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IS*6110* Mediates Increased Transcription of the *phoP* Virulence Gene in a Multidrug-Resistant Clinical Isolate Responsible for Tuberculosis Outbreaks

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Drug resistance in *Mycobacterium tuberculosis* complex strains is solely due to chromosomal mutations that could affect bacterial virulence. Molecular epidemiology studies have shown that resistant strains are less likely to be clustered than susceptible strains. However, a few multidrug-resistant (MDR) *M. tuberculosis* complex strains have been described as causing outbreaks, suggesting that they have restored virulence or increased transmission. One of the biggest MDR tuberculosis outbreaks documented to date was caused by the B strain of *M. bovis*. Restriction fragment length polymorphism fingerprinting revealed that the B strain contains two copies of IS*6110*. Here, we mapped and sequenced the regions flanking the two copies of IS*6110* in the B strain. Ligation-mediated PCR showed that one of these IS*6110* copies is located within the promoter region of *phoP*, a transcriptional regulator that is essential for *M. tuberculosis* virulence. We used PCR to screen 219 MDR *M. tuberculosis* complex strains (90.4% of all MDR isolates) isolated in Spain between 1998 and 2002 and found that the B strain was the only strain that contained a copy of IS*6110* in the *phoP* promoter. To determine whether IS*6110* affects *phoP* promoter activity in the B strain, we individually cloned the *phoP* gene and its promoter region (including IS*6110* from the B strain and the equivalent region from *M. tuberculosis* without IS*6110* as a control) into a mycobacterial replicative plasmid and transformed *M. smegmatis* with the resulting plasmid. Primer extension analysis showed that *phoP* transcription was strongly upregulated when the promoter region contained IS*6110*, as in the case of the B strain.

Tuberculosis (TB) is currently one of the leading causes of mortality throughout the world (8, 25, 27). The human immunodeficiency virus-AIDS pandemic, the deterioration of public health systems in developing countries, and the emergence of multidrug-resistant (MDR) *Mycobacterium tuberculosis* complex strains have further contributed to the spread of TB. Knowledge of the molecular mechanisms involved in the bacillus-host cell interaction is essential for developing adequate strategies for TB control. Recent advances in the genetic manipulation of mycobacteria (2, 29) combined with the publication of the complete *M. tuberculosis* genome sequence (6) have made it possible to study the contribution of individual genes to *M. tuberculosis* virulence (5, 7). However, little is known about the regulatory and expression mechanisms that determine the virulence of clinical isolates of *M. tuberculosis*.

Insertion sequence (IS) *6110* has been extensively used for molecular typing of *M. tuberculosis* strains. Restriction fragment length polymorphism (RFLP) analysis using IS*6110* as a probe is currently the most common molecular method used to type *M. tuberculosis* complex strains. However, the physiological role and impact of specific IS*6110* insertions on the biology of bacilli are not well known. IS*6110* fingerprinting studies have demonstrated heterogeneity between virulence and transmissibility of different *M. tuberculosis* clinical isolates, as judged by rates of skin test conversion and onset of clinical disease among exposed individuals (34, 43). In some strains of *M. tuberculosis*, drug resistance is associated with low growth rates and reduced persistence in mice and guinea pigs (19). Although drug-resistant *M. tuberculosis* strains appear to be less transmissible than susceptible isolates (45), MDR strains of *M. tuberculosis* have caused several outbreaks (4, 38). For example, the widely distributed W strain, which belongs to the Beijing family of isolates (1, 4), and the MDR *M. bovis* B strain (35, 38, 39) have caused large outbreaks in the United States and Spain, respectively. The high transmission rate of some MDR strains suggested that their fitness and virulence are unaffected, making these strains especially adapted to their hosts. The study of the genetic mechanisms involved in the transmissibility of these epidemic MDR strains could provide valuable information about the genes implicated in *M. tuberculosis* virulence.

Many IS elements affect the expression of neighbor genes by generating new promoter sequences capable of driving their expression. The presence of IS*6110* in *M. tuberculosis* clinical isolate 210, which belongs to the Beijing family, provides a promoter sequence that enhances gene transcription (3).

MDR TB strains isolated in Spain have been systematically typed since 1998. The typing results showed that most MDR

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M. tuberculosis complex strains are not further transmitted to other patients (reference 38 and our unpublished data). In contrast, the B strain was found to cause severe MDR TB outbreaks (35, 39). RFLP analysis showed that the B strain contains two copies of IS*6110* (39).

An *M. tuberculosis phoP* mutant was recently constructed by gene disruption (30). This mutant exhibits impaired growth in vitro within mouse-grown bone marrow-derived macrophages, and it was also attenuated in vivo in a mouse model of infection. Thus, *phoP* may act as a virulence regulator in *M. tuberculosis*. The *phoP* locus from pathogenic bacterial species is a member of the PhoP/PhoQ two-component regulatory system (12, 42). This system is required in *Salmonella* for the expression of virulence factors in mice and for survival within macrophages and confers resistance to killing by certain defensins (13).

In this work, we found that one copy of IS*6110* is located within the *phoP* promoter (6) in the MDR epidemic B strain. We used PCR to screen more than 200 MDR *M. tuberculosis* complex clinical isolates and showed that the B strain is the only strain to contain IS*6110* in its *phoP* promoter region. Primer extension (PE) analysis showed that IS*6110* increased the expression of the *phoP* virulence gene in the B strain causing MDR tuberculosis outbreaks.

