Variable-number tandem repeat 3690 polymorphism in Indian clinical isolates of *Mycobacterium tuberculosis* and its influence on transcription

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Variable-number tandem repeat (VNTRs) occur throughout the chromosome of *Mycobacterium tuberculosis*. Although these polymorphic VNTRs, also known as mycobacterial interspersed repetitive units (MIRUs), have proved to be useful tools in molecular epidemiology, their biological significance is less well understood. This study investigated the polymorphism of the VNTR 3690 locus located in the intergenic region between *rv3304* and *rv3303c* (encoding the *gpID2* and *lpdA* genes, respectively) and its possible function in the regulation of gene expression. The copy number of VNTR 3690 was found to vary among Indian clinical isolates of *M. tuberculosis* (one to twelve copies), *M. tuberculosis* H37Rv TMC102 (four copies), *M. tuberculosis* H37Ra (two to four copies), *Mycobacterium bovis* BCG (one copy). The expression of *lpdA* as measured by quantitative RT-PCR was 12-fold higher in *M. tuberculosis* H37Rv than in *M. bovis* BCG. Using a GFP reporter system in which the 5'-flanking region of *lpdA* was fused to the *gfp* gene, the effect of VNTRs on gene expression was measured in an *M. bovis* BCG host background by real-time PCR. Compared with one VNTR repeat, a 12.5-fold upregulation of GFP expression was found with a flanking region containing four VNTR 3690 repeats, indicating that there is a good correlation between VNTR copy number and transcription of *lpdA*.

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INTRODUCTION

The genomes of eukaryotes and prokaryotes are laden with tandem-repeated DNA sequences occurring in several up to thousands of copies, dispersed throughout the genome. Some of these repeats show inter-individual length variability and are designated variable-number tandem repeats (VNTRs). VNTR loci have been of interest because of hypervariability in the number of repeats at each locus and hence have emerged as useful markers to allow individual identification, and tracking of genetic drift and speciation in populations, in both eukaryotes and prokaryotes (Jeffreys *et al.*, 1985; Van Belkum *et al.*, 1998). Contiguous repeats have been characterized in various prokaryotic genomes (Frothingham & Meeker-O'Connell, 1998; Keim *et al.*, 2000; Le Flèche *et al.*, 2002; Supply *et al.*, 2000; Van Belkum *et al.*, 1997) and although they are found most often in intergenic regions, they can also form part of coding sequences. Microsatellites with short-sequence repeats are more

common and have been characterized in various prokaryotic genomes in genes encoding products as diverse as microbial surface components recognizing adhesive matrix molecules and specific virulence factors such as LPS-modifying enzymes or adhesions (Van Belkum *et al.*, 1998). Certain types of bacteria are known to use microsatellite-containing genes called contingency genes, which can confer survival advantages on a subset of the bacterial population under changing environmental conditions; for example, in *Neisseria gonorrhoeae*, they are used as a means of evading the immune system (Van Belkum *et al.*, 1998).

Minisatellite structures composed of 40–100 bp repetitive units reported in *Mycobacterium tuberculosis* are VNTRs (Frothingham & Meeker-O'Connell, 1998), also known as mycobacterial interspersed repetitive units (MIRUs), originally reported in 41 locations throughout the chromosome (Supply *et al.*, 1997). Twelve of these have been shown to display a polymorphism in MIRU copy number among non-related *M. tuberculosis* isolates (Supply *et al.*, 2000). The number of characterized polymorphic loci has since been expanded, and variations in the number of

Abbreviations: EAI, East African-Indian; MIRU, mycobacterial interspersed repetitive unit; SNP, single-nucleotide polymorphism; VNTR, variable-number tandem repeat.

the repeated units have been evaluated in diverse collection of *M. tuberculosis* isolates (Smittipat *et al.*, 2005; Supply *et al.*, 2006). These VNTRs have been located in both coding and non-coding regions, and the polymorphism resulting from their inclusion within coding regions has been suggested as a source of antigenic variation for evading the immune response to provide bacterial populations with evolutionary flexibility to adapt to unpredictable environmental changes in situations in which classical regulation of gene expression is not sufficient (Skuce *et al.*, 2002; Supply *et al.*, 2000). The presence of VNTRs in intergenic regions has been widely reported in eukaryotes and shown to influence gene expression; however, this role has not been addressed in *M. tuberculosis*.

