Published in final edited form as:

Microbiology. 2004 May ; 150(Pt 5): 1519–1527.

Genome structure in the vole bacillus, *Mycobacterium microti*, a member of the *Mycobacterium tuberculosis* complex with a low virulence for humans

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Abstract

Mycobacterium microti, a member of the *Mycobacterium tuberculosis* complex, is phylogenetically closely related to *M. tuberculosis*, differing in a few biochemical properties. However, these species have different levels of virulence in different hosts; most notably *M. microti* shows lower virulence for humans than *M. tuberculosis*. This report presents genomic comparisons using DNA microarray analysis for an extensive study of the diversity of *M. microti* strains. Compared to *M. tuberculosis* H37Rv, 13 deletions were identified in 12 strains of *M. microti*, including the regions RD1 to RD10, which are also missing in *Mycobacterium bovis* BCG. In addition, four new deleted regions, named MiD1, RD1 β , MiD2 and MiD3, were identified. DNA sequencing was used to define the extent of most of the deletions in one strain. Although RD1 of *M. bovis* BCG and *M. microti* is thought to be crucial for attenuation, in this study, three of the four *M. microti* strains that were isolated from immunocompetent patients had the RD1 deletion. In fact, only the RD3 deletion was present in all of the strains examined, although deletions RD7, RD8 and MiD1 were found in almost all the *M. microti* strains. These deletions might therefore have some relation to the different host range of *M. microti*. It was also noticeable that of the 12 strains studied, only three were identical; these strains were all isolated from immunocompetent humans, suggesting that they could have arisen from a single source. Thus, this study shows that it is difficult to ascribe virulence to any particular pattern of deletion in *M. microti*.

INTRODUCTION

Mycobacterium microti belongs to the *Mycobacterium tuberculosis* complex, which is composed of closely related species that are difficult to distinguish using biochemical properties alone (Tsukamura *et al.*, 1985; Rastogi *et al.*, 2001). Wells first discovered *M. microti* in the 1930s, and named it the 'vole bacillus' (Wells, 1937). It was later designated

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M. tuberculosis var. *muris*, because it could not be differentiated from *M. tuberculosis*. At that time, the bacillus was described as the causative agent of an epizootic disease in the wild English field vole (*Microtus agrestis*) (Brooke, 1941). *M. microti* causes disease in a variety of mammalian species, including wood mice (*Apodemus sylvaticus*), bank voles (*Clethrionomys glareolus*) and shrews (*Sorex araneus*). Disease is rare in llamas (*Lama vicugna molina*), cats and pigs, and very rare in humans (Huitema & Jaartsveld, 1967; Wayne & Kubica, 1986; Kremer *et al.*, 1998; Cavanagh *et al.*, 2002). *M. microti* differs from other members of the *M. tuberculosis* complex in its curved cell morphology (Pattyn *et al.*, 1970), extremely slow growth *in vitro*, and distinct, host-specific pathogenicity for laboratory animals (van Soolingen *et al.*, 1998).

M. microti has also been employed as a vaccine against tuberculosis and was reported to give a high degree of protection, similar to that seen with BCG (*Mycobacterium bovis* bacille Calmette-Guérin) (Sula & Radkovsky, 1976). An attenuated strain of *M. microti* was used as a vaccine in Czechoslovakia from 1951 to 1969, and about 500 000 people, mostly newborn, were vaccinated intradermally. In a separate study in the UK between 1950 and 1952, a vaccine was prepared from non-attenuated *M. microti* and used in vaccination trials organized by the Medical Research Council (Hart & Sutherland, 1977). The trials in Czechoslovakia and the UK gave similar results, with the *M. microti* vaccine conferring about 75 % protection. Furthermore, the *M. microti* vaccine showed low allergenic potency, making it less likely than BCG to compromise the tuberculin test in the vaccinated population. The *M. microti* vaccine induced fewer than 30 % positive skin test conversions in response to Purified Protein Derivative (PPD) from *M. tuberculosis* (Brooke, 1941; Sula & Radkovsky, 1976; Bloom & Fine, 1994). A recent comparison of efficacy between the Pasteur substrain of BCG and the *M. microti* vaccine showed that both could provide protection against tuberculosis in rabbits (Dannenberg *et al.*, 2000) and mice (Manabe *et al.*, 2002). Despite conferring good protection against tuberculosis, *M. microti* can still cause clinical disease in immunocompetent patients, and therefore its use in vaccination may constitute a health hazard (van Soolingen *et al.*, 1998).