MATERIALS AND METHODS

Bacterial strains, culture media, and growth conditions. All MDR *M. tuberculosis* complex clinical isolates were sent to our laboratory as part of the *M. tuberculosis* genotyping network of the University of Zaragoza (38). The *M. bovis* B strain was selected for this study (39). *M. tuberculosis* Mt14323, *M. bovis* BCG Pasteur, and *M. smegmatis* mc^{2}155 (41) were also used. Mycobacterial strains were routinely grown in Middlebrook 7H10 Bacto agar supplemented with oleic acid-albumin-dextrose-catalase or in Middlebrook 7H9 broth supplemented with albumin-dextrose-catalase (Difco Laboratories, Detroit, Mich.) and 0.05% Tween 80 (17). *M. smegmatis* mc^{2}155 was grown in Dubos broth containing 0.1% Tween 80 and albumin (Difco Laboratories) for RNA extraction.

Escherichia coli XL1-blue (37) was grown on solid medium or in liquid Luria-Bertani medium for cloning and plasmid propagation.

All the strains were grown at 37°C. When required, kanamycin (20 μg/ml) or hygromycin (50 μg/ml for *M. smegmatis* and 200 μl/ml for *E. coli*) was added to the culture medium.

IS*6110* fingerprinting. MDR *M. tuberculosis* strains isolated in Spain since 1998 have been systematically typed by standard RFLP and spoligotyping in our laboratory (38, 44, 46, 47). For RFLP, cetyltrimethylammonium bromide-NaCl and chloroform-isoamyl alcohol are used as described previously to extract chromosomal DNA from the isolates (46). Extracted DNA is digested with *Pvu*II (Boehringer-Mannheim), separated in 0.8% agarose gels in Tris-borate-EDTA buffer, and vacuum blotted onto nylon membranes. Blotted DNA was hybridized with a 0.8-kb fragment corresponding to the PCR-amplified fragment between the *Pvu*II restriction site and the 3′ end of IS*6110*. The probe was labeled and RFLP patterns were visualized by use of an ECL direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, England). Autoradiograms of IS*6110* fingerprints were scanned, analyzed, and compared by use of Bionumerics software (version 3.0; Applied Maths, Kortrijk, Belgium).

Mapping of the regions flanking IS*6110* in the B strain. Ligation-mediated PCR (LM-PCR) was used as described by Prod'hom et al. (32) to determine the location of the two copies of IS*6110* in the *M. bovis* B strain. Briefly, genomic DNA was digested with *Sal*I (Boehringer-Mannheim) and ligated to a linker containing a *Sal*I restriction site at its 3′ end and the Salgd primer sequence (5′-tagcttattcctcaaggcacgagc-3′). The resulting template was then digested with *Sal*I. PCR was performed using ISA1 (5′-cctgacatgaccccatcctttcc-3′) and ISA3 (5′-gaggctgcctactacgtcaacg-3′), specific primers for IS*6110* directed outwards from this element (22), and the linker primer Salgd (1 μM). The template was initially denatured by incubation at 95°C for 9 min and amplified by 35 cycles of PCR (95°C for 30 s, 70°C for 30 s, and 72°C for 90 s) followed by a final extension at 72°C for 10 min in a Gene Amp PCR System 9700 thermocycler (PE Applied Biosystems, Foster City, Calif.). Amplified products were separated by standard horizontal gel electrophoresis in a 1.5% agarose gel in Tris-borate-EDTA buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA) and stained with ethidium bromide. PCR products were purified using GFX PCR DNA and a gel band purification kit (Amersham Pharmacia Biotech). The amplified products were sequenced on an ALF DNA sequencer using a Thermo Sequenase fluorescent labeled primer cycle sequencing kit (Amersham Pharmacia Biotech).

Detection of IS*6110* upstream from *phoP* in *M. tuberculosis* complex isolates. We used PCR (10) to seek IS*6110* in the *phoP* promoter region of 219 of the 241 MDR *M. tuberculosis* complex strains previously genotyped by RFLP or spoligotyping. We used the BCG2B (5′-ccatgttcaaaccggtgtc-3′) and BCG2A (5′-gccg tccatcccgggcatc-3′) primers, which annealed 136 bp upstream and 293 bp downstream from IS*6110* in the B strain, respectively. The PCR conditions were as follows: 35 cycles of 95°C for 60 s, 62°C for 60 s, and 72°C for 60 s followed by a final extension at 72°C for 10 min. When IS*6110* was present upstream of *phoP*, an 1,828-bp amplification product was obtained. However, when the insertion element was absent, a 463-bp PCR product was obtained.

