Identification of one insertion site of IS6110 in Mycobacterium tuberculosis H37Ra and analysis of the RvD2 deletion in M. tuberculosis clinical isolates

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Mycobacterium tuberculosis H37Rv and the attenuated strain H37Ra were used as a model to investigate the virulence properties of M. tuberculosis at the genetic level. To test whether transposition of the insertion element IS6110 might be involved in the loss of virulence of strain H37Ra, the nucleotide sequence of a differential IS6110-positive restriction fragment detected in strain H37Ra, but not in strain H37Rv, was determined. The region flanking the 3' end of the IS6110 element showed partial sequence homology with internal sequences of M. tuberculosis H37Rv genes plcA, plcB and plcC, each one coding for phospholipase C, a well-known bacterial virulence factor. A 100% homology was found between the IS6110-flanking region and an internal sequence of M. bovis plcD, a further phospholipase C gene that is truncated and partly lost in strain H37Rv in the so-called RvD2 deletion. This result indicates that the differential restriction fragment of strain H37Ra originally stems from the plcD gene interrupted by the insertion of the IS6110 element. The occurrence of the RvD2 deletion was then investigated in 45 clinical isolates of M. tuberculosis by Southern blot. The deletion was demonstrated in 15 isolates; the entire RvD2 region (including the undisturbed plcD gene) was detected in 29 isolates, whereas only one isolate showed the RvD2 region in which the plcD gene was interrupted by an IS6110 insertion. It is concluded that disruption of the plcD gene and deletion of the RvD2 region by IS6110 insertion have no consequence for the virulence of M. tuberculosis, although the role of phospholipase C as a virulence factor of M. tuberculosis remains debatable.

Introduction

The mechanisms by which Mycobacterium tuberculosis establishes progressive disease in man are not well understood, although various strategies have been developed to identify M. tuberculosis virulence factors [1–5]. A promising experimental model for the investigation of M. tuberculosis virulence factors at the gene level is represented by the virulent strain H37Rv and the attenuated mutant H37Ra, originally derived from the classical M. tuberculosis strain H37 [6, 7]. Several studies have tried to identify the genetic basis for the attenuation of M. tuberculosis H37Ra but the reason(s) for the decreased virulence of M. tuberculosis H37Ra have not been determined.

Transposition of the insertion element IS6110 is a major force in generating genome plasticity of the tuberculosis bacillus [8, 9], including strains H37Rv and H37Ra. By comparing the IS6110-based fingerprints of strains H37Rv and H37Ra, an earlier study reported that the strain H37Ra differs from strain H37Rv in a number of IS6110-positive restriction fragments, thus demonstrating that novel insertions of the IS6110 element exist in the attenuated strain H37Ra [10]. Moreover, by a combination of clone mapping, sequence analysis and comparative genomics, Brosch et al. [11] showed that IS6110 transpositions are responsible for genomic polymorphisms and deletions that generate the genomic diversity in strains H37Rv and H37Ra. In these and other studies [12, 13], it has been hypothesised that the transpositions of IS6110...
might have involved virulence genes. Although in M. tuberculosis H37Rv most of the insertion sequences are located in intergenic or non-coding regions and many are clustered, suggesting the existence of insertional hot-spots that prevent genes from being inactivated [14], it becomes important to identify the differential insertion sites of IS6110 in strain H37Ra to verify whether IS6110 transposition actually interrupted any reputed virulence gene.

The present study determined the nucleotide sequence flanking the 3' end of one differential IS6110-positive PvuII II-fragment of the attenuated strain H37Ra to identify the insertion site of the IS6110 element in the chromosome. Moreover, as these experiments led to the identification of a phospholipase C-coding gene in the so-called RvD2 region as target of IS6110 insertion, this region was investigated in a number of clinical isolates.

Materials and methods

Bacterial strains

M. tuberculosis strains H37Rv and H37Ra, M. bovis P3 and M. bovis BCG (Pasteur strain) were from a collection maintained in this department. The 45 clinical strains were isolated from clinical specimens by the radiometric BACTEC system according to standard procedures and identified by molecular probes (Gen-Probe, San Diego, CA, USA).