Identification of *M. microti* by traditional methods is not easy, so the prevalence, geographical distribution and clinical importance of *M. microti* may have been underestimated (van Soolingen, 2001). *M. microti* grows slowly on solid media supplemented with pyruvate, and shows similar biochemical properties to *M. tuberculosis*, giving variable results for pyrazinamidase and urease activity and niacin accumulation (Levy-Frebault & Portaels, 1992; van Soolingen *et al.*, 1998). Two separate studies have shown the usefulness of novel genetic markers (*IS6110*) to characterize *M. microti* isolates (Kremer *et al.*, 1998; van Soolingen *et al.*, 1998). Moreover, cases of *M. microti* derived tuberculosis in both immunocompromised and immunocompetent human patients have been identified using molecular methodologies (Foudraine *et al.*, 1998; Niemann *et al.*, 2000).

The virulence mechanisms of *M. microti* in its natural hosts, and its low virulence in humans, are not well understood. Like that of *M. tuberculosis*, the *M. microti* cell wall contains 34 % by weight of mycolates. In both species, exponential-phase *in vitro* cultures and bacteria harvested from mouse lungs contain a high proportion of ketomycolates, whereas in stationary-phase cultures, ketomycolates decrease rapidly to give proportions similar to those of methoxymycolates (Davidson *et al.*, 1982; Watanabe *et al.*, 2001). Although there is no clear relationship between possession of a particular mycolate and strain pathogenicity, the same mycolate types are present in all *M. tuberculosis* complex strains, differing only in chain length and other structural features (Daffé & Draper, 1998; Kremer *et al.*, 2000).

In this study, comparative genomics, by microarray assay methods, were employed to delineate the genetic differences between *M. tuberculosis* and *M. microti*, and to relate these to virulence. DNA microarrays were used to study a group of 12 strains classified as *M. microti*, including the strain OV254, originally isolated from voles in the 1930s (Wells, 1937). *M. microti* strains isolated from other mammals, such as pig, llama and hyrax, were also included. Lastly, *M. microti* strains have more recently been isolated from human tuberculosis infections (van Soolingen *et al.*, 1998), from both immunocompromised and immunocompetent individuals, and we included these strains in our investigation. These strains were identified as *M. microti* using the spacer oligonucleotide typing, 'spoligotyping', DNA fingerprinting method (van Soolingen *et al.*, 1998).

METHODS

Bacterial strains

The 12 strains of *M. microti* used in the work described here are listed in Table 1. *M. tuberculosis* H37Rv was used as a comparison in genomic experiments. All strains were grown in Dubos broth (Beckton Dickinson) supplemented with 0.05 % (w/v) Tween 80 (Sigma) and 0.1 % (v/v) Dubos medium albumin (Beckton Dickinson). All mycobacterial liquid cultures were grown in 1 litre rolling bottles and incubated in a rolling incubator (2 r.p.m.) at 37 °C. Growth was monitored by measurement of OD₆₀₀ using a Cecil CE 1010 spectrophotometer. All *M. microti* isolates in this study had been subjected to identification by spoligotyping DNA fingerprint analysis (van Soolingen *et al.*, 1998).

DNA extraction

Chromosomal DNA was isolated by the standard lysozyme method of Larsen (2000).

Synthesis of labelled DNA

Genomic DNA was labelled with dCTP coupled to Cy3 or Cy5 dyes (Amersham Pharmacia Biotech). DNA (3 µg) was mixed with 3 µl random hexamer primers (1 µg ml⁻¹, Invitrogen) in a total volume of 41.5 µl, and the primers were annealed to the DNA by incubating the mix at 95 °C for 5 min, followed by rapid cooling on ice. The tubes were centrifuged briefly, and the following were added: 5 µl 10× Klenow polymerase buffer (Promega), 1 µl dNTP mix (5 mM dA/G/TTP and 2 mM dCTP, Amersham Pharmacia Biotech), 1.5 µl Cy3- or Cy5-dCTP (25 nM, Amersham Pharmacia Biotech) and 1 µl Klenow DNA polymerase (5 U µl⁻¹, Promega). The reaction mixture was incubated at 37 °C for 90 min, and labelled cDNA was eluted in 13 µl distilled water after extraction in a MiniElute column (Qiagen). The probe was mixed with 3.2 µl 20× SSC (0.15 M NaCl, 0.015 M sodium citrate), and 0.3 µl 20 % (w/v) SDS, and then denatured by heating at 95 °C for 2 min. The mix was allowed to cool briefly before being added to the array.