Construction of pSO5 and pSO7. pSO5 has been described previously (30). This recombinant plasmid contains a 2-kb genomic fragment containing both the promoter region and the entire *phoP* coding region of *M. tuberculosis* MT103. The *phoP* nucleotide sequence was cloned into pNBV1 (kindly provided by W. R. Bishai) (16). pNBV1 is a shuttle plasmid that contains the origin of replication from *E. coli* and the origin of replication from *M. fortuitum* pAL5000 (33). pSO7 contains the *phoP* promoter region from the B strain, including a complete copy of IS*6110*. To construct pSO7, primers phoPF (5′-aatctagatcaagcatcagccgaggta c-3′) and phoPR (5′-aatctagacccgaacgtagaacc) were used to amplify a 3,411-bp fragment from the *M. bovis* B strain. PhoPF annealed 1,000 and 929 bp upstream from the *phoP* start codon and the IS*6110* insertion site, respectively. PhoPR annealed 305 and 1,117 bp downstream from the *phoP* termination codon and IS*6110*, respectively. As PhoPF and PhoPR contained an *Xba*I restriction site, the PCR product was ligated to pNBV1 that had been cut with *Xba*I. The purified plasmids (pSO5 and pSO7) were then electroporated into *M. smegmatis* mc^{2}155 (17). The pSO5 and pSO7 plasmids were sequenced using an ABI 3700 DNA sequencer with a BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems). Primers SePho1 (5′-gagagcacccgctcgataag-3′), BCG2B (5′-ccatgttcaaaccggtgtc-3′), PhoExp (5′-cccatatgcggaaaggggttgatctct-3′), T3, and T7 were used for sequencing of pSO5. Primers BCG2A, ISA3 (see Fig. 2), PhoRF (5′-aatctagagggcaagggcaacaaggaac-3′), PhoPR (5′-aactagagatcacccga acgtagaagg-3′), SePho8 (5′-gttccttgttgcccttgccc-3′), T3, and T7 were used for sequencing of pSO7. DNA sequences were used to search the TubercuList database.

Isolation of total RNA. RNA was isolated from the *M. smegmatis* mc^{2}155 wild-type strain, and transformants were grown to middle exponential phase (optical density at 600 nm, 0.4 to 0.6) as described previously (11). Further DNase treatment was performed for the RNase protection assays.

Identification of transcription starting site. PE and RNase protection (RPA) experiments were carried out as described previously (24). Briefly, PE studies was carried out using primer phoBO4.1 (5′-aacgttaccttcacagtcat-3′), which anneals immediately upstream from the *phoP* ATG start codon (see Fig. 2). PhoBO4.1 was end labeled with [γ-^{32}P]ATP by a T4 polynucleotide kinase reaction. A total of 100 fmol of labeled primer was annealed to 30 μg of total RNA and extended by using avian myeloblastosis virus reverse transcriptase (11, 24). The polymerization products were ethanol precipitated and resuspended in an appropriate volume.

The RPA method required the construction of a minigene corresponding to the region under analysis. This minigene was used as a template for the synthesis of a radiolabeled antisense RNA probe complementary to the RNA transcript. Minigenes were synthesized by using PCR to amplify the region upstream from the *phoP* gene of the *M. bovis* B strain and of *M. tuberculosis.* The phoBO4.1-T7 (phoBO4.1 plus T7 promoter sequence) and ISA3 primers were used for *M. bovis*, and the phoBO4.1-T7 and BCG2B primers were used for *M. tuberculosis* (see Fig. 2). Minigenes of 245 and 181 bp were obtained, respectively. Transcription products from minigenes were radiolabeled by using a Riboprobe kit (Promega, Madison, Wis.) according to the manufacturer's recommendations. The products were purified on a 6% (wt/vol) polyacrylamide gel; appropriate bands of the expected sizes were excised and eluted as described previously (24). Hybridization was carried out with 20 μg of DNA-free RNA and 2.5×10^5 cpm of radiolabeled samples and digested with an RNase cocktail containing both RNase T_1 (20 U/μl) and RNase A (1 U/μl) (Ambion). The optimum RNase concentration for RPA was established.

Products obtained in both PE and RPA were separated on 8% (wt/vol) polyacrylamide-urea sequencing gels. Radioactive products were located by autoradiography using an intensifying screen. Radioactivity corresponding to the sev-

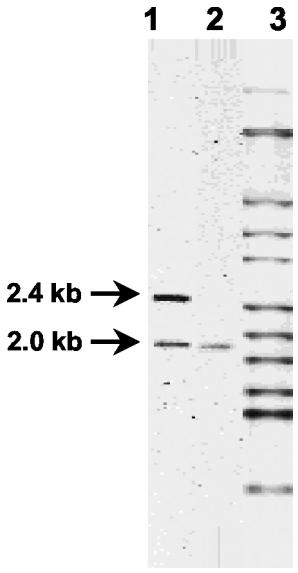

FIG. 1. RFLP analysis showing the IS*6110* fingerprint of the B strain. Fingerprints of strains B (lane 1), *M. bovis* BCG Pasteur (lane 2), and *M. tuberculosis* Mt14323 (lane 3) are shown. The 2.0- and 2.4-kb bands represent copies of IS*6110* in DRs and upstream from *phoP*, respectively.

eral transcriptional products detected by PE was measured by using an Instant Imager system (Packard-Izasa, Barcelona, Spain).