IS6110-based restriction fragment length polymorphism (RFLP)

The IS6110-based RFLP assay was run as described previously [10]. Briefly, the bacterial cells from cultures on Lowenstein-Jensen medium were suspended in saline and heated at 80°C for 20 min. Genomic DNA, extracted by the N-cetyl-N,N,N-trimethyl ammonium bromide method, was digested with restriction endonuclease PvuII and electrophoresed on an agarose 0.8% gel. DNA fragments were blotted on to a nylon filter and hybridised by addition of a 245-bp probe specific for the IS6110 insertion element. The probe, which was prepared from M. bovis P3 DNA by PCR with oligonucleotides INS-1 (6'TCGTGAAGGC ATCCAGGTGGCCG) and INS-2 (6'TGCGTAGGGCG TCGGTGACAAA) as primers, hybridises to a target sequence located to the right of the PvuII site. Hybridisation was detected on autoradiographic films by the enhanced chemiluminescence gene detection system (Hyperfilm-ECL, Amersham). The RFLP patterns were scanned and fingerprints were compared by GelCompar 4.1 software (Applied Maths, Belgium).

Cloning of IS6110-specific PvuII restriction fragment and sequencing procedures

Molecular cloning techniques were performed according to standard protocols [15]. In particular, a narrow slice around the selected band of IS6110-specific PvuII restriction fragments from M. tuberculosis H37Rv genomic DNA was excised and purified from the gel with the JetSeq-Gel extraction kit (Genomed). The DNA was inserted into the dephosphorylated EcoRV site of the plasmid pBluescript SK+ (Stratagene) by the T4 DNA ligase (Pharmacia Biotech) and the ligation product was used to transform Escherichia coli JM109 cells. Bacterial clones containing inserts were identified as white colonies from which the plasmid DNA was purified by standard alkaline lysis procedures. Samples of recombinant plasmid DNA were screened for the presence of IS6110 by PCR with INS-1 and INS-2 primers as described previously [10].

Sequencing of inserted DNA fragments was performed with an automated apparatus (ALFexpress DNA sequencer, Pharmacia Biotech) according to the T7 DNA polymerase method. The Bluescript T3 and T7 universal primers and the oligonucleotide TN1, designed from the IS6110 sequence (6TGCTACTA CGCTCAAGGC) (120T), were used as sequencing primers.

![Fig. 1. IS6110-based fingerprints of M. tuberculosis strains H37Rv and H37Ra. Arrows indicate the IS6110-positive restriction fragments differentially generated in the study strains (see text for more details); lane M shows the markers of molecular size expressed on the left in kb.](https://www.microbiologyresearch.org/assets/fiG1.jpg)
Southern blot analysis of clinical isolates for RvD2 deletion

For the analysis of clinical isolates, DNA extraction, restriction endonuclease PvuII digestion, electrophoretic separation, blotting on to nylon filter and autoradiographic detection were performed as described above. Three PCR-generated, peroxidase-labelled probes, prepared from *M. bovis* BCG DNA, were used: a 153-bp probe, coded *a*, specific for the cloned 237-bp restriction fragment; a 277-bp probe, coded *b*, external to the *plcD* gene and hybridising to a sequence of the RvD2 region from position 1566 to 1842 and a 422-bp probe, coded *c*, external to the *plcD* gene and hybridising to a sequence of the RvD2 region from position 5383 to 5804 (nucleotide positions refer to GenBank sequence Y18606). Oligonucleotide primer pairs were: 5'--TCTGGACGCCTACTTGTGGT--3' and 5'--TGGTCCACGCCAATCTCC--3' for probe *a*; 5'--AACTTCCACCGCATTCAG--3' and 5'--CACACGCAGTGCACAAAA--3' for probe *b*; 5'--GCGCTGCAACACCTCTCTCAAA--3' and 5'--CCTCATGGAATGCT--3' for probe *c*. PCR was performed in 0.5-ml micro-centrifuge reaction tubes in a final volume of 50 μl containing 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 50 mM KCl, Triton X-100 0.1%, 0.25 μM primers, 200 μM dNTPs. Taq polymerase (DynaZyme) 0.8 U and *M. bovis* BCG DNA 20 ng.

