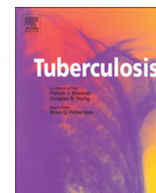
Tuberculosis 91 (2011) 117–126

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: http://intl.elsevierhealth.com/journals/tube



MOLECULAR ASPECTS

Deciphering the role of IS*6110* in a highly transmissible *Mycobacterium tuberculosis* Beijing strain, GC1237

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ARTICLE INFO

Article history:
Received 29 October 2010
Received in revised form
20 December 2010
Accepted 28 December 2010

Keywords:
Beijing genotype
IS*6110*
Promoter activity
Rv2179c

SUMMARY

The capacity of infection and the ability of *Mycobacterium tuberculosis* strains belonging to the Beijing family to spread rapidly probably result from genetic advantages and unidentified mechanisms of virulence not yet thoroughly investigated. Among the mechanisms proposed to be responsible for the varying virulence phenotypes of *M. tuberculosis* strains we find IS*6110* insertions, genetic reorganizations and deletions, which have strong influences on fitness.

Beijing family is one of the lineages with the highest number of copies of IS*6110*. By studying genetic markers characteristic for this lineage, here we have characterized the clinical isolate *M. tuberculosis* GC1237 strain responsible for important epidemic outbreaks in the Gran Canary Island. We have identified and analyzed each point of insertion of IS*6110* using a bacterial artificial chromosome (BAC) library of this strain, in addition to the use of other approximations.

Nineteen copies of IS*6110* have been localized in GC1237 genome of which, four copies of IS*6110* can act as a promoter and we have focused in the characterization of one copy located 31 bp upstream of the essential gene Rv2179c and compared to the reference strain H37Rv.

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1. Introduction

Strains of the Beijing genotype were first described in China and neighboring countries in 1995.[1] They are widespread in many regions of the world and frequently cause epidemic outbreaks. Different studies have indicated that one-third of global tuberculosis (TB) cases is caused by Beijing family strains assigning this lineage to one of the most successful mycobacterial families in terms of morbidity and mortality.[2] The predominance of the Beijing lineage probably results from genetic advantages, including unidentified virulence factors and the modulation of specific host responses not yet thoroughly investigated. There are some studies that relate hypervirulence of W-Beijing strains with production of phenolic glycolipid PGL,[3,4] which is a putative virulence factor that attenuates the host's innate immune response and ability to control infection.[4] The *pks15/1* locus, described to be polymorphic among members of the *Mycobacterium tuberculosis* complex,[5] is involved in the biosynthesis of the glycolipid PGL and although it is characteristic of Beijing strains to have an intact *pks15/1*, not all members of this family are producers of PGL.[5–7]

Nowadays, Beijing strains are currently attracting considerable worldwide attention because they display important pathogenic features.[8,9] These strains are often associated with multi-drug resistance as the well-known case in New York in the 1990s caused by the W strain.[10–12] The clinical isolate *M. tuberculosis* GC1237 which belongs to the Beijing family has been responsible for epidemic outbreaks in the Gran Canary Island since its first introduction in the community in 1993.[13] Nowadays, this strain continues being predominant in the area due to its rapid and successful dissemination within the community. The increased capacity of infection and the high success rate of the Beijing family to spread rapidly could be a consequence of genetic advantages and unidentified mechanisms of virulence not yet thoroughly investigated.

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doi:10.1016/j.tube.2010.12.007

IS*6110* insertions, genetic reorganizations and deletions are some of the mechanisms proposed to be responsible for differences in the virulence phenotypes of *M. tuberculosis*.

It has been demonstrated that IS*6110* may increase the expression of neighboring virulence genes by generating new promoter sequences capable of driving their expression.[14,15] IS*6110* can upregulate downstream genes through an outward-directed promoter in its 3′ end. This activity has been demonstrated for upregulation of the two-component system PhoP/PhoR.[15] Promoter activity was orientation dependent and was localized within 110-bp fragment adjacent to the right terminal inverted repeat.[14] The fact that the Beijing lineage contains a larger number of IS*6110* copies than other lineages[16] could be related with the special characteristics of this family in terms of virulence and capacity for rapid dissemination.

In this study we have classified the *M. tuberculosis* GC1237 within the Beijing family.[6,17] We also identified the locations of IS*6110* insertions in this strain and compared them to the IS*6110* insertions in two Beijing strains, 210 and W.[18] We studied the orientation and distance to neighboring genes of each copy focusing our study in one copy of IS*6110* that is acting as a promoter located 31 bp upstream the hypothetical essential gene Rv2179c.

2. Material and methods

2.1. Bacterial strains, culture media, and growth conditions

The clinical isolate *M. tuberculosis* GC1237 and the reference *M. tuberculosis* H37Rv strains were used. Mycobacterial strains were grown in Middlebrook 7H9 broth supplemented with albumin-dextrose-catalase and 0.05% Tween 80 or in Middlebrook 7H10 medium Bacto agar supplemented with oleic acid-albumin-dextrose-catalase (Difco Laboratories, Detroit, Mich.) and 0.05% Tween 80.[19] Liquid cultures were grown to logarithmic phase to be used for macrophage infection in vitro and for mycobacterial RNA extraction. *Escherichia coli* DH10B cultures were grown in Lurina-Bertani (LB) medium supplemented with chloramphenicol (12.5 μg/ml) in order to isolate the BACs. All of the strains were grown at 37 °C pBeloBAC11 plasmid was used for the construction of the BAC library of *M. tuberculosis* GC1237. pFPV27-int, derived from pFPV27,[20] was used for the construction of the GFP strains.