RESULTS

IS*6110* genotyping of the MDR *M. bovis* B strain. A total of 241 *M. tuberculosis* complex strains isolated in Spain between 1998 and 2002 from patients with MDR TB were analyzed by RFLP and spoligotyping. The IS*6110* fingerprints are included in the Spanish MDR TB database (University of Zaragoza). A total of 26 (10.8%) of these isolates displayed a characteristic RFLP pattern, with two IS*6110* bands of 2.4 and 2.0 kb (39) (Fig. 1, lane 1). These isolates belong to the previously described *M. bovis* B strain (39). The B strain was responsible for a severe nosocomial outbreak of MDR TB in Spain (35). The rate of reinfection with the B strain is high among human immunodeficiency virus-coinfected patients (35). The 2.4-kb IS*6110* band was found only in B strains (whereas the 2.0-kb band was also found in *M. bovis* isolates [such as *M. bovis* BCG isolates] containing a single copy of IS*6110*) (Fig. 1, lane 2).

Localization of the two copies of IS*6110* in the B strain. LM-PCR was used to localize the two copies of IS*6110* in the B strain genome. The regions flanking IS*6110* were amplified by LM-PCR and sequenced.

The IS*6110* corresponding to the 2.0-kb band of the B strain (Fig. 1) was inserted in a genomic region characterized by the presence of multiple direct repeats (DRs) (located between positions 3119182 and 3122372) in the *M. tuberculosis* H37Rv genome. (This region is composed of 36-bp DRs separated by 36- to 41-bp nonrepetitive segments. DRs are hotspots for IS*6110* transposition in most *M. tuberculosis* and *M. bovis* strains.) In *M. bovis* BCG, which contains a single copy of IS*6110* (Fig. 1), the insertion sequence is located in an identical position (3, 14).

The nucleotide sequence of the genomic region flanking the second IS*6110* copy in the B strain, corresponding to the 2.4-kb band (Fig. 1), showed that IS*6110* was inserted at nucleotide 851531 upstream from open reading frame Rv*0757* (annotated as *phoP* in the H37Rv genome) (6). This insertion site was located 75 bp upstream from the ATG start codon of *phoP* in the B strain (Fig. 2). IS*6110* was flanked by a 4-bp DR (TGGC) on both sides (Fig. 2).

The copy of IS*6110* located upstream from the *phoP* gene is specific to the *M. bovis* B strain from the MDR *M. tuberculosis* complex strains studied. To determine whether the presence of the second copy of IS*6110* in the promoter region of *phoP* was specific to the B strain, we used PCR to screen DNAs from different MDR *M. tuberculosis* complex strains.

We screened 218 of the 241 (90.4%) MDR TB strains isolated in Spain since 1998 for the presence of the IS*6110* upstream from *phoP*. The specific primers BCG2A and BCG2B (10) were used to amplify the *phoP* promoter region from genomic DNA (Fig. 2B). A total of 21 (9.6%) of the MDR *M. tuberculosis* complex strains tested generated a 1,828-bp PCR product (Fig. 3, lanes 13 to 15), indicating that they contained IS*6110* upstream from *phoP* as in the case of the B strain (Fig. 1 and 2A). According to our fingerprint databank, all of these strains were B strains.

A 463-bp amplification product (signifying that IS*6110* was not present upstream from *phoP*) was obtained for 191 (87.6%) clinical isolates (Fig. 3, lanes 4 to 12). No amplification product was obtained with DNAs from five strains (1.8%), three of which were shown by RFLP to be B strains. We believe that the DNA preparations were contaminated in the two other cases (0.9%). All these results strongly suggest that only the outbreak-causing B strains contain a copy of IS*6110* in the promoter region of *phoP* in the MDR TB strains present in our database.

Transcriptional level of *phoP*. To study the effect of IS*6110* on the transcription of *phoP*, we constructed the recombinant plasmids pSO5 and pSO7 (Fig. 4) in a pNBV1 background (16). We used *M. smegmatis* mc^2155 as a surrogate host to study the role of IS*6110* in *phoP* transcription, because it is difficult to manipulate the B strain due to its resistance to most antituberculosis drugs. The differences between the *M. smegmatis* and *M. tuberculosis* complex *phoP* gene sequences allowed the design of specific primers for PE.

We carried out PE analysis with RNA isolated from *M. smegmatis* mc^2155 harboring pSO5 and pSO7, which contain the *phoP* promoter regions of *M. tuberculosis* wild-type and B strains, respectively. We detected two putative transcription start points (tsp1 and tsp2) in both cases (Fig. 5 and 2B). In *M. smegmatis* harboring the wild-type promoter (pSO5), tsp1 and tsp2 were located 121 and 57 bp upstream from the ATG start codon of *phoP*, respectively; the position of tsp1 was confirmed by RPA (data not shown). In *M. smegmatis* harboring the B strain promoter (pSO7), tsp1 and tsp2 were located 136 and 57 bp upstream from the ATG starting codon of *phoP*, respectively (Fig. 5A and 2B). Thus, tsp1 from the B strain (tsp1∗) was located inside IS*6110* (Fig. 5A). The locations of tsp1∗ and tsp2 in pSO7 were confirmed by RPA (Fig. 5B).

