (Papa *et al.* 1989a; Torgal-Garcia *et al.* 1989).

Initial sudies indicated that about 95~98% of confirmed pulmonary tuberculosis patients had significant level of antibodies to the antigen (Torgal-Garcia *et al.* 1988 & 1989; Martin-Dasabona *et al.* 1989). However, only about 20% of tuberculosis patients had IgM antibodies to PGL-Tb, and only one of 11 isolates expressed the antigen detectable by thin-layer chromatography (TLC) (Daffe *et al.* 1991). In another study, however, all clinical isolates produced the PGL-Tb antigen detectable by immunoassays using anti-PGL-Tb antibodies, but the antigen was not detectable in the *M. tuberculosis* H37Rv strain even with the immunoassay (Papa *et al.* 1989b).

These results suggests that there might be differences in the ability to produce the PGL-Tb antigen among clinical isolates of *M. tuberculosis.* One possible explanation for the phenomenon would be the genotypic variation by mutation among strains of *M. tuberculosis.* This study was thus initiated to

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compare DNA fragment patterns after digestion of DNA with various restrction endonucleases between the PGL-Tb producers and non-producers.

## MATERIALS AND METHODS

### Organisms

Three strains of *M. tuberculosis* (No. 293, 343, 413) were selected as the PGL-Tb producers by TLC among clinical isolates. The clinical isolates of *M. tuberculosis* were confirmed based on the colony morphology and growth characteristics, followed by the presence of the 123 bp DNA fragment specific to *M. tuberculosis* by polymerase chain reaction (PCR) (Eisenach *et al.* 1990). *M. tuberculosis* H37Rv and *M. bovis* BCG strains were obtained from the Korean Institute for Tuberculosis Research, Seoul, Korea and used as PGL-Tb non-producers. All organisms were maintained in the Ogawa solid egg medium until used.

### Polymerase chain reaction

The primers and protocols reported by Eisenach *et al.* (1990) were employed for detecting 123 bp sequence of *M. tuberculosis* DNA from the clinical isolates, with minor modifications as described previously (Cho *et al,* 1990). Briefly, the primers were synthesized by a DNA synthesizer (Applied Biosystems, Foster City, Calif.) and the sequences were as follows: 5'-CCTGCGAGCGTAGGC-GTCGGT'3 (P1); 5'-CTCGTCCAGCGCCGCTTCGG-3' (P2). The reaction mixture (final volume: 50 $\mu$l) contained 10 mM of Tris-HCl (pH 8.3), 50 mM of KCl, 1.5 mM of $MgCl_2$, 0.01% (w/v) of gelatin, 100 $\mu$M of dNTP, 1.25 unit of *Taq* polymerase (Perkin Elmer, Co., Norwalk, Conn.), 100 *p*M of each primer, 100 *p*g of target DNA (below). Three steps were repeated 30 times in an automated thermal cycler (Easy Cycler; Ericomp, Inc., San Diego, Calif.); denaturation at 95°C for 1 min, annealing at 60°C for 2 min, and extension at 72°C for 3 min. After the reaction, the PCR product was examined by polyacrylamide gel electrophoresis and subsequent ethidium bromide staining of the gel.

### Detection of the PGL-Tb antigen

*M. tuberculosis* H37Rv and clinical isolates were grown in a Sauton medium for 4~6 weeks and harvested by centrifugation after heat-killing. The whole cells were washed twice by centrifugation with phosphate buffered solution, pH 7.2, and lyophilized. Lipids were then extracted with the $CHCl_3$: $CH_3OH$ (2:1) solution (30 ml/g dry weight) at 50°C for 48 h and dried under a $N_2$ flow. The lipid extracts dissolved in $CHCl_3$ were applied to florisil column and eluted with two-bed volumes of $CHCl_3$, 2%, 5%, and 10% $CH_3OH$ in $CHCl_3$, respectively. The lipid fraction eluted with 10% $CH_3OH$ in $CHCl_3$ was then examined by TLC. The standard PGL-Tb antigen was kindly provided by M. Daffe (Centre de Recherche de Biochimie et Genetique Cellulaires du CNRS, Toulouse, France) and was included in TLC analysis. The glycolipid spot was visualized with 10% $H_2SO_4$ spray.

