

BACTERIAL PATHOGENICITY

# 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 undisrupted *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 tubercle 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

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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 *Pvu* 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)

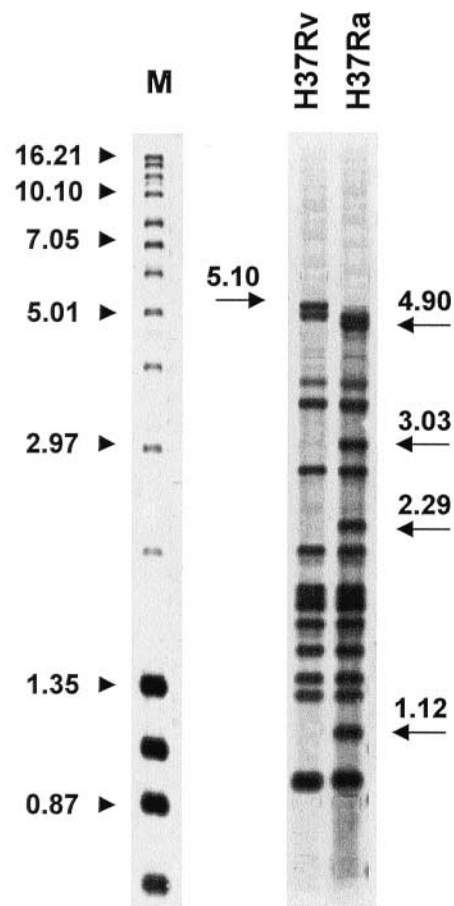
The *IS6110*-based RFLP assay was run as described previously [10]. Briefly, the bacterial cells from cultures on Lowenstein-Jensen medium were suspended in saline and heated at 80°C for 20 min. Genomic DNA, extracted by the N-cetyl-N,N,N-trimethyl ammonium bromide method, was digested with restriction endonuclease *Pvu*II 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 probing sequence specific for the *IS6110* insertion element. The probe, which was prepared from *M. bovis* P3 DNA by PCR with oligonucleotides INS-1 (<sup>633</sup>CGTGAGGGC ATCGAGGTGGC<sup>652</sup>) and INS-2 (<sup>858</sup>GCGTAGGCG TCGGTGACAAA<sup>877</sup>) as primers, hybridises to a target sequence located to the right of the *Pvu*II 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 *Pvu*II 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 *Pvu*II restriction fragments from *M. tuberculosis* H37Ra genomic DNA was excised and purified from the gel with the JETsorb-Gel extraction kit (Genomed). The DNA was inserted into the dephosphorylated *Eco*RV 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 (<sup>1276</sup>TGCCTACTA CGCTCAACGCCAG<sup>1297</sup>), 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.

### Southern blot analysis of clinical isolates for RvD2 deletion

For the analysis of clinical isolates, DNA extraction, restriction endonuclease *Pvu*II 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'-TCTGCGACCGCTACTTTTGTCT-3' and 5'-TGATGCGCCAACCGAATCTCC-3' for probe *a*; 5'-AACCTCCAGCCGCATTTTCAG-3' and 5'-CACACGCAGTCCGCACAA-3' for probe *b*; 5'-GCCGTGCCAACCTCTCAAA-3' and 5'-CCCCGCCGATGCTGTCAA-3' for probe *c*. PCR was performed in 0.5-ml micro-centrifuge reaction tubes in a final volume of 50  $\mu$ l containing 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl<sub>2</sub>, 50 mM KCl, Triton X-100 0.1% 0.25  $\mu$ M primers, 200  $\mu$ M dNTP, Taq polymerase (Dynazyme) 0.8 U and *M. bovis* BCG DNA 20 ng.