The PE and RPA results showed that the B strain (pSO7) synthesized more *phoP* transcripts than *M. tuberculosis* (pSO5). Due to the use of end-labeled oligonucleotide in the PE ex-

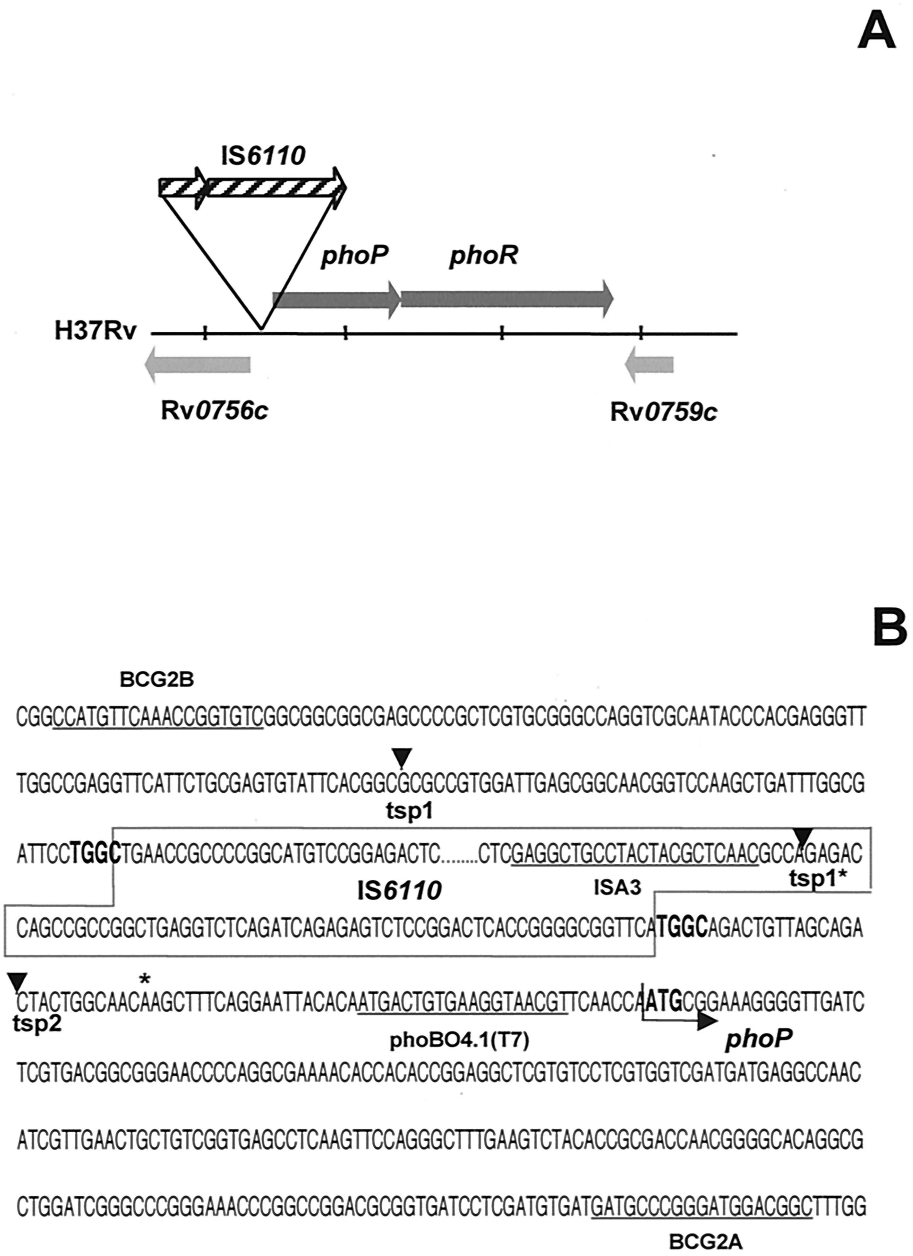

FIG. 2. Location of IS*6110* upstream from *phoP* in the *M. bovis* B strain. (A) Schematic representation showing the position of IS*6110* in the *phoP* promoter region. (B) Nucleotide sequence surrounding the second copy of IS*6110*. The IS*6110* nucleotide sequence is boxed. The TGGC DRs flanking the IS*6110* insertion site are shown in boldface characters. The ATG start codon of *phoP* is indicated by an arrow. The nucleotide sequences of primers BCG2A, BCG2B, phoBO4.1(T7), and ISA3 are underlined. ▼, transcription starting points tsp2 and tsp1 from pSO5 and pSO7 (tsp1*). tsp1*, tsp1 position in pSO5 wild-type *M. tuberculosis*. The nucleotide change (A for G) 46 bp upstream from the *phoP* ATG start codon of *M. bovis* with respect to *M. tuberculosis* H37Rv is indicated by an asterisk.

periments (see Materials and Methods), the relative radioactivity of the products reflects their relative abundance irrespective of their lengths. Thus, based on the amount of radioactivity incorporated into the cDNA, the B strain (pSO7) contained approximately 10-fold more *phoP* transcripts than the wild type (pSO5) (Fig. 5), indicating that differences in *phoP* expression occur at the transcription level. These results strongly suggest that *phoP* is overexpressed due to the presence of IS*6110* in the gene promoter region.