2.2. Cell culture and infections

MH-S murine alveolar macrophages were obtained from HPA culture collections. Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 4 mM L-glutamine. Infections were performed during 4 h at a multiplicity of infection (MOI) of 10 bacteria per cell. After incubating with bacteria, cells were washed three times with PBS, and cultured in complete medium during the time indicated for each experiment.

2.3. Isolation of genomic DNA

Genomic DNA of mycobacterial strains was isolated using the CTAB method as previously described by van Soolingen et al.[21]

2.4. Construction and characterization of BAC library of M. tuberculosis GC1237 strain

The construction of BACs was carried out as previously described by Brosch et al.[22] Briefly, the *M. tuberculosis* GC1237 library was constructed by ligation of partially digested *Hin*dIII fragments (50–125 Kb) into pBeloBAC11 plasmid. From almost 10,000 clones obtained 2000 were placed into 96-well plates and stored at −80 °C. Plasmid preparations of recombinant clones for sequencing reactions were prepared in 96-well plates containing an overnight culture in 250 μl of 2X yeast-tryptone medium with 12.5 μg/ml of chloramphenicol.

BAC DNA extraction was done as previously described by Birnboim et al.[23] with minor modifications. Briefly, 100 ml of BAC-transformed *E. coli* was prepared in LB medium containing 12.5 μg/ml chloramphenicol, and the cultures were grown overnight at 37 °C with vigorous and continuous agitation. Then, the bacterial cells were collected by centrifugation. The bacterial pellet was softly resuspended in a solution of 5 ml of 50 mM glucose 10 mM EDTA, 25 mM Tris pH 8, 200 mg lysozime was added to the tube and a solution of 4 ml of ice-cold NaAc pH 4.8 was added to the mixture. The tube was placed on ice and the precipitated debris was removed by centrifugation. Then, 14 ml of chloroform/isoamilic alcohol (24/1) were added to the supernatant and after centrifugation the aqueous phase was transferred to a new microfuge tube. DNA of the BACs was precipitated adding isopropanol and finally obtained by centrifugation.

End-sequencing reactions were performed with Taq DyeDeoxy Terminator cycle sequencing kit (Applied Biosystems) by using a mixture of 13 μl of BAC DNA solution, 2 μl of primer SP6-BAC1 or T7-BAC1 (supplementary material), 2.5 μl of Big Dye, and 2.5 μl of a 5X buffer (50 mM $MgCl_2$, 50 mM Tris). Thermal cycling was performed on a thermocycler (MJ Research Inc.) with an initial denaturation step of 60 s at 95 °C, followed by 26 cycles of 15 s at 96 °C, 15 s at 56 °C, and 4 min at 60 °C. DNA was precipitated with 70 μl of 70% ethanol, centrifuged, rinsed with 70% ethanol, dried and resuspended in 2 ml of formamide-EDTA buffer. The sequencing was performed on a model 373A automatic DNA sequencer (Applied Biosistems) for 12–16 h. Sequence data were transferred to Digital workstations and edited with TED software from the Staden package.[24] Edited sequences were compared using BLAST programs to the *M. tuberculosis* H37Rv sequence database.

2.5. Identification of genomic deletions and analysis of pks15/1 region

The study of the genomic deletions of the regions of difference RD105, RD142, RD150, RD181 and RD207 in *M. tuberculosis* GC1237 strain, which identify and phylogenetically sub-classify the Beijing lineage, was performed by PCR. Other genomic deletions (RD108, RD110a, RD127, RD129, RD139BW, RD149, RD152, RD165, RD166 and RD182a) also were analyzed. The primers used in these amplifications were as described elsewhere.[6] The PCR was carried out in a total volume of 50 μl, containing 0.5 μg of DNA, 5 μl of 10x PCR buffer, 200 μM dNTPs, 12.5 pmol of each primer and 1 U Taq Gold polymerase (Roche). Before the amplification, the template was initially denatured by incubation at 94 °C for 9 min then the amplification was performed for 35 cycles of 94 °C for 30 s, corresponding annealing temperature for 30 s, and 72 °C for 1–2 min depending on the amplified product. After the last cycle, the samples were incubated at 72 °C for 10 min. The RD deletions were confirmed by DNA sequencing using H37Rv as reference genome.

The pks15/1 polymorphism in this strain was determined by PCR and sequencing as previously described in Ref.[6]

2.6. Location of the copies of IS6110 insertion sequence

The study of the presence of IS*6110* insertion sequence in GC1237 BACs library was carried out by PCR as previously described in this study with the specific primers of this sequence, Gab 1 and Gab 2 (supplementary material).

We used Ligation-mediated PCR (LMPCR) to locate the copies of IS*6110* in GC1237 strain as described by Prod'hom et al.[25] This

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