The VNTR 3690 locus, described in this paper, is located in the intergenic region between *rv3303c* and *rv3304*, in the 5'-flanking region of the *lpdA* (*rv3303c*) gene. *LpdA* is a flavoprotein disulfide reductase originally annotated as a probable lipamide dehydrogenase in *M. tuberculosis* (Argyrou *et al.*, 2004). Whilst the laboratory *M. tuberculosis* H37Rv strain TMC102 contains four tandem repeats, *Mycobacterium bovis* BCG has been found to have only one repeat unit. The copy number of VNTR 3690 has been reported to vary extensively, ranging from one to eight copies in one study (Supply *et al.*, 2006) and from one to 22 in another (Le Flèche *et al.*, 2002). As such, the VNTR 3690 locus has been included as one of the polymorphic VNTRs utilized in the 15 and 24 subsets of VNTRs utilized in molecular epidemiological investigations of tuberculosis (Supply *et al.*, 2006). The presence of one imperfect repeat (with two nucleotide differences) present in all isolates and included as one of the copies in molecular typing is noteworthy. Overall, VNTR 3690, also referred to as Mtub39 (Fabre *et al.*, 2004) or allele no. 7, is 58 bp long and has been found to have an allelic diversity of 0.64 (Le Flèche *et al.*, 2002; Supply *et al.*, 2006). Among 65 clonally and epidemiologically related isolates and single-colony cultures, only one variant was identified. The presence and variability of VNTR 3690 in the intergenic region led us to investigate the hypervariability of this VNTR locus in Indian clinical isolates of *M. tuberculosis* and to address the functional role of the repeat variability in transcription by measuring *lpdA* transcription by quantitative RT-PCR (qRT-PCR) in *M. tuberculosis* H37Rv (four copies) and *M. bovis* BCG (one copy), and by fusing intergenic regions containing four and one repeats of VNTR 3690 with a reporter GFP gene (*gfp*) and assaying for GFP expression.

METHODS

M. tuberculosis clinical and reference strains and plasmids.

Seventeen H37 variants were obtained from the American Type Culture Collection (ATCC), including H37Rv isolates 25618, 27294, 35820–35830, 35837 and H37Ra isolates 25177, 35835 and 35836 (Bifani *et al.*, 2000). Other reference strains included *M. bovis* BCG ATCC 35737 and *Mycobacterium microti* ATCC 19422. Clinical isolates included CDC1551 (Valway *et al.*, 1998), W17, W1 and W4 (Bifani *et al.*, 1996), AF9 and AF13 belonging to the same H37 strain

family (Bifani *et al.*, 1999; Gutacker *et al.*, 2006), strain Erdman belonging to the Haarlem family and an ancestral East African–Indian (EAI) isolate originating from India. Clinical isolates were a kind gift of B. Kreiswirth (Tuberculosis Center, Public Health Research Institute, NJ, USA). All samples were molecularly characterized by RFLP, spoligotyping, MIRU–VNTR analysis, principal genetic grouping and single-nucleotide polymorphism (SNP) analysis, as reported previously (Bifani *et al.*, 1996, 2000; Gutacker *et al.*, 2006; Sreevatsan *et al.*, 1997). The Indian clinical isolates were obtained from the All India Institute of Medical Sciences, New Delhi, India, and identified by biochemical characterization and 16S rRNA gene sequencing. Mycobacteria were grown in Sauton's liquid medium (0.5 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 2.0 g citric acid, 0.05 g ferric ammonium citrate, 35 ml glycerol, 4.0 g L-asparagine and 2.5 ml 20 % Tween 80 in 1 litre distilled water, pH adjusted to 7), Middlebrook 7H9 medium (Difco) supplemented with ADC (0.2 % glucose, 0.5 % BSA fraction V, 0.0003 % beef catalase) or solid Middlebrook 7H10 agar (Difco) with OADC enrichment (Becton Dickinson). All cultures were grown at 37 °C.