DISCUSSION

A number of molecular epidemiology studies have shown that resistant and MDR *M. tuberculosis* strains are less transmissible than wild-type susceptible strains (references 38 and 45 and our unpublished data). The mutations responsible for drug resistance in bacteria are frequently associated with adaptive changes correlated with decreased growth rates in comparison with wild-type strains (40). Nevertheless, particular

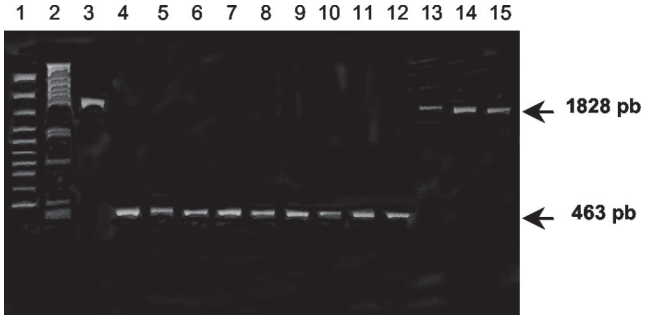

FIG. 3. The copy of IS*6110* in the *phoP* promoter region is specific to B strains. Genomic DNA was PCR amplified with primers BCG2A and BCG2B (Fig. 2B). Lanes 3 and 13 to 15, B strains; lane 4, *M. tuberculosis* H37Rv; lanes 5 to 12, other MDR *M. tuberculosis* complex strains; lane 1, Gene Ruler 100-bp DNA Ladder Plus (MBI Fermentas, Vilnius, Lithuania); lane 2, λ/*Pst*I. The presence of a 1,828-bp PCR product means that IS*6110* was present in the promoter region of *phoP* in the analyzed strain.

MDR TB strains cause outbreaks. Over the last 6 years, the *M. bovis* B strain was the strain most often isolated that caused MDR TB in Spain (35, 38, 39). Even though the sequences of *M. bovis* strains are very similar to those of *M. tuberculosis*, *M. bovis* is rarely transmitted between humans. No MDR *M. bovis* strains other than the B strain were isolated during this period. We focused on the B strain with the aim of elucidating the molecular mechanism of *M. tuberculosis* complex virulence.

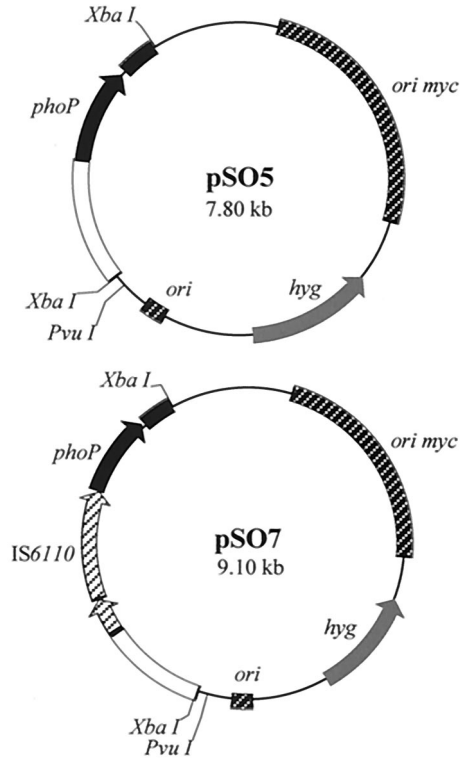

FIG. 4. pSO5 and pSO7 mycobacterial plasmids. The *phoP* genes (including the promoter region) of *M. tuberculosis* and *M. bovis* B were amplified by PCR and ligated to pNBV1 that had been cut with *Xba*I to obtain pSO5 and pSO7, respectively. pSO5 contained a 2,046-bp *M. tuberculosis* fragment containing a 1-kb region upstream from the ATG start codon plus the entire nucleotide sequence of *phoP* (30). pSO7 contained the equivalent region from the B strain (see text for more details). *hyg*, hygromycin-resistant gene; *ori myc*, mycobacterial origin of replication; *ori*, *E. coli* origin of replication.

RFLP fingerprinting showed that two copies of IS*6110* are present in the B strain. Analysis of the nucleotide sequence of the genomic regions flanking the IS*6110* in this strain revealed that an IS*6110*, corresponding to a 2.0-kb RFLP band, was inserted in a homologous region of DR, as is the case for most *M. bovis* isolates (including BCG isolates) (3). DRs are hotspots for IS*6110* insertion that have been detected in 94% of *M. tuberculosis* clinical isolates (14). Common insertion of IS*6110* in the DR region should represent an ancient IS*6110* transposition event. The second copy of IS*6110*, corresponding to the 2.4-kb RFLP band in the B strain, was inserted 75 nucleotides upstream from the ATG start codon of a DR annotated as being *phoP* in *M. tuberculosis* H37Rv (6). The *phoP*/*phoR* locus of *M. tuberculosis* is a two-component system that is similar to the *phoP*/*phoQ* locus, a global virulence regulator with pleiotropic effects on gene expression in intracellular pathogens such as *Salmonella* spp. (15, 23). In the B strain, IS*6110* was flanked on both sides by a 4-bp DR (TGGC). The generation of 3- to 4-bp DRs after a transposition is one of the characteristics of members of the IS*3* family, particularly IS*6110* in *M. tuberculosis* (22). Thus, the presence of 4-bp DRs flanking IS*6110* strongly suggests that IS*6110* was inserted upstream from *phoP* as a consequence of a transposition event in the B strain.