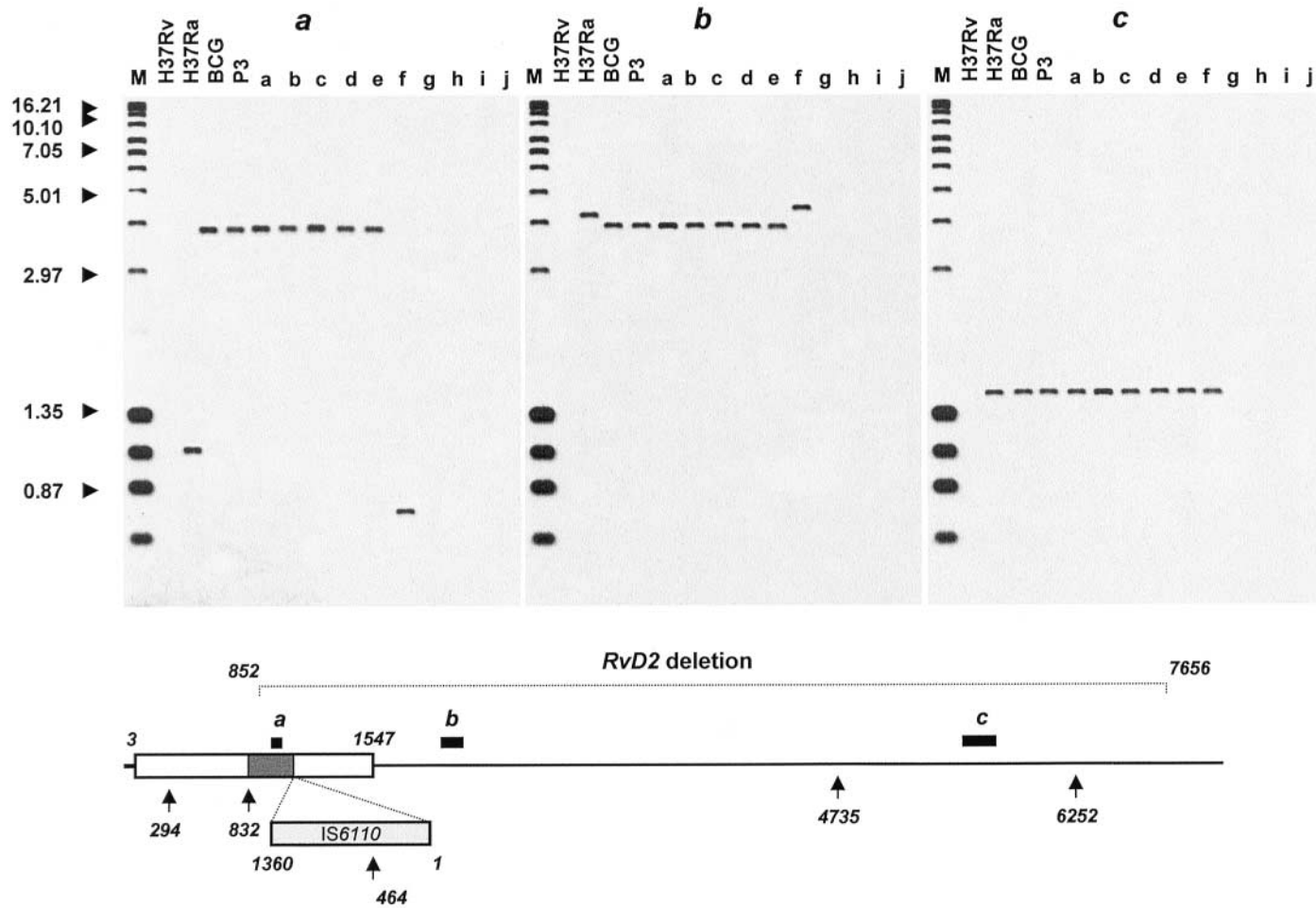
After an initial denaturation step of 94°C for 2 min, fragment amplification was performed with a PCR Sprint Thermal cycler (Hybaid UK), set for 1 min at 94°C, 1 min at primer annealing temperatures (63°C, 58°C and 60°C for probes *a*, *b* and *c*, respectively), 2 min at 72°C for 30 cycles, followed 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