Plasmid pLL192 is an *Escherichia coli*–mycobacterium shuttle promoter probe vector containing *gfp* and kanamycin resistance reporter genes for cloning of mycobacterial promoters (Srivastava *et al.*, 2007b).

PCR amplification, cloning and nucleotide sequencing. For genomic DNA extraction, mycobacteria were cultured in Lowenstein–Jensen medium for 3 weeks. The cells were harvested and chromosomal DNA was extracted as described previously (Akhtar *et al.*, 2006). The intergenic region between *rv3304* and *rv3303c* (coordinates 3689457 to 3690938 on the *M. tuberculosis* genome; www.sanger.ac.uk) was PCR-amplified from the genomic DNA of *M. tuberculosis* H37Rv, clinical isolates and other mycobacterial strains by using the primers LpdN (5'-CGGGATCCACGGGGGAAAGCT-TAGAC-3', forward primer) and LpdC (5'-CGGGATCCAGCCCA-TAACCTAGCTC-3', reverse primer). The PCR-amplified products were cloned in TOPO TA vector (Invitrogen) and transformed into *E. coli* TOP10 strain (Invitrogen) following the manufacturer's protocol.

For cloning in the pLL192 vector (Srivastava *et al.*, 2007), the intergenic regions were PCR-amplified from *M. tuberculosis* H37Rv TMC102 and *M. bovis* BCG using the LpdN and LpdC primers and subcloned into the *Bam*HI site of pLL192. Recombinant plasmids containing inserts in the correct orientation with respect to the *gfp* gene were selected by *Hind*III restriction digestion. The constructs pLL192::P1 and pLL192::P4 containing one and four copies of VNTR3690, respectively, were electroporated into *M. bovis* BCG. The positive control contained the *hsp60* promoter cloned in the vector and the negative control consisted of vector alone. The nucleotide sequence of the cloned intergenic regions was confirmed with an Automated Sequence Analyser (Applied Biosystem Genetic Analyser model 310) using the M13 primers supplied in the kit.

RNA isolation from mycobacteria and real-time PCR. *M. tuberculosis* H37Rv, *M. bovis* BCG, BCG[pLL192::P1] and BCG[pLL192::P4] cells were grown in Middlebrook 7H9 broth containing ADC and 0.05 % Tween 80 to exponential growth phase (1×10^8 c.f.u. ml⁻¹). Total RNA was isolated using a Qiagen Total RNA isolation kit. Bacterial cells were harvested by centrifugation at 6000 g at 4 °C for 10 min. The bacterial pellets (containing up to 1×10^9 c.f.u.) were resuspended in 1050 µl RLT buffer (Qiagen) containing 0.1 % β-mercaptoethanol. Cell suspensions were transferred to 2 ml screw-capped tubes containing 0.5 ml glass beads (diameter 0.1 mm). The mixture was shaken in a Mini Bead Beater six times for 30 s at maximum speed (~5000 r.p.m.) before performing the rest of the extraction. The tubes were centrifuged for 1 min at 8000 g and 4 °C. Supernatants were transferred to microcentrifuge tubes, 0.7 vols ethanol was added and the

supernatant was applied to two RNeasy columns (Qiagen) per sample, according to the manufacturer's instructions.