Slide processing

Spotted microarray slides, representing 100 % of the genes of the *M. tuberculosis* H37Rv genome, were prepared at the Bacterial Microarray Group, St George's Hospital Medical School, London. The rehydration and blocking steps were carried out as described by Eisen & Brown (1999).

Hybridization and washing

The processed array slides were placed in prehybridization solution (3.5× SSC, 0.1 %, w/v, SDS and 0.1 % fraction V BSA [Sigma]) at 60 °C for 20 min, to block non-specific probe-binding sites. The slides were then rinsed sequentially for 1 min in distilled water and 1 min in 2-propanol (Merck), and then centrifuged in 50 ml centrifuge tubes at 433 g for 5 min.

Samples of denatured, labelled probe (16 µl) were applied to a dried, prehybridized slide. Post-hybridization and slide-washing steps were performed as described by Eisen & Brown (1999).

Data collection and analysis

Slides were scanned in a dual-laser microarray scanner (Genepix 4000A, Axon Instruments) and the images obtained were analysed using GenePix Pro software (Axon Instruments). The software calculated the mean signal intensity and local background for each spot on the array and subtracted the local background from the signal intensity of each spot. Spots showing a high background or poor hybridization were eliminated from the analysis. Data obtained from six slides, including dye swaps, were analysed with GeneSpring software, version 4.1.5 (Silicon Genetics). To account for dye swap, the signal channel and control channel measurements for dye swap samples were reversed. Each gene's measured intensity was divided by its control channel value in each sample; if the control channel was below 10, then 10 was used instead. If the control channel and the signal channel were both below 10 then no data were reported. Each measurement was divided by the 50.0th percentile of all measurements in that sample. The percentile was calculated using only genes marked present.

PCR amplification and DNA sequencing of deletions

Primers were designed to flank known deletion sites (Table 2). DNA was amplified using HotStar *Taq* polymerase (Qiagen), and the products purified using a Qiaquick PCR purification kit (Qiagen). DNA sequencing was carried out on an ABI377 DNA sequencer using a Big Dye terminator kit (ABI).

RESULTS

Microarray hybridization of genomic DNA

Each of the 12 strains of *M. microti* was compared with *M. tuberculosis* H37Rv by carrying out competitive hybridization with *M. microti* and *M. tuberculosis* DNA. Each comparison was carried out in triplicate. The Cy5/Cy3 fluorescence ratios, calculated for each ORF represented on the array, were normalized using GeneSpring software, and the genes were considered to be significantly differentially hybridized compared with the *M. tuberculosis* control if they displayed at least a twofold difference in the ratio. Fig. 1 shows the hybridization ratio between the two species, indicating that several genes are deleted from the *M. microti* genome. The extent of the deletions in one strain, OV254, was determined by sequencing the PCR products obtained using flanking oligonucleotide primers (Table 2).

Genomic analysis also suggested that the sequence of individual genes was similar in *M. tuberculosis* and *M. microti*, giving equivalent hybridization by *M. tuberculosis* and *M. microti* DNA to genes present in both species. Mismatched hybridization signals probably reflected non-equivalent representation in the genome, as a result of repeated elements or deletions in the *M. microti* genes. However, it should be emphasized that deletions represent only a subset of the total genetic variability; single nucleotide polymorphisms would not be detected using the microarray technique used in this study. Nevertheless, the identification of regions that are present in *M. tuberculosis* and consistently absent from *M. microti* strains may provide insights into the phenotypic differences between the two species (Brodin *et al.*, 2002; Brosch *et al.*, 2000).

Table 3 shows genes deleted in *M. microti* and demonstrates that, apart from three strains isolated from humans, the pattern of deleted sequences was different in every strain examined. In comparison with *M. tuberculosis* H37Rv, each *M. microti* strain had an

average of 9.4 deleted regions. Among the 12 strains, a total of 13 different deleted sequences were detected.