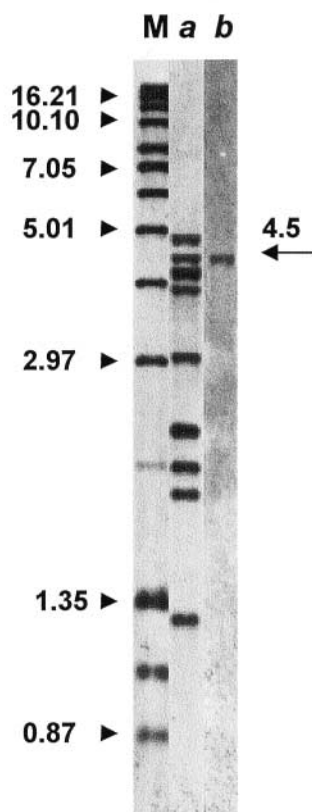
Fig. 1 shows the IS6110-based fingerprints of *M. tuberculosis* H37Rv and H37Ra obtained by the use of the restriction endonuclease *Pvu*II and an IS6110-specific DNA probe homologous to a sequence located at the right of the *Pvu*II site; the banding patterns seem to correspond to genotypes Rv4 and Ra1 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 *Pvu*II

<i>M. bovis</i>	gtaagaaccg aggagatggg cccgagtgtc ttgttgcat acaccttcca gctgacgccg	840
237-bp	.....	9
H37Ra	.....	
H37Rv	.....	
<i>M. bovis</i>	gcatcgctaa gggtctgccc catgatgcgc caaccgaatc tccgcaccgg ttggaaagtg	900
237-bp	.....	69
H37Ra	.....	
H37Rv	..... ← RvD2 deletion →	
<i>M. bovis</i>	ggactctgca gctccggccc accatcttgg ccgtcggggc cgatggtggc gctcagccaa	960
237-bp	.....	129
H37Ra	.....	
H37Rv	..... ← RvD2 deletion →	
<i>M. bovis</i>	taaaggcggc tgggcagggt gggaccaat accgagcaaa agtagcggtc gcagacggcg	1020
237-bp	.....	189
H37Ra	.....	
H37Rv	..... ← RvD2 deletion →	
<i>M. bovis</i>	aacgcgtcgg ccaacagata gtggatcgga atgtcttggc gcgtgtag tagccatcacc	1080
237-bp	.....	237
H37Ra	..... IS6110	
H37Rv	..... ← RvD2 deletion →	
<i>M. bovis</i>	gtgggagtgt gggccgccga gcgagtcttg gcctgcgctg gtagccagtt gtcggtgacg	1140
H37Ra	..... IS6110	
H37Rv	..... ← RvD2 deletion →	

**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 *Pvu*II restriction fragment cloned from *M. tuberculosis* H37Ra, *M. tuberculosis* H37Ra (accession no. AJ242907) and *M. tuberculosis* H37Rv (accession no. Z95890). Only alignment from position 781 to 1140 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.



**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. *Pvu*II-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 *Pvu*II 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.