IS*6110* transposes randomly within the *M. tuberculosis* genome, although it has a preference for particular sites such as DRs or the *ipl* locus (9, 14). We used PCR to search for IS*6110* upstream from *phoP* in 221 MDR *M. tuberculosis* complex clinical isolates. IS*6110* was found at this position in 21 strains, all B strains, indicating that this event is exclusive to this particular MDR isolate. Thus, the unusually high transmission rate of the B strain in comparison to those of most MDR *M. tuberculosis* complex clinical isolates suggests that the insertion of IS*6110* in the promoter region of *phoP* confers some advantage allowing transmission.

Transposable elements frequently alter bacterial gene expression, either by inactivating the gene or by acting as a portable promoter for gene activation mediated by transcriptional regulation. This phenomenon can affect the regulation of the surrounding genes (20). For example, the insertion of IS*1186* upstream from the *cfiA* gene induces resistance to carapenin in *Bacteroides fragilis* clinical isolates (31). The transcription of *cfiA* is driven from a promoter sequence located at the 3′ end of IS*1186*. Moreover, high- and low-level trimethoprim resistance in *Staphylococcus aureus* is mediated by genomic deletions adjacent to a copy of IS*257* inserted upstream from the dihydrofolate reductase gene, *dfrA*. In this case, the insertion sequence encodes a −35 sequence that is necessary for full promoter activity (18). IS*6110* can promote gene expression in *M. tuberculosis* 210 (3). This strain belongs to the Beijing family and has an increased capacity for transmission. According to reverse transcriptase PCR experiments, the insertion of IS*6110* within *ctpD* generates a promoter sequence that upregulates the transcription of the gene (3).

The insertion of IS*6110* upstream from *phoP* in the B strain may affect gene expression given that *phoP* is an important