Nine of the deleted sequences were previously identified in BCG (Gordon *et al.*, 1999); therefore the nomenclature for these deletions was based on that of BCG (from RD1 to RD10). The deleted regions RD3, RD7, RD9 and RD10 were identical to the ones in BCG described by Behr *et al.* (1999) and Gordon *et al.* (1999). The RD3 and RD9 deletions have also been identified in clinical isolates of *M. tuberculosis*, where they are referred to as DS5 and DS21, respectively (Kato-Maeda *et al.*, 2001). In addition, four new deletions, MiD1, MiD2, MiD3 (standing for microti deletion, named by Brodin *et al.*, 2002) and RD1 β (to differentiate it from RD1) were identified. Of the 13 deleted regions, only the RD3 deletion was found in all *M. microti* strains. The RD7, RD8 and MiD1 deletions were found in 11 strains, and RD4, RD6 and MiD2 were found only in a few strains.

Presence of *esat6* within the RD1 deletion in *M. microti*

esat6 (Rv3875) appears in a cluster region along with *lhp* (Rv3874) in *M. tuberculosis* and other GC-rich bacteria, including *M. bovis* and *Mycobacterium leprae* (Gey van Pittius *et al.*, 2001). Recent results analysing the function of the ESAT-6 and CFP-10 proteins, known to form a tight 1 : 1 complex (Renshaw *et al.*, 2002), have shown them to be required for invasion of lung interstitial tissue (Hsu *et al.*, 2003). The extent of the RD1 deletion, where *esat6* is located, was found to be highly variable in *M. microti* (Fig. 2). Besides the deletion of genes Rv3871–Rv3876, most strains had the additional, contiguous RD1 β deletion, which meant that the entire deleted region extended from Rv3864 through to Rv3876, compared with Rv3871 to Rv3879c for BCG (Gordon *et al.*, 1999). However, one strain, vole scab15, had the RD1 β deletion in the absence of RD1, and in two strains RD1 resulted in the deletion of a single gene only: Rv3871 in strain vole 15498, and *esat6* (Rv3875) in strain human 97-0770.

Recently, it was reported that *M. microti* is a natural *esat6* deletion mutant (Brodin *et al.*, 2002), but in the present study *esat6* (Rv3875) and *lhp* (Rv3874) were present in two *M. microti* isolates (vole 15498 and vole scab15). However, recent evidence shows that loss of function of ESAT-6 can also result from the loss of the secretion apparatus (Hsu *et al.*, 2003) responsible for its extracellular transport. This has been shown to be coded by genes Rv3870, Rv3871 and Rv3877, which flank *esat6* and *cfp10* (Stanley *et al.*, 2003). Such a loss of ESAT-6 function would result from the deletion of Rv3871 in strain vole 15498. In strain vole scab15, however, where only RD1 β (Rv3864–Rv3869) was deleted, the situation is unclear with regard to ESAT-6 function.

The *plcABC* gene cluster

The RD5 region, which includes the phospholipase C operon, was variable among the *M. microti* strains, with Rv2350c (*plcB*) being the most frequently deleted gene (Fig. 3). In BCG, RD5 deletion was different, deleting from Rv2344 to Rv2354. Interestingly, *plcD* appeared to be deleted in two *M. microti* strains (hyrax and human 97-0770). In *M. tuberculosis*, *plcD* is not located close to *plcA*, *B* or *C*; at the time of writing the precise location of *plcD* in *M. microti* is not known.

Phospholipase activity is known to be involved in virulence in *Pseudomonas aeruginosa* (Johansen *et al.*, 1996; Gomez *et al.*, 2001; Wilderman *et al.*, 2001). Moreover, mutants of *M. tuberculosis* with deletions of *plcA*, *B* and *C* exhibited reduced virulence in a mouse model of infection, although no information was available for the virulence of individual mutants (Raynaud *et al.*, 2002). It is possible, therefore, that the *M. microti* strains from immunocompetent human patients had retained the *plc* genes. Nonetheless, all four isolates

from immunocompetent patients had at least one gene encoding phospholipase C deleted, while the isolate from an immunocompromised patient (human 94-2272) had retained all of the RD5 genes, suggesting that there is no simple relationship between phospholipase C activity and virulence of *M. microti* in man.