The purity of the RNA was determined by measuring absorbance at 260 and 280 nm and quantified by using a Qubit fluorimeter (Invitrogen). For real-time PCR analysis, each RNA sample was treated with Qiagen RNase-free DNase and heat-inactivated according to the manufacturer's instructions. RT-PCR experiments without prior reverse transcription were performed to ensure exclusion of DNA contaminants. RNA (300–400 ng) was used for cDNA synthesis with random hexamer primers by using a RevertAid First-strand cDNA synthesis kit (Fermentas). Internal primers to amplify a region of around 200–250 bp were designed for the *lpdA*, *sigA*, *gfp* and streptomycin resistance (*str*) genes using DNASTAR primer designing software.

Real-time quantitative PCR for the *lpdA* and *gfp* genes was carried out by using a LightCycler RNA Master SYBR Green kit (Roche). *sigA* and *str* RNA were used for normalization. The primers used are listed in Table 1. All primer sets were tested prior to use to ensure that only a single product of the correct size was amplified for each set. Each reaction was repeated three times with three independent RNA samples in a LightCycler 480 II machine. Negative controls consisting of no-template reaction mixtures were run with all reactions. RT-PCR conditions were an initial denaturation step of 10 min, followed by 40 amplification cycles of 10 s at 95 °C, 20 s at 60 °C and 30 s at 72 °C. Melting-curve analysis was carried out to confirm the specificity of the amplified product. Each reaction was repeated three times with three independent RNA samples. After baseline correction and determination of threshold settings, calculations were carried out using the $2^{-\Delta\Delta Ct}$ method of Livak & Schmittgen (2001), as PCR efficiencies were found to be similar. Statistical analysis was carried out using PRISM software. The results are expressed as fold induction, which denotes fold increase in expression of the gene.

RESULTS

VNTR polymorphism in *M. tuberculosis*, *M. bovis* BCG and clinical isolates of *M. tuberculosis*

The intergenic region between *rv3303c* and *rv3304* was PCR-amplified from the genomic DNA of *M. tuberculosis* reference strains, clinical isolates and *M. bovis* BCG using the LpdN and LpdC primers. Agarose gel analysis of PCR amplicons followed by nucleotide sequencing revealed four tandem repeats of VNTR 3690 in *M. tuberculosis* H37Rv TMC102 (laboratory strain), one repeat in *M. bovis* BCG and between one and 12 copies in clinical isolates of *M. tuberculosis* (Fig. 1). The clinical isolates were isolated from tuberculosis patients and characterized as described in Methods. The distribution of repeats in H37Rv variants obtained from the ATCC is shown in Table 2, including H37Rv isolates 25618, 27294, 35820–35830 and 35837, H37Ra isolates 25177, 35835 and 35836, reference strains *M. bovis* BCG ATCC 35737 and *M. microti* ATCC 19422, clinical isolates CDC1551, W17 and W1, AF9 and AF13 belonging to the same cluster as H37Rv/Ra, and strain Erdman. The strains and their sources are described in Methods. In all samples, PCR-amplified sequences were cloned in the TOPO TA vector followed by nucleotide sequencing. The sequences flanking the 58 bp repeat were identical in all cases. The nucleotide sequences of the

Fig. 1. Agarose (2%) gel electrophoresis of the PCR-amplified intergenic region from *M. tuberculosis* H37Rv (lane 2), *M. bovis* BCG (lane 3) and representative Indian *M. tuberculosis* clinical isolates containing three, two, four, twelve and one copy of VNTR 3690 (lanes 4–8, respectively). Lane 1, 50 bp DNA ladder (MBI Fermentas).

intergenic region between *rv3303c* and *rv3304* for *M. tuberculosis* H37Rv and *M. bovis* BCG are given in Fig. 2. Variation among the different isolates was only in copy number of VNTR 3690. All samples contained one imperfect repeat (5'-AGCTGTGCTTG*ACTGCTCGGCT-CCTCCTCACGCCGCTGCGCGGCGTGCATCGTCGCCG-3') with two SNPs, followed by a varying number of tandem repeats of VNTR 3690 (Fig. 2). The imperfect repeat carried an extra C and one G was missing (*) compared with VNTR 3690. The single copy of this imperfect repeat with an identical sequence was present in the same location in all of the strains and isolates described in Table 2.