### Preparation of chromosomal DNA

Each strain of *M. tuberculosis* and *M. bovis* BCG grown in a Sauton medium for 2~4 weeks was harvested by centrifugation and washed with Tris-EDTA-NaCl (TEN) buffer. The whole cells (about 3g, wet weight) were then treated with lysozyme (final concentration; 5 mg/ml) at 37°C for 1 h, followed by the proteinase K (2 mg/ml) and 1% (w/v) sodium dodecyl sulfate (SDS) treatment at 55°C for 16 h. The chromosomal DNA was extracted by phenol /chloroform and precipitated with ethanol at -20°C by the standard method (Sambrook *et al.* 1989). The precipitated DNA was pooled with a glass rod, dried, and resuspended with Tris-EDTA (TE) buffer (pH 8.0). Each DNA solution was treated with ribonuclease A and precipitated by ethanol at -20°C after phenol/chloroform extraction. The DNA pellets were dried and resuspended in 500 $\mu$l of TE buffer (pH 7.4) before storage at -20°C.

### Restriction endonuclease digestion and analysis

The purified DNA from each organism was digested in a 100 $\mu$l reaction mixture under conditions specified by the manufacturer with the follwing restriction enzymes: *Eco* RI, *Sau3A I, Bam* HI, *Xho* I, Sma I *Pst* I, *Hinc* II, and *Bst* EII (New England Biolabs, Inc., Beverly, Mass). The DNA fragments were separated by submarine horizontal gel electrophoresis in a horizontal gel box of 10x18 cm (Hoefer Scientific Instruments, San Francisco,Calif.). The gel contained 1% (w/v) agarose (Bio-Rad, Richmond, Calif.) in Tris-acetate-EDTA buffer. The gels were stained for 30 min in an ethidium bromide sholution (0.4 $\mu$g/ml in distilled water) and photographed on the UV transilluminator through Kodak 23A wratten gelatin filter.

## RESULTS

### RCR for *M. tuberculosis* isolates

The purified DNA from three clinical isolates was examined by PCR for the presence of the 123 bp sequence, which is specific to *M. tuberculosis*. As shown in Fig. 1, *M. tuberculosis* H37Rv and the clinical isolates strain 413, 343, and 293 of *M. tuberculosis* gave the 123 bp DNA products, thus confirming the clinical isolates as *M. tuberculosis* ; in contrast, *M. kansaii* gave no amplification of the 123 bp DNA fragment.

### Detection of the PGL-Tb antigen

When the lipid extracts from the three clinical isolates and *M. tuberculosis* H37Rv were examined for the presence of the PGL-Tb antigen, the clinical isolates had the antigen detectable by TLC (Fig. 2). However, the *M. tuberculosis* H37Rv strain did not produce the PGL-Tb antigen at the same culture conditions as the other clinical isolates.

### Comparison of cultivation time for chromosomal DNA

Since *M. tuberculosis* requires a long incubation period to grow, there may be a spontaneous release of nuclease(s) from dying cells, which degrades the chromosomal DNA. Therefore, the cells were havested at two different times, and the purified DNA was examined by agarose gel electrophoresis for the nuclease activity. As shown in Fig. 3, DNA from 6~8 week old cultures had a smear along the lanes of agarose gel, indicating that an autologous nuclease digested part of chromosomal DNA. The optimal incubation period for DNA preparation was two-weeks for *M. bovis* BCG and 3~4 weeks for *M. tuberculosis*.