RD6 and RD8 deletions

The RD6 region, which encodes PE/PPE proteins, was described by Brodin *et al.* (2002) as not being deleted in *M. microti* strains. In the present study, the situation was variable, with the region being deleted in three of the twelve strains. The RD8 region was deleted in the majority of the strains, but in each case the deletion was not continuous, with two genes (Rv3619c and Rv3620c, encoding proteins of unknown function) present in all the analysed strains, as evidenced by hybridization to the Rv3619c and Rv3620c microarray probes. However when the microarray probes for these two genes were analysed for potential cross-hybridization with other *M. tuberculosis* genes, each gave very high ^{BLAST} homology scores with two other genes (data not shown). Thus the presence of Rv3619c and Rv3620c within the *M. microti* genome could be an artefact due to cross-hybridization. We believe, therefore, that the RD8 deletion is probably continuous in *M. microti*. The RD8 deletion in BCG extends from Rv3617 to Rv3622c.

Newly identified deletions: MiD1, MiD2 and MiD3

Two of the newly identified deletions, MiD1 and MiD3, were deleted in the majority of the *M. microti* strains; MiD1 encodes conserved hypothetical proteins and MiD3 encodes insertion sequences and PE/PPE proteins. The MiD2 region was deleted only in two vole isolates (OV254 and vole 15498), both being strains originally isolated from voles in the 1960s. Therefore, this deletion could have occurred during multiple passages of these strains in culture media. The Rv3345c, Rv3346c and Rv3348 microarray probes from the variable MiD3 region also show cross-hybridization with other genes encoding PE/PPE and PGRS proteins; nevertheless the deleted region from *PE PGRS50* (Rv3345c) to Rv3349c was confirmed by PCR amplification and sequencing (see Table 2).

DISCUSSION

The genomic analysis using DNA microarrays, allied to the confirmation by sequencing across the deleted regions, have been shown to be useful, rapid and simple techniques to identify which genes are missing in *M. microti* relative to *M. tuberculosis*. Since it is based on a *M. tuberculosis* DNA microarray, the technique does not provide information about sequences present in *M. microti* but absent from *M. tuberculosis*. The genomic deletions identified in this study provide insights into mycobacterial diversity, and suggest that many genes in the genome are non-essential. Although the results are mostly compatible with *M. tuberculosis* and *M. microti* having a common ancestor (Brosch *et al.*, 2002), there are some discrepancies. For example, we found that not all *M. microti* strains had RD9 deleted; we have no ready explanation for this apparent disagreement.

With regard to deleted regions and virulence, this study shows that it is difficult to ascribe virulence to any particular pattern of deletion. Although RD1 of BCG and *M. microti* is thought to be crucial for attenuation (Mahairas *et al.*, 1996; Brodin *et al.*, 2002; Pym *et al.*, 2002), in this study three of the four *M. microti* strains from immunocompetent patients had the RD1 region deleted. It is also noticeable that of the 12 strains studied, only three were identical in deletion pattern; these strains were all isolated from immunocompetent humans, suggesting that they may have arisen from a single source.

The genomic comparison of *M. tuberculosis* with *M. microti* identified 13 deletion regions in *M. microti*, compared to *M. tuberculosis*. Nine deleted regions (RD1 to RD10) had already been reported in BCG (Behr *et al.*, 1999; Gordon *et al.*, 1999), although five of these (RD1, RD4, RD5, RD6 and RD8) were not identical to those described for BCG. Although *M. microti* mainly causes disease in small rodents, it can also, rarely, cause disease in man and other large mammals, indicating that the deleted regions do not totally eliminate virulence (van Soolingen *et al.*, 1998; Horstkotte *et al.*, 2001). RD1 is thought to be the principal deletion resulting in attenuation of BCG and *M. microti* (Mahairas *et al.*, 1996; Brodin *et al.*, 2002; Pym *et al.*, 2002); even so, one of the *M. microti* strains investigated here did not have an RD1 deletion and two more had only a single gene deleted within this region. For this reason, although RD1 deletions undoubtedly contribute to attenuation of *M. microti* (Pym *et al.*, 2002, 2003), they are not the only mechanism of attenuation. In fact, only deletion RD3 was present in all of the strains examined, while RD7, RD8 and RD1 were found in almost all *M. microti*, and may therefore have some relation to the host range of *M. microti*. Interestingly, the human isolate 97-0770 has an exceptional and divergent deletion pattern, and since this isolate caused pulmonary tuberculosis in an immunocompetent 34 year old male (van Soolingen *et al.*, 1998), it may have unknown extra genes enabling it to cause disease in immunocompetent individuals.