Expression of *lpdA* in *M. tuberculosis* H37Rv and *M. bovis* BCG

The expression of *lpdA* (*rv3303c*) was monitored in *M. tuberculosis* H37Rv TMC102 and *M. bovis* BCG grown *in vitro* by qRT-PCR using *lpdA*-specific primers (Table 1). RNA was isolated from *M. tuberculosis* and *M. bovis* BCG strains grown *in vitro* and the *sigA* gene was used as an internal control for normalization of transcriptional analysis. The expression of *lpdA* in *M. tuberculosis* was found to be 12-fold higher than that observed in *M. bovis* BCG and this was statistically significant ($P < 0.05$).

Cloning of the intergenic region in pLL192 and the influence of repeat number on transcriptional activity

As the intergenic region formed the 5'-flanking region of *rv3303c*, it was of interest to see whether the region could drive the expression of a heterologous gene cloned downstream from the intergenic region and whether the varying number of tandem repeats influenced

Fig. 2. Nucleotide sequence of the PCR-amplified intergenic region between *rv3303c* and *rv3304* from the genomic DNA of *M. tuberculosis* H37Rv TMC102 (378 bp) (a) and *M. bovis* BCG (204 bp) (b) containing VNTR 3690 and its flanking sequences. The arrows show the location and orientation of the *rv3304* and *rv3303c* genes and their initiation codons (boxed above the arrows) as annotated by Cole *et al.* (1998). In all samples, VNTR 3690 repeats (boxed) were preceded by an imperfect copy (red) of the repeat containing two SNPs. The imperfect repeat contained an extra C residue (boxed), and a G residue (*) was missing.

transcriptional activity. To check this, two intergenic regions with four and one repeat units were PCR-amplified from their respective genomic DNAs and cloned in the TOPO TA vector. After confirmation of the nucleotide sequence, the inserts were excised from the TOPO vector and subcloned in the *Bam*HI site of the promoter cloning vector pLL192 (Srivastava *et al.*, 2007). pLL192 contains the *gfp* and kanamycin resistance reporter genes expressed as a transcriptional fusion preceded by a unique *Bam*HI site, the *str* gene for selection and the mycobacterial and *E. coli* origins of replication (Srivastava *et al.*, 2007). Transformants with the intergenic region cloned in the correct orientation with respect to the *gfp* gene were selected and electroporated into *M. bovis* BCG for

expression analysis. BCG transformants containing pLL192::P1 (one repeat) or pLL192::P4 (four repeats) were grown to exponential phase and total RNA was isolated for *gfp* expression analysis by qRT-PCR, using *str*-specific primers for normalization. Both constructs expressed GFP and were kanamycin resistant, suggesting the presence of a putative promoter in the intergenic region, i.e. 5'-flanking region of the *lpdA* gene. The expression of *gfp* in BCG[pLL192::P4] was 12.5-fold higher than in BCG[pLL192::P1] and this difference was statistically significant (Fig. 3). The differential expression of *gfp* in the two constructs was not related to plasmid copy number as it was normalized with *str* (plasmid marker) as an internal control. No difference in *lpdA* expression was observed by qRT-PCR of *lpdA* using *sigA* for normalization (Fig. 3). The results of these qRT-PCR experiments thus demonstrated a direct association between repeat number and transcription of the gene.