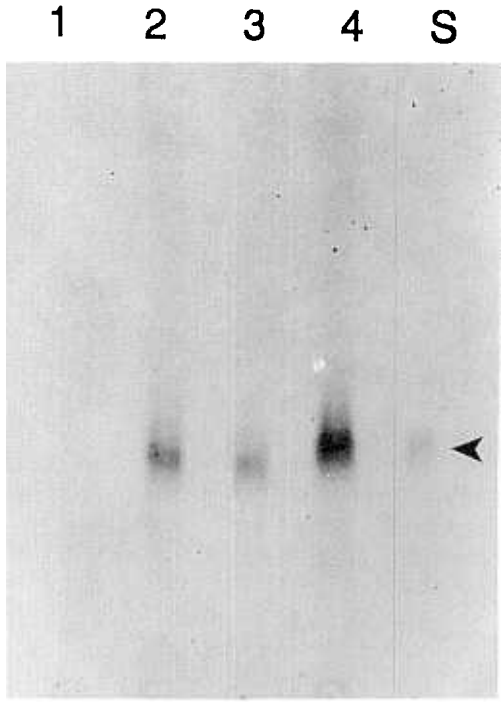


*Fig. 2. TLC of lipids purified from clinical isolates and H37Rv strain of* M. tuberculosis. *Chromatogram was developed in $CHCl_3$: $CH_3OH$ (95:5) and lipids were located with 10% $H_2SO_4$. Lanes 1.:* M. tuberculosis *H37Rv; 2: strain 293; strain 343; 4: strain 413; and S: standard PGL-Tb.*

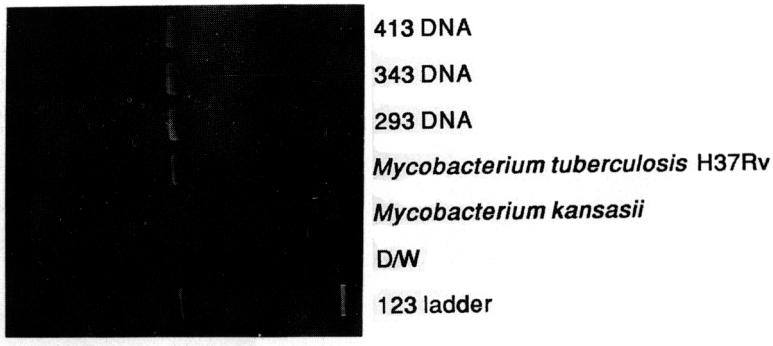


*Fig. 1. Amplification of a 123 bp fragment in purified DNA from clinical isolates and the H37Rv strain of* M. tuberculosis, *and M. Kansasii.*

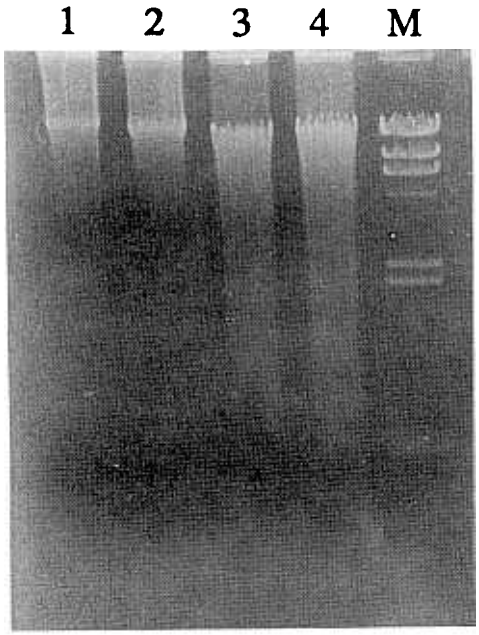


*Fig. 3. Agarose gel electrophoresis pattens of DNA from* M. bovis *BCG (lanes 1 and 3) and* M. tuberculosis *(lanes 2 and 4) of a fresh culture (2 weeks for lane 1 and 3~4 weeks for lane 2) and of an old culture (8 weeks for lanes 3 and 4). Lane M: size marker contains λDNA digested with Hind III; 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kb from top.*