In general, although the results support the evolutionary scenario proposed for the *M. tuberculosis* complex by Brosch *et al.* (2002), in that most isolates of *M. microti* had the RD7, RD8, RD9 and RD10 deletions, some isolates did not appear to fit into this neat picture. In particular, one isolate did not have the RD7, RD8, RD9 or RD10 deletions, another did not have the RD9 deletion and a third lacked both the RD9 and RD10 deletions – an apparent discrepancy for which at present there is no explanation.

Acknowledgments

We should like to thank Dr Philip Butcher for providing the facilities of the Bacterial Microarray Group, St George's Hospital Medical School (BμG@S), and also for the supply of the *M. tuberculosis* DNA microarrays and for advice. We should also like to thank the Wellcome Trust for funding the Multi-Collaborative Microbial Pathogen Microarray Facility, under its Functional Genomics Resources Initiative. C. C. F. gratefully acknowledges the support of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil). K. K. and D. v. S. gratefully acknowledge EC grant 2000-0630, on Molecular Epidemiology of Tuberculosis. Work at NIMR was funded by the Medical Research Council.

Abbreviation

BCG *Mycobacterium bovis* bacille Calmette-Guérin.

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Fig. 1. Scatter plot of genomic DNA microarray hybridization of *M. tuberculosis* H37Rv versus *M. microti* OV254. The deleted genes in *M. microti* fall below the diagonal line, which denotes equivalent hybridization in both species. The plot was generated by GeneSpring software; the upper and lower lines mark two-fold hybridization ratio.

Fig. 2. Overview of the RD1 and RD1 β regions, showing the genes deleted in the various *M. microti* strains, as well as *M. bovis* BCG. Only in strain OV254 was sequencing performed across the deletion. Accordingly, for the other strains, the extent of the deletion is approximate, being based solely on DNA microarray data.

Fig. 3. Overview of the RD5 region, showing the genes deleted in the various *M. microti* strains, as well as *M. bovis* BCG. Only in strain OV254 was sequencing performed across the deletion (see legend to Fig. 2).

Table 1

Source of the *M. microti* strains used in the study

NIMR, derived from the NIMR, UK, strain collection; D. van Soolingen, derived from the National Mycobacteria Reference Laboratory, The Netherlands, strain collection.

Source host	Strain	Source or reference
Vole (UK)	OV254	NIMR
	15498	Cavanagh <i>et al.</i> , 2002; D. van Soolingen
	scab15	Cavanagh <i>et al.</i> , 2002; D. van Soolingen
	00-0421	Cavanagh <i>et al.</i> , 2002; D. van Soolingen
Pig	15911	Cavanagh <i>et al.</i> , 2002; D. van Soolingen
Llama	15912	Cavanagh <i>et al.</i> , 2002; D. van Soolingen
Hyrax	15499	Cavanagh <i>et al.</i> , 2002; D. van Soolingen
Human (immunocompromised; Netherlands)	94-2272	van Soolingen <i>et al.</i> , 1998
Human, immunocompetent	97-770	van Soolingen <i>et al.</i> , 1998
	99-1853	D. van Soolingen
	2001-1205	D. van Soolingen
	2001-1206	D. van Soolingen

Table 2
Comparison of deleted regions in *M. microti* OV254 and *M. tuberculosis* H37Rv

Deleted region *	ORF and/or genes deleted	Deletion in [†]		Region start/end [‡]	Primer name	Primer sequence (5' to 3')	Annealing temp. (°C)
		<i>M. tuberculosis</i> H37Rv	<i>M. microti</i> OV254				
RD1 and RD1β	Rv3864-Rv3876	No	Yes	4340416-4354536	RD1-For	TGCAGATAATGATGGCG	55
					RD1-Rev	CAGTCCAAACGGACCGA	
RD3	Rv1573-Rv1586c	No	Yes	1779267-1788515	RD3-For	GGACTGCGTGATCGGGCG	60
					RD3-Rev	CATGGCCGATGTGATCC	
RD5	Rv2346c-PPE39 (Rv2346c-Rv2353c)	ND	ND	ND	RD5-For	CGTCGTTCCGGCGGTTCG	60
					RD5-Rev	GCAGTAACAACATCGGC	
RD7	yrbE34-Rv1977 (Rv1964-Rv1977)	No	Yes	2207995-2220725	RD7-For	ATGCTAACTCGTGGCCGAC	60
					RD7-Rev	CGTCGGGTCGAGGTACT	
RD8	ephA - lpgG (Rv3617-Rv3623)	No	Yes	4056839-4062734	RD8-For	GCACAATCACCTCGCCG	55
					RD8-Rev	CTCGGAGATCGAGATCA	
RD9	cobL - Rv2075 (Rv2072c-Rv2075)	No	Yes	2330072-2332101	RD9-For	GCATGCCAGAGCGGC	60
					RD9-Rev	CGGCACGCACAACTCTCGT	
RD10	Rv0221-Rv0223c	No	Yes	264754-266656	RD10-For	CTCAACTACACACATCCG	60
					RD10-Rev	CGGTCAGGCGCTGACGT	
Mid1	Rv2816c-Rv2818c	No	Yes	3121881-3126685	Mid1-For	GTACCTCTCTCGATGAAC	55
					Mid1-Rev	TCTACAGCTACGCGCGA	
Mid2	Rv3188-Rv3189	No	Yes	3554066-3555259	Mid2-For	GAGCTGATCAAAACCCGG	60
					Mid2-Rev	GACAGCAGAGGTGCCAA	
Mid3	PE PGRS50-Rv3349c (Rv3345c-Rv3349c)	No	Yes	3741139-3755777	Mid3-For	GACGCCGCGCTCGCCGT	60
					Mid3-Rev	CTTGCTCTTCACCGCGG	