After an initial denaturation step of 94°C for 2 min, fragment amplification was performed with a PCR Sprint Thermal cycler (Hybaid UK), set for 1 min at 94°C, 1 min at primer annealing temperatures (63°C, 58°C and 60°C for probes *a*, *b* and *c* respectively), 2 min at 72°C for 30 cycles, followed by one final 4-min extension at 72°C. The amplicons were purified by Sephadex G-50 chromatography column, precipitated with ethanol 96%, solubilised with TE buffer and covalently labelled with horseradish peroxidase (HRP) by glutaraldehyde according to the procedure of the ECL Direct System (Amersham).

Results and discussion

Fig. 1 shows the IS6110-based fingerprints of *M. tuberculosis* H37Rv and H37Ra obtained by the use of the restriction endonuclease PvuII and an IS6110-specific DNA probe homologous to a sequence located at the right of the PvuII site; the banding patterns seem to correspond to genotypes Rv4 and RvA1 of the H37Rv/Ra variants recently defined by Bifani and colleagues [16]. The RFLP analysis shows one differential band of c. 5.1 kb in strain H37Rv and four differential bands of c. 1.12, 2.29, 3.03 and 4.90 kb in strain H37Ra. As each IS6110-positive restriction fragment includes a part of the IS6110 element, spanning from the PvuII

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**Fig. 2.** Alignment of the nucleotide sequence of gene *plcD* of *M. bovis* BCG-Pasteur (accession no. Y18606) with the 237-bp sequence fragment flanking the 3' end of IS6110 in the PvuII restriction fragment cloned from *M. tuberculosis* H37Ra, *M. tuberculosis* H37Ra (accession no. A2242907) and *M. tuberculosis* H37Rv (accession no. Z95890). Only alignment from position 781 to 1145 of the 1545-bp *plcD* gene is shown. Dots indicate nucleotide identity; RvD2 deletion in strain H37Rv and IS6110 element in strain H37Ra are indicated by arrowhead lines and light grey boxes, respectively.