DISCUSSION

Mycobacteria contain highly polymorphic VNTR elements in their genome (Le Flèche *et al.*, 2002; Supply *et al.*, 2006). In this investigation, we characterized the VNTR 3690 polymorphism located in the intergenic region between *rv3304* and *rv3303c*, the latter having been annotated as *lpdA*, and analysed its role in the regulation of expression of *lpdA*. *LpdA*, encoded by *rv3303c*, appears to be a virulence factor of *M. tuberculosis*. The protein has been characterized as encoding a new member of the flavoprotein disulfide reductase catalysing the NAD(P)H-dependent reduction of alternative electron acceptors such as 2,6-dimethyl-1,4-benzoquinone and 5-hydroxy-1,4-naphthoquinone (Argyrou *et al.*, 2004), and thus may contribute to the energy requirements under anaerobic conditions. *LpdA* was one of the major antigens of *M. tuberculosis* identified in clinical sera through the *in vivo*-induced antigen technology approach (Deb *et al.*, 2002). *LpdA* has been shown to protect *M. tuberculosis* from oxidative stress in a mouse model and to contribute to enhanced survival of the tubercle bacillus (Akhtar *et al.*, 2006). NADPH quinone reductase in *Helicobacter pylori* has been shown to play a similar role in oxidative stress and host colonization (Wang & Maier, 2004). It was intriguing to determine whether the presence of more than one copy of VNTR in the 5'-flanking region of *rv3303c* correlated with expression of *lpdA*.

VNTR 3690 is highly polymorphic (Le Flèche *et al.*, 2002; Supply *et al.*, 2006) and exists with variable copy number in *M. tuberculosis* H37Rv TMC102 and *M. bovis* BCG; there are four copies in the former and one in the latter. Determination of the presence of VNTR 3690 was extended to a collection of clinical isolates from northern India and reference strains belonging to the H37Rv/RA family including H37Rv, H37Ra variants, *M. bovis* BCG, representative *M. tuberculosis* strains of the W/Beijing genotype and CDC1551. These reference strains have been typed

Table 1. Primers used for real-time PCR

Gene	Primer sequence (5'–3')
<i>gfp</i>	TTGCACTACTGGAAAACCTACCTG TCCATCTTCTTTAAATCAATACC
<i>str</i>	TTCAGGAACCGGATCAAGAGT GACATCATTCCGTGGCGTTATC
<i>lpdA</i>	GCTCAGCATGGGGTTTCAG CGCGTCCAAGTCGTAGAGC
<i>sigA</i>	CCGGGGCATGGCGTTTCTC ATGCGGCCAGCTTGTGAT

Table 2. Distribution of VNTR 3690 in *M. tuberculosis* reference strains, clinical isolates and other mycobacteria

ND, Not determined.

<i>M. tuberculosis</i> IS6110 fingerprint	Reference code*	No. of repeats	Strain family	Cluster group†	Amplicon size (bp)‡
H37 strains					
Rv9	ATCC 25618	4	H37 reference	VIII	378
Rv7	ATCC 27294	4	H37 reference	VIII	378
Rv1	ATCC 35820	6	H37 reference	VIII	494
Rv2	ATCC 35821	3	H37 reference	VIII	320
Rv8	ATCC 35822	4	H37 reference	VIII	378
Rv3	ATCC 35823	4	H37 reference	VIII	378
Rv5	ATCC 35824	3	H37 reference	VIII	320
Rv6	ATCC 35825	4	H37 reference	VIII	378
Rv8	ATCC 35826	4	H37 reference	VIII	378
Rv8	ATCC 35827	4	H37 reference	VIII	378
Rv8	ATCC 35828	4	H37 reference	VIII	378
Rv4	ATCC 35829	4	H37 reference	VIII	378
Rv8	ATCC 35830	4	H37 reference	VIII	378
Rv7	ATCC 35837	3	H37 reference	VIII	320
Ra1	ATCC 25177	2	H37 reference	VIII	262
Ra1	ATCC 35835	4	H37 reference	VIII	378
Ra1	ATCC 35836	4	H37 reference	VIII	378
H37Rv-25618§	TMC 102	4	H37 reference	VIII	378
AF9	TN 5705	4	H37 clinical	VIII	378
AF13	TN 3095	4	H37 clinical	VIII	378
W/Beijing strains					
W17	TN 2015	1	W/Beijing	II	204
W1	TN 7759	1	W/Beijing	II	204
W4	TN 10775	1	W/Beijing	II	204
KY	TN 8717	1	W/Beijing	II	204
Other strains					
HR102	TN 19546	1	EAI	I	204
Erdman strain	TMC 107	4	Haarlem	III	378
CDC1551	TN 5970	2	CDC	V	260
P	TN 1618	1	LAM	VI	204
<i>M. tuberculosis</i>-complex strains					
<i>M. bovis</i> BCG	ATCC 35737	1	Reference	I	204
<i>M. microti</i>	ATCC 19422	3	Reference	I	320
<i>Mycobacterium africanum</i>	TMC 5122	1	Reference	I	204
<i>M. tuberculosis</i> Indian clinical isolates					
Six isolates	ND	1	Clinical	ND	204
58 isolates	ND	2	Clinical	ND	262
28 isolates	ND	3	Clinical	ND	320
Four isolates	ND	4	Clinical	ND	378
One isolate	ND	12	Clinical	ND	842