ND, Presence or absence of deletion not determined (PCR product not obtained).

*The RD nomenclature used in this table is based on Gordon *et al.* (1999).

[†]Yes, deletion found in the strain; no, deletion not found.

[‡]Position in relation to the *M. tuberculosis* H37Rv genome sequence.

Distribution of deletions in the *M. microti* strains
The figures in each column indicate the numerical designation of each ORF in the genome of *M. tuberculosis* H37Rv, omitting the ‘Rv’ prefix. The ‘c’ that follows some numbers indicates that the gene has a reverse orientation. It should be noted that the extent of the RD1, 4, 5, 6 and 8 deletions is not identical in BCG.

<i>M. microti</i> strain	Region deleted												
	RD1	RD3	RD4	RD5*	RD6	RD7	RD8	RD9	RD10	Mid1	RD1β	Mid2	Mid3
OV254	3871–3876	1573–1585c	-	2350c–2355	-	1964–1977	3617–3622c	2073c–2074	0221–0223c	2816c–2818c	3864–3870	3188–3189	3345c–3349c
Vole 15498	3871	1573–1585c	-	2350c–2351c	-	1964–1977	3617–3622c	2074	0221–0223c	2816c–2818c	3864–3870	3188–3189	3349c
Vole scab15	-	1575–1585c	-	-	3425–3426	1964–1977	3618–3622c	-	-	2816c–2818c	3864–3869	-	3347c
Vole 00-0421	3871–3876	1575c–1585c	1501–1507c	2350c–2351c	3425–3428c	1964–1977	3617–3622c	2074	0221–0223c	2816c–2818c	3864–3870	-	3346c–3349c
Pig	3871–3876	1573–1585c	-	-	-	1964–1977	3617–3622c	2073c–2074	0221–0223c	2816c–2818c	3864–3870	-	3345c–3349c
Hyrax	3874–3876	1573–1585c	-	2349c–2351c*	-	1964–1977	3617–3622c	2073c–2074	0221–0223c	2816c–2818c	-	-	-
Llama	3871–3876	1573–1585c	-	-	-	1964–1977	3617–3622c	2073c–2074	0221–0223c	-	3864–3870	-	3346c–3349c
Human 94-2272	3871–3876	1574–1585c	-	-	-	1964–1977	3617–3622c	-	0221–0223c	2816c–2818c	3864–3870	-	3347c–3349c
Human 97-0770	3875	1573–1585c	1505c–1511	2347c–2351c*	3425–3426	-	-	-	-	2816c–2818c	-	-	3343c
Human 99-1853	3871–3876	1573–1585c	-	2350c	-	1964–1977	3617–3621c	2073c–2074	0221–0223c	2816c–2818c	3864–3870	-	3345c–3349c
Human 2001-1205	3871–3876	1573–1585c	-	2350c	-	1964–1977	3617–3622c	2073c–2074	0221–0223c	2816c–2817c	3864–3870	-	3345c–3349c
Human 2001-1206	3871–3876	1573–1585c	-	2350c	-	1964–1977	3617–3622c	2073c–2074	0221–0223c	2816c–2818c	3864–3870	-	3345c–3349c

* Strains human 97-0770 and hyrax also have *plcD* (Rv1755c) deleted.