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Fig. 3. Southern blot analysis of control strains (M. tuberculosis H37Rv, and H37Ra, M. bovis BCG and M. bovis P3) and 10 representative clinical isolates of M. tuberculosis out of 45 tested. Clinical isolates included six RvD2-positive (lanes a–f) and four RvD2-negative (lanes g–j) strains. PvuII-digested genomic DNA from the strain indicated at the top of each lane was electrophoresed, blotted on to nylon filter and then hybridised with probes a, b and c indicated above each panel. M, mol. wt markers. The drawing at the bottom of the panels represents the physical map of the RvD2 region of M. bovis BCG-Pasteur (accession no. Y18606) with the cleavage positions of restriction endonuclease PvuII indicated by arrows. The white box represents the plcD gene in which the sequence 100% homologous to the 237-bp restriction fragment cloned from M. tuberculosis H37Ra is in dark grey. The lines to the left and right of plcD denote chromosomal DNA. The thick black bars and the respective letters at the top represent the probing sequences a, b and c. The insertion site of the IS6110 element (indicated as a light grey box) in plcD of M. tuberculosis H37Ra is also shown. The RvD2 deletion in M. tuberculosis H37Rv is shown as a dotted line.
restriction endonuclease analysis. Inserted DNA fragments from three positive clones were sequenced by using the Bluescript T3 and T7 universal primers and an oligonucleotide designed from the IS6110 sequence as sequencing primers. The cloned mycobacterial DNA fragment was found to consist of a 1133-bp fragment containing (i) an 896-bp sequence homologous to the IS6110 sequence from the PvuII restriction site to the 3' end of the IS sequence (as expected) and (ii) a 237-bp sequence flanking the 3' end of IS6110. When analysed in the M. tuberculosis H37Rv database [14], the sequence showed partial nucleotide identity (72–78%) with the genes plcA, plcB and plcC, all coding for phospholipase C (PLC), a well-known bacterial virulence factor not only for extracellular pathogens, such as Clostridium perfringens, Pseudomonas aeruginosa and Staphylococcus aureus [17–20], but also for facultative intracellular pathogens, such as Listeria monocytogenes [21, 22]. On the other hand, the M. tuberculosis H37Rv genome is known to contain a truncated open-reading frame of 840 bp of a further plc gene, i.e., plcD', separated from the plcA, B, C locus by c. 640 kb [14]. According to recent reports, plcD' originally resulted from the insertion of IS6110 in the plcD gene [23], an event that disrupted the plcD gene and determined the deletion of a 7.9-kb fragment, known as RvD2, which also includes a sugar transferase, an oxidoreductase and a membrane protein [11, 24]. The unbroken plcD gene, without IS6110 insertion, is present in the genome of M. bovis, including the BCG strain [24]. Therefore, this study aligned the 237-bp sequence from the cloned PvuII restriction fragment with the RvD2 region of M. bovis BCG-Pasteur (GenBank accession no. Y18060) and found 100% nucleotide homology to an internal sequence of the plcD gene, from position 832 to 1068 (Fig. 2). This indicates that the differential 1133-bp restriction fragment of strain H37Ra stems from the plcD gene interrupted by the insertion of the IS6110 element, part of the gene being then lost in strain H37Rv in the RvD2 deletion; as shown in Fig. 2, the last 14 bases of the truncated plcD' of strain H37Rv, just before the RvD2 deletion, are also found at the end of the 237-bp fragment of strain H37Ra.

The occurrence of the RvD2 deletion was then sought in 45 clinical isolates of M. tuberculosis by Southern blot analysis. For this purpose, PvuII-cleaved genomic DNA of each isolate was electrophoresed, blotted on to nylon filters and then hybridised with three probing sequences, named a, b and c, prepared by PCR from M. bovis BCG DNA, and specific for the RvD2 region (see drawing at bottom of Fig. 3); in particular, probe a hybridised to the above-described 237-bp sequence of the plcD gene in a 3903-bp PvuII restriction fragment; probe b hybridised to a sequence located externally to the plcD gene in the same 3903-bp restriction fragment; probe c hybridised to the contiguous 1517-bp PvuII restriction fragment in the RvD2 region. As expected, no hybridisation signal was obtained with any

![Fig. 4](image-url) Southern blot analysis of the M. tuberculosis isolate indicated as f in Fig. 3. Blots, prepared as described in Fig. 3, were hybridised with a IS6110-specific probe (lane a) or with the RvD2-specific probe b (see legend to Fig. 3 for details) (b). The position of bands in each lane was normalised by equating external molecular size markers between the films, so that band positions of the fingerprints are mutually comparable. Banding patterns were compared with GelComp4.1 software by the UPGMA clustering method using the Dice coefficient, following the instructions of the GelComp4.1 manufacturer. M, mol. wt markers.
of the molecular probes in *M. tuberculosis* H37Rv, thus confirming the RvD2 deletion. *M. tuberculosis* H37Ra yielded the expected bands of 1133 bp, 4131 bp and 1517 bp with probes *a*, *b* and *c*, respectively (the 4131-bp band includes a 464-bp fragment of the IS6110 element plus a fragment of RvD2 from position 1068 – the insertion site of IS6110 – to the *Pvu*II site at position 4735). *M. bovis* BCG and *M. bovis* P3 yielded the expected bands of 3903 bp with probes *a* and *b*, and a band of 1517 bp with probe *c*. Fifteen (33.3%) of the 45 clinical isolates gave negative results with all three probes, thus demonstrating the deletion of the entire RvD2 region, while 30 isolates (66.7%) yielded a single band with each probing sequence. Of these, 29 isolates behaved as *M. bovis*, which indicates that these strains possess the complete RvD2 region, including the undispersed *plcD* gene without IS6110 insertion. One isolate, shown in lane *f* of Fig. 3, yielded a low-size (approximately 700 bp) restriction fragment with probe *a* and a large-size fragment of c. 4.5 kb with probe *b*; as shown in Fig. 4, the 4.5-kb fragment of isolate *f* hybridised with the IS6110 probe and with the RvD2-specific probe *b*. These results are compatible with the presence of an IS6110 element inserted in the *plcD* gene with an inverted orientation as compared with strain H37Ra. In fact, the nucleotide sequence of the RvD2 region of isolate *f* confirmed the inverted orientation of the IS6110 element and also showed that the IS6110 insertion site was located 29 bases downstream compared with the H37Ra strain (data not shown). The polymorphism of the RvD2 region in *M. tuberculosis* H37Rv, H37Ra and clinical isolates is outlined in Fig. 5.