*TMC, Trudeau Mycobacterial Collection; TN, tracking number, Public Health Research Institute (NJ, USA).

†Genetic cluster according to Gutacker *et al.* (2006).

‡Includes 146 nt comprising one 58 bp imperfect repeat and flanks. Each additional repeat is 58 bp long.

§Same strain as ATCC 25618 but from a different source.

||Two bases missing on second repeat.

previously by various molecular techniques including IS6110, principal genetic grouping, genetic clustering (SNPs) and spoligotyping (Bifani *et al.*, 2000). The 17 ATCC H37 variants have been discriminated into nine

distinct yet similar patterns (Rv1–Rv9) with a single pattern for all H37Ra (Ra1) isolates. These variants could not be discriminated by polymorphic GC-repetitive sequences and VNTR analysis (Bifani *et al.*, 2000).

Fig. 3. Transcriptional analysis of *gfp* expression in BCG[pLL192::P1] and BCG[pLL192::P4] in culture. P1 and P4 are the intergenic regions between *rv3304* and *rv3303c* containing one and four copies, respectively, of VNTR 3690 cloned into the pLL192 vector. Recombinant *M. bovis* BCG was grown to exponential phase and RNA was isolated. The transcriptional levels of *gfp* and *lpdA* were determined by qRT-PCR, as described in Methods, using *str* and *sigA* as internal controls for normalization, respectively. (1) and (4) represent the copy number of VNTR repeats. The recombinant BCG containing four VNTR repeats showed 12.5-fold enhanced expression of *gfp* expression compared with rBCG containing one VNTR repeat, whilst there was no difference in *lpdA* transcription between the constructs. The fold induction observed with four repeats was statistically significant ($P < 0.05$). Error bars, sd.

PCR amplification of genomic DNA of different strains using the LpdN and LpdC primers and subsequent nucleotide sequencing revealed that 13 Rv variants had varying repeat numbers ranging from three to six, with ten strains having four repeats, three having three repeats and one having six repeats. Out of three Ra strains analysed, two had four repeats, whilst one had two repeats (Table 2). The genome sequence of *M. tuberculosis* sequence published by Cole *et al.* (1998) (www.sanger.ac.uk) contained only one copy of VNTR 3690, as found in *M. bovis* BCG (Table 1). The variability encountered in the strains belonging to all three principal genetic groupings (Sreevatsan *et al.*, 1997) suggested that the repeat can be either lost or duplicated. This is particularly evident when examining the H37 variants. The original H37Rv strain was isolated from a patient at the Trudeau Sanatorium in 1905 and later dissociated into the H37Rv (virulent) and H37Ra (attenuated) strains in 1934 (Bifani *et al.*, 2000; Steenken & Gardner, 1946). All other variants evolved from these two original isolates. As such, the data suggest that both Rv and Ra strains possessed a 378 bp fragment (four repeats) at the time of dissociation, as several of its descendents still have four copies (Rv 35822, 35823, 35825, 35826, 35827, 35828, 35829, 35830, 25618 and 27294, and Ra 35835 and 35836). This observation is further supported by analysis of the recently isolated clinical isolates AF9 and AF13, which share the 378 bp amplicon (Table 2). Only isolate 35820 was found to have duplication, whilst others had deletions (Rv 35821, 35824 and 35837, and Ra 25177). However,