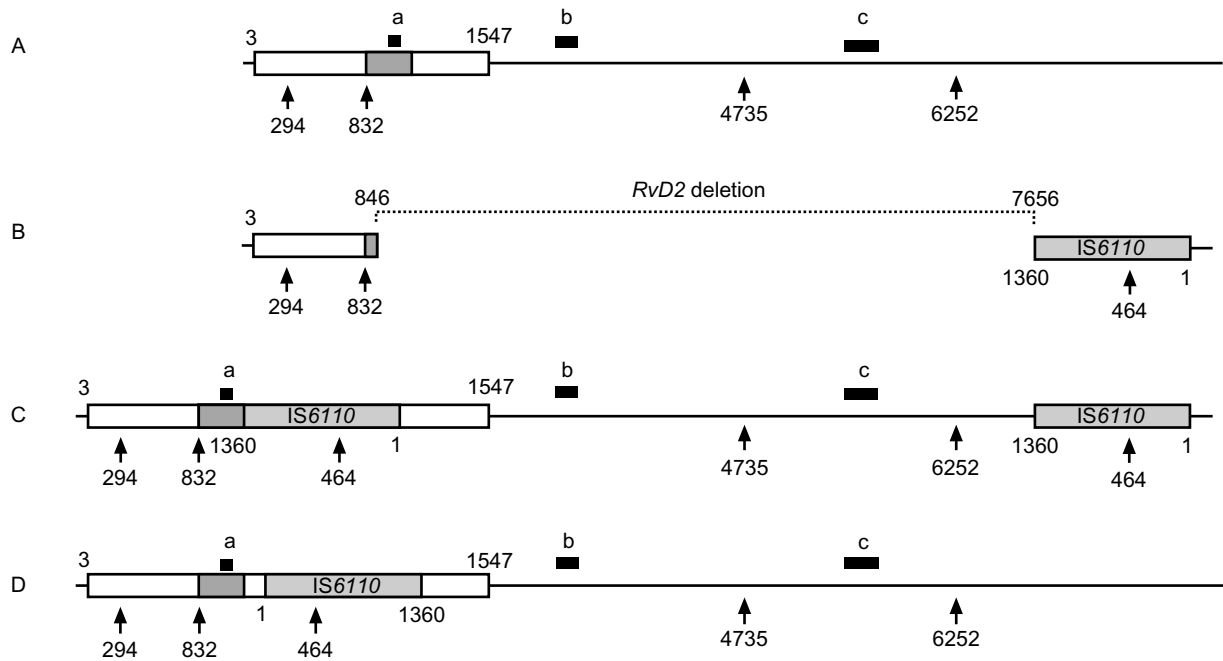
**Fig. 4.** Southern blot analysis of the *M. tuberculosis* isolate indicated as **f** in Fig. 3. Blots, prepared as described in Fig. 3, were hybridised with a IS6110-specific probe (lane **a**) or with the RvD2-specific probe **b** (see legend to Fig. 3 for details). The position of bands in each lane 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 GelCompar 4.1 software by the UPGMA clustering method using the Dice coefficient, following the instructions of the GelCompar manufacturer. M, mol. wt markers.

restriction site CAG↓CTG located at IS position 464 to the 3' terminus of the IS element at position 1360, and an unknown sequence flanking the 3' end of IS6110, it becomes important to identify such a flanking sequence in the H37Ra strain to identify the insertion sites of the IS6110 element in the chromosome and verify whether IS6110 transposition actually interrupted any putative virulence gene. This paper reports the results relative to the 1.12-kb IS6110-positive, *Pvu*II restriction fragment from *M. tuberculosis* H37Ra.

A narrow slice around the 1.12 kb fragment was excised from a gel of *Pvu*II-digested genomic DNA and the DNA fragment was purified and cloned into the plasmid pBluescript SK+ in *E. coli* JM109 cells. Bacterial clones containing the specific insert were identified by screening samples of recombinant plasmid DNA for the presence of IS6110 by PCR and by

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 *Pvu*II 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 *Pvu*II restriction fragment with the RvD2 region of *M. bovis* BCG-Pasteur (GenBank accession no. Y18606) 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, *Pvu*II-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 *Pvu*II 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 *Pvu*II restriction fragment in the RvD2 region. As expected, no hybridisation signal was obtained with any



**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.

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 *PvuII* 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 undisrupted *plcD* gene without IS6110 insertion. One isolate, shown in lane f of Fig. 3, yielded a low-size (apparently 700 bp) restriction fragment with probe *a* and a large-size fragment of 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 *plcD* 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 hot-spot 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 RvD5 deletion, but they have an undisrupted *plcD* gene [24]; *M. africanum* 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

PLC in mycobacterial virulence. Studies from this and another laboratory have shown that cloning and expression of *plcA* and *plcB* 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 *plcD* 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 *plcD* expression in the attenuated strain *M. tuberculosis* H37Ra and the disruption of the *plcA*, *B*, *C* locus in the virulent strain *M. tuberculosis* H37Rv will probably provide conclusive evidence.

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