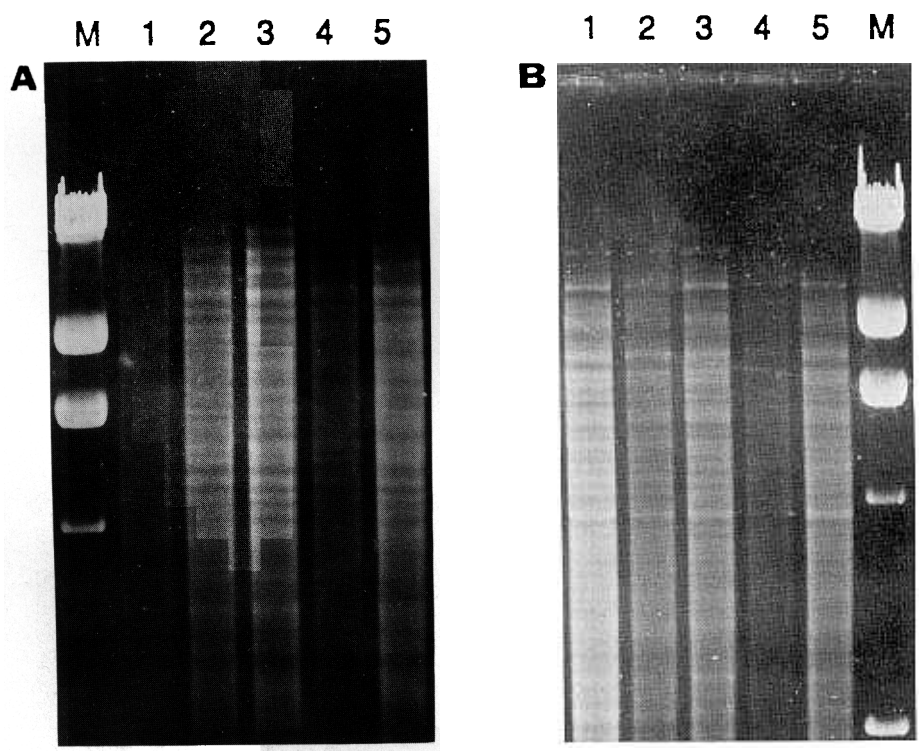


*Fig. 4. Comparison of agarose gel electrophoresis patterns of chromosomal DNA digested with* Xho *I (A) and* Sma *I (B) between PGL-Tb non-producers,* M. bovis *BCG (lane 1) and* M. tuberculosis *H37Rv (lane 2) and producers,* M. tuberculosis *strains 293 (lane 3), 343 (lane 4), and 413 (lane 5). Lane M: size marker contains λDNA digested with* Hin *d III; 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kb from top.*

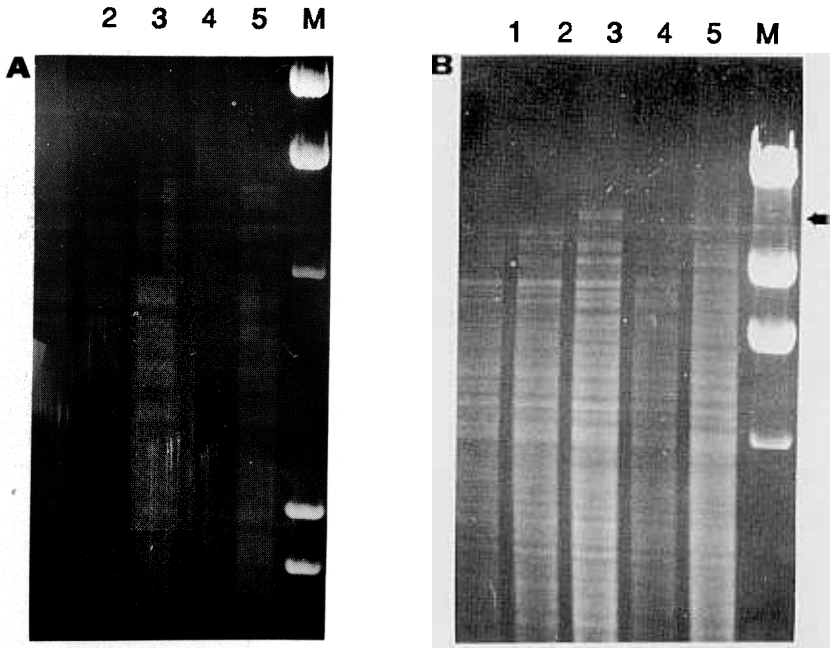


***Fig. 5.*** *Comparison of agarose gel electrophoresis patterns of chromosomal DNA digested with* Hinc *II (A) and* Bst *EII (B) between PGL-Tb non-producers,* M. bovis *BCG (lane 1) and* M. tuberculosis *H37Rv (lane 2) and producers,* M. tuberculosis *strains 293 (lane 3), 343 (lane 4), and 413 (lane 5). A 13 kb DNA fragment present only among PGL-Tb producing strains of* M. tuberculosis *is indicated by an arrow. Lane M: size marker contains λDNA digested with* Hin *d II; 23.1, 9.4, 6.6, 4.4, 2.3, 2.0, and 0.56 kb from top.*