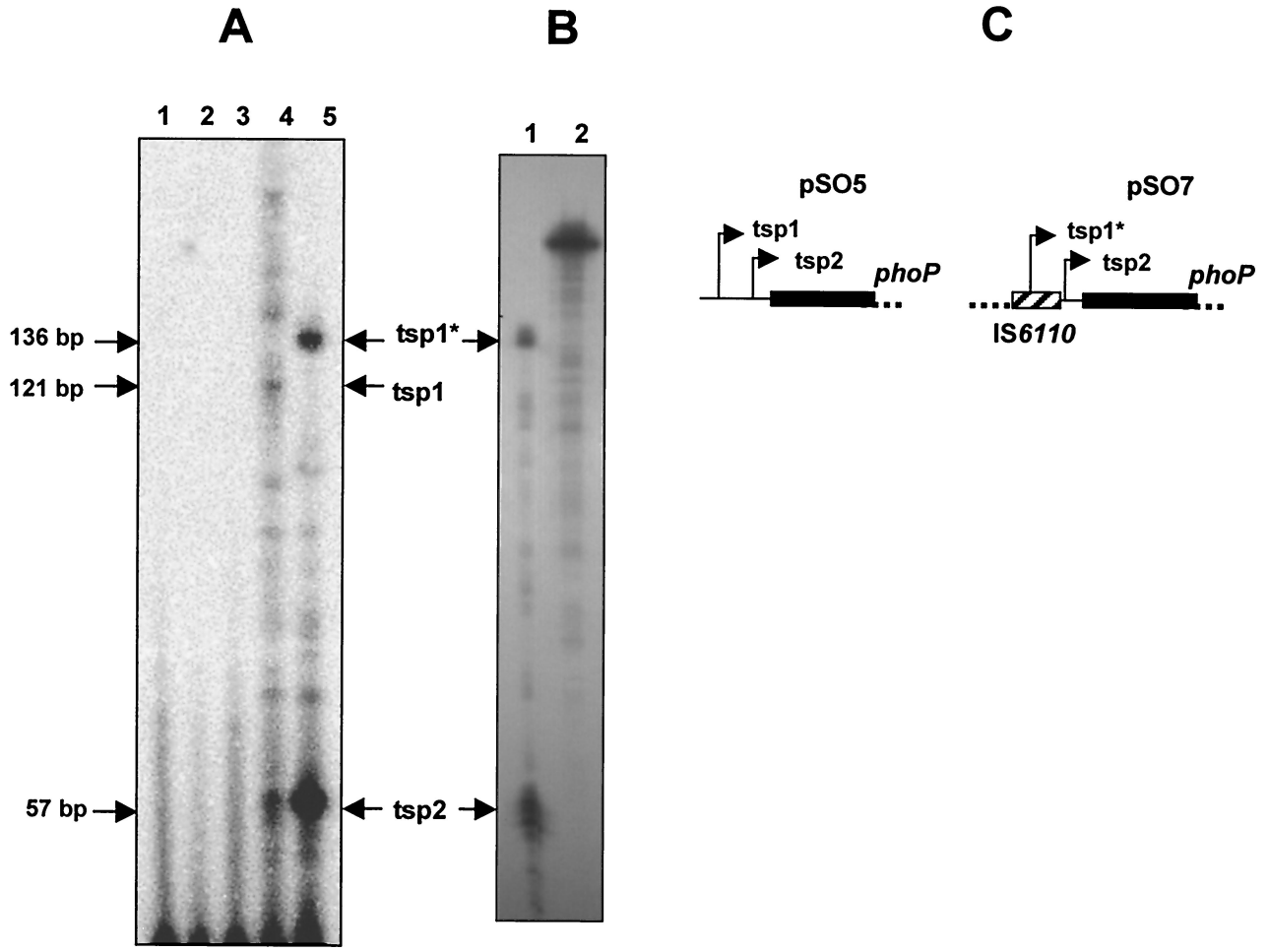

FIG. 5. Mapping the *phoP* transcription starting points. The PE and RPA products were subjected to electrophoresis in 6% (wt/vol) polyacrylamide–8 M urea gels and then to autoradiography. (A) Products of PE analysis using total RNA from *M. smegmatis* mc^2155 (lane 2), *M. smegmatis* mc^2155:pNBV1 (lane 3), *M. smegmatis* mc^2155:pSO5 (lane 4), *M. smegmatis* mc^2155:pSO7 (lane 5) and using no RNA (lane 1). tsp1, tsp1∗, and tsp2 are the putative transcription start points of potential *phoP* promoters for *M. tuberculosis* (pSO5) and the B strain (pSO7). The tsp1 for the B strain (tsp1∗) lies within the IS*6110* nucleotide sequence. No *phoP* transcripts were detected with the control strains (*M. smegmatis* mc^2155 and the same strain transformed with pNBV1). (B) Products of RPA of *M. smegmatis* mc^2155:pSO7 (lane 1) and a probe (lane 2). The locations of the transcription starting points (tsp1∗ and tsp2) are indicated. (C) Schematic figure showing the locations of tsp1, tsp1∗, and tsp2 in the promoter regions of *M. tuberculosis* (pSO5) and the *M. bovis* B strain (pSO7).

transcriptional regulator in intracellular pathogens such as *Salmonella* spp. and *Yersinia* spp. (13, 26, 28). The B strain grows slowly (mean growth time in Lowenstein medium, 57 days) and displays peculiar colony morphology (smaller colonies than other *M. bovis* strains) (35). The insertion of IS*6110* at this particular locus could be responsible for these changes. As a consequence, the variation in the levels of *phoP* expression might affect the genes regulated by PhoP, which has pleiotropic effects in bacteria (13, 30). *M. tuberculosis phoP* mutants are a dramatically different shape and size compared to their wild-type parent when grown in liquid or on solid medium, which is consistent with PhoP being involved in global regulatory circuits in mycobacteria (reference 30 and our unpublished data).

In *Corynebacterium diphtheriae* (which is phylogenetically related to *M. tuberculosis*) iron depletion results in the derepression of virulence genes such as the diphtherial toxin (*tox*) gene mediated by DtxR (diphtheria toxin regulator). The corynebacterial DtxR has an homologue in *M. tuberculosis*, IdeR (iron-dependent regulator) (36). In six genes containing potential IdeR-binding sites, iron-box consensus sequences have been found. One of theses potential IdeR-binding sites is present immediately upstream from the ATG start codon of *phoP* in *M. tuberculosis* (21).

The molecular mechanisms possibly involved in the regulation of *phoP* transcription mediated by IS*6110* in mycobacteria are unknown. The nucleotide sequences of the *phoP* promoter region in *M. bovis* and *M. tuberculosis* are well conserved, differing by just one nucleotide (Fig. 2B). The insertion of IS*6110* into the *phoP* promoter region did not produce nucleotide sequence changes or deletions in the genomic region between the IS*6110* insertion site and the *phoP* ATG start codon in the B strain. PE experiments detected two putative transcription start points (tsp1 and tsp2) for *phoP* in the B and *M. tuberculosis* strains. A new tsp1 promoter region (tsp1∗) was

detected in the B strain within the IS*6110* nucleotide sequence. In addition, the amount of transcripts detected from tsp2 was much higher in the B strain than in the wild-type region, which does not contain IS*6110*. The increase in *phoP* transcription rates in *M. bovis* B is clearly a consequence of the IS*6110* insertion. This suggests that the insertion of IS*6110* upstream from tsp2 altered its promoter sequence, thus generating a new strong promoter region and increasing the *phoP* transcription rate from tsp2.

RFLP analysis using IS*6110* as a probe is a powerful tool for differentiating *M. tuberculosis* strains, making it extremely useful for molecular studies of tuberculosis. Here, we show that IS*6110* upregulates the transcription of a gene implicated in *M. tuberculosis* virulence in a particular clinical isolate that causes MDR TB outbreaks. Another example of an increase in gene transcription mediated by IS*6110* has been shown for a Beijing strain (3). Further studies are necessary to determine how IS*6110* mediates *M. tuberculosis* clinical-isolate physiological changes that can alter the expression of genes determining the infection or transmission rates of certain *M. tuberculosis* isolates. In addition to the most common use of IS*6110* as an RFLP tool, results presented here show one example of the role of IS*6110* in increasing expression of virulence genes in a clinical isolate. We suggest a new view of IS*6110*, according to which it plays a dynamic role in changing the expression of a gene or a network of genes that orchestrate transmissibility in conjunction with epidemiological, environmental, and host factors.

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