In summary, the demonstration of the IS6110 insertion in the *plcD* gene of strain H37Ra and isolate *f*, and the occurrence of the RvD2 deletion in approximately one-third of clinical isolates further support previous observations indicating that the *plcD* gene is a hotspot for IS6110 insertion [11]. However, the RvD2 deletion has no consequence for the virulence of *M. tuberculosis*. Nevertheless, the role of PLC as a virulence factor of *M. tuberculosis* remains debatable. It has been proposed, in analogy with *L. monocytogenes* [25, 26], that mycobacterial PLC may disrupt the integrity of the phagolysosomal membrane with subsequent escape of the bacterium into the cytoplasm [27, 28]. Moreover, some data suggest that PLC is associated with the most virulent mycobacterial species. In particular, *M. tuberculosis*, including strain H37Rv and clinical isolates, possesses the three genes *plcA*, *plcB* and *plcC* [24], in *M. bovis* and *M. microti* the region encompassing these genes is absent, being deleted in the so-called RvDS deletion, but they have an undispersed *plcD* gene [24]; *M. africanaum* possesses all four *plc* genes [24]; DNA sequences homologous to the *plc* genes of *M. tuberculosis* have been reported in the pathogenic *M. ulcerans* [29], but not in other *Mycobacterium* spp, including *M. smegmatis, M. avium* and *M. intracellulare*. PLC enzymic activity also seems to correlate with mycobacterial virulence, as it has been reported in *M. tuberculosis*, including strains H37Rv and H37Ra, *M. bovis, M. microti, M. ulcerans* and *M. marinum*, but not in *M. bovis* BCG, in spite of the presence of the undisrupted *plcD* gene [29–31]. However, the available experimental data failed to demonstrate a direct role of

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**Fig. 5.** Polymorphism in the RvD2 region of *M. tuberculosis*. Drawings represent the proposed physical map of the RvD2 region in (A) *M. bovis* and 29 clinical isolates; (B) *M. tuberculosis* H37Rv and 15 clinical isolates; (C) *M. tuberculosis* H37Ra; (D) clinical isolate shown in lane *f* of Fig. 3. Refer to legend to Fig. 3 for symbols of drawings. Distances between nucleotide positions are not to scale.
PLC in mycobacterial virulence. Studies from this and another laboratory have shown that cloning and expression of pldA and pldB genes of M. tuberculosis into the non-pathogenic, fast-growing bacterium M. smegmatis do not confer growth advantage in macrophages or in experimental animals [32; Lari et al., unpublished results].

In conclusion, although IS6110-mediated disruption of the pldD gene apparently does not influence the virulence properties of M. tuberculosis, the role of PLC as a mycobacterial virulence factor remains to be clarified. Complementation experiments aimed at restoring pldD expression in the attenuated strain M. tuberculosis H37Ra and the disruption of the pldA, B, C locus in the virulent strain M. tuberculosis H37Rv will probably provide conclusive evidence.

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References


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