**Restriction endonuclease analysis**

The purified DNA from *M. tuberculosis* H37Rv, 293, 343, and 413 strains and *M. bovis* BCG were digested with various restriction enzymes to compare the DNA fragment patterns between the strains. When DNA was digested with *Eco* RI, *Sau* 3AI, and *Bam* HI, the DNA fragments gave no clear bands on agarose gel with which to compare the patterns between the organisms examined (data not shown). The digestion of DNA with *Xho* I (Fig. 4A), *Sam* I (Fig. 4B), and *Pst* I (data not shown), gave clear DNA fragments in agarose gel, but there was no difference in DNA fragment patterns between PGL-Tb producing and non-producing *M. tuberculosis* strains.

Although there were minor differences in DNA band patterns between the organisms after digestion with *Hinc* II (Fig. 5A), no common DNA band was found among the PGL-Tb producing clinical isolates of *M. tuberculosis*. Interestingly, however, a 13 Kb DNA fragment was common among the PGL-Tb antigen producers and not present in non-producers when the DNAs were digested with *Bst* EII (Fig. 5B); thus, indicating that there might be difference in DNA fragment patterns between the two groups of *M. tuberculosis*.

## DISCUSSION

The isolation of the PGL-Tb antigen from the Canetti strain of *M. tuberculosis* by Daffe *et al.* (1987) happened to be an unusual finding, because many efforts have been made and failed to isolate such a specific antigen from *M. tuberculosis* H37Rv or H37Ra, the two most widely used prototype strains. The major question became then why the H37Rv or H37Ra strains did not have the ability to produce the PGL-Tb antigen under the same culture conditions in which the Canetti strain and

some clinical isolates of *M. tuberculosis* had. The two possible explanations would be the phenotypic or genotype variation between strains of *M. tuberculosis*.

Changes in colonial morphology or growth characteristics of mycobacterial culture have not been unusual. In *M. tuberculosis*, colonies forming extensive serpentine cords, in which cord factor is a major chemical component, were associated with greater virulence (Yegian and Kurung 1952). Barrow and Brennan (1982) reported that C-mycoside antigen was not found in rough colonies of *M. intracellulare*, although smooth colonies had the antigen in the surface; this suggests that a variation in colonial morphology might be due to a change in certain components of the cell wall. This assumption was supported by a recent study from Bellisle *et al.* (1989) who reported that lipooligosaccharides were associated with smooth colonial morphology in *M. kansasii*. Since the rough colonies of *M. avium* complex and *M. kansasii* were stable in a subsequent culture, this might be due to a mutation of the smooth type of isolates. However, no study has yet addressed the genetic basis of the smooth and rough variations in colonies and growth characteristics or the absence or presence of certain antigens in mycobacteria.

The recent development in DNA hybridization and restriction endonuclease analysis made it possible to closely examine the genetic relationship between species or strains of mycobacteria. Within strains of the *M. tuberculosis* complex, the DNA homology was greater than 95% (Baess 1972; Bradley 1973; Imaeda 1985), and DNA fragment patterns after digestion with various restrction endonuclease were indistinguishable in agarose gel electrophoresis (Eisenach 1986; Imaeda 1985). In this study, we also tried to correlate the ability to produce the PGL-Tb antigen with a certain genetic element by restriction endonuclease analysis. However, we could not find any significant difference in DNA fragment patterns between the PGL-Tb producing and non-producing strains after digestion with 8 restriction enzymes except *Bst* EII. Although the *Bst* EII digestion of DNA from the PGL-Tb producers showed a distinct 13 Kb DNA fragment which was not found in the PGL-Tb non-producers, the significance of the DNA fragment in relation to the PGL-Tb synsthesis was still unknown. Only cloning the DNA fragment into a suitable host would answer the question of whether or not it is associated with the biosynthesis of the PGL-Tb antigen. Recent success in cloning genes for the biosynthesis of glycopeptidolipid antigen of *M. avium* complex (Belisle *et al.*1990) would make it possible in the future to clone the genes coding molecules required for the synthesis of phenolic glycolipid antigens including the PGL-Tb antigen of *M. tuberculosis*.

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