analysis of the Indian clinical isolates revealed a different picture. Only four isolates shared the 378 bp fragment (four repeats), 28 isolates had three repeats (320 bp), 58 had two repeats (262 bp), six had one repeat and one had 12 repeats (842 bp). The clinical isolates were from patients belonging to different regions. If the original isolates had four repeats, then most have undergone deletion with only one having undergone duplication. Further analysis of repeat variability in clinical isolates from different geographical regions will provide an insight into strain polymorphism related to this VNTR locus.

The dispersal of repetitive sequences throughout the genome indicates a possible functional role which is yet to be explored. The location of VNTR loci in promoter regions and their influence on gene activity has been reported in higher eukaryotes. Some examples include VNTR polymorphism in the promoter region of the thiopurine methyltransferase gene (Spire-Vayron de la Moureyre *et al.*, 1999), the human insulin 5'-flanking region influencing tissue-specific expression of insulin and insulin-like growth factor (Eerligh *et al.*, 2004) and the 5'-upstream promoter region of the prostacyclin synthase gene affecting transcriptional activity (Amano *et al.*, 2004). In the case of prokaryotes, in *M. tuberculosis*, VNTR loci have been found located both in the intergenic regions and within genes (Supply *et al.*, 2000, 2001). VNTR 3690 is located in the 5'-flanking region of the *lpdA* gene, which led us to assess the role of repeats in modulating the expression of *lpdA*. The first evidence was derived from transcriptional analysis of the *lpdA* gene in *M. tuberculosis* and *M. bovis* BCG by qRT-PCR. The results demonstrated ~12-fold enhancement of expression in *M. tuberculosis* with four repeats compared with *M. bovis* BCG with one repeat. These results are supported by recent reports of a differential expression phenotype of *lpdA* transcription in *M. tuberculosis* H37Rv and H37Ra strains as determined by qRT-PCR (Zheng *et al.*, 2008), where the 58 bp tandem repeat unit of VNTR 3690 was repeated twice in *M. tuberculosis* H37Rv but three times in *M. tuberculosis* H37Ra, and in exponential-phase cultures, where the transcription of *lpdA* in H37Ra was ~3-fold higher than in H37Rv.

The intergenic region between *lpdA* (*rv3303c*) and *rv3304*, when cloned in the promoter probe vector pLL192 (Saxena *et al.*, 2008; Srivastava *et al.*, 2007, 2008), drove the expression of both *gfp* and kanamycin resistance reporter genes, indicating the presence of a putative promoter, and variable repeat number was found to influence the level of *gfp* expression. *M. bovis* BCG harbouring pLL192 with a cloned intergenic region containing one or four copies of the VNTR showed differences in GFP fluorescence and kanamycin resistance. These results allow us to conclude that VNTR copy number has an effect on transcription of *lpdA* and suggest the presence of a putative promoter within the intergenic region. A hairpin structure (CACGCCGCTGCGGGCGTG) present within the sequence of the repeat unit has been suggested as a

potential binding site for regulatory proteins (Zheng *et al.*, 2008). Regulation of promoter activity through VNTRs may be a way of using transcriptional regulation to adapt to the environmental changes to which *M. tuberculosis* is exposed both outside and inside its human host.

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