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Mutations in *Mycobacterium tuberculosis Rv0444c*, the gene encoding anti-SigK, explain high level expression of MPB70 and MPB83 in *Mycobacterium bovis*

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Summary

It has recently been advanced that *Mycobacterium tuberculosis* sigma factor K (SigK) positively regulates expression of the antigenic proteins MPB70 and MPB83. As expression of these proteins differs between *M. tuberculosis* (low) and *Mycobacterium bovis* (high), this study set out to determine whether *M. bovis* lacks a functional SigK repressor (anti-SigK). By comparing genes near *sigK* in *M. tuberculosis* H37Rv and *M. bovis* AF2122/97, we observed that *Rv0444c*, annotated as unknown function, had variable sequence in *M. bovis*. Analysis of *in vitro mpt70/mpt83* expression and *Rv0444c* sequencing across *M. tuberculosis* complex (MTC) members revealed that high-level expression was associated with a mutated *Rv0444c*. Complementation of *M. bovis* bacillus Calmette-Guerin Russia, a high producer of MPB70/MPB83, with wild-type *Rv0444c* resulted in a significant decrease in *mpb70/mpb83* expression. Conversely, a *M. tuberculosis* H37Rv mutant which expressed *sigK* but not *Rv0444c* manifested the *M. bovis* phenotype of high-level MPB70/MPB83 expression. Further support that *Rv0444c* encodes the anti-SigK was obtained by yeast two-hybrid studies, where the N-terminal region of *Rv0444c*-encoded protein interacted with SigK. Together these findings indicate that *Rv0444c* encodes the regulator of SigK (RskA) and mutations in this gene explain high-level MPT70/MPT83 expression by certain MTC members.

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Introduction

The *Mycobacterium tuberculosis* complex (MTC) comprises pathogenic organisms for humans (*M. tuberculosis sensu stricto*), cattle (*Mycobacterium bovis*) and a number of other hosts, including seals, voles and goats (*Mycobacterium pinnipedii*, *Mycobacterium microti* and *Mycobacterium caprae* respectively) (Mostowy *et al*., 2005; Smith *et al*., 2006). Post-genomic study of these naturally occurring variants of the tubercle bacillus has revealed limited genetic variability, with 99.9% similarity at the nucleotide level and about 2% variability in genome content through insertion/deletion events (Sreevatsan *et al*., 1997; Fleischmann *et al*., 2002; Gutacker *et al*., 2002; Garnier *et al*., 2003). Nonetheless, an important difference between these closely related organisms is observed in their antigenic protein repertoire. For instance, both *M. microti* and the dassie bacillus are natural mutants of the RD1-region encoding ESAT-6 (Pym *et al*., 2002; Mostowy *et al*., 2004a), a region also implicated in the attenuation of bacillus Calmette–Guerin (BCG) vaccine strains (Pym *et al*., 2002; Lewis *et al*., 2003). Furthermore, sequence comparison of *M. bovis* AF2122/97 to *M. tuberculosis* H37Rv has revealed a number of differences in the coding sequences of antigenic PE/PPE PGRS proteins (Garnier *et al*., 2003).

A long-recognized and striking phenotypic difference between *M. bovis* and *M. tuberculosis* is in the production of antigenic proteins MPB70 and MPB83. *M. bovis* produces these proteins at high levels *in vitro*, whereas, for *M. tuberculosis*, there is a very low *in vitro* production of the corresponding proteins, MPT70 and MPT83 (by convention, '*mpt*' refers to a gene in *M. tuberculosis* while '*mpb*' refers to the corresponding gene in *M. bovis*) (Hewinson *et al*., 1996). Further studies demonstrated that with the exception of *M. bovis*, which had elevated expression of MPB70, all MTC members tested, including *M. tuberculosis*, *Mycobacterium africanum* and *M. microti*, were low producers of this antigen (Liebana *et al*., 1996; Cousins *et al*., 2003). Interestingly, production of these proteins by *M. bovis* BCG strains is also variable (Wiker *et al*., 1996). BCG strains obtained from the Pasteur Institute before 1927 ('early strains') produce these proteins at high levels unlike strains

obtained in 1931 or later ('late strains') where production is low (Charlet *et al*., 2005). Recently our laboratory has demonstrated that a mutation in *sigK,* the gene encoding the extracytoplasmic function (ECF) sigma factor K, explains the difference in MPB70/MPB83 expression between the two groups of BCG strains (Charlet *et al*., 2005). Given that SigK is a positive regulator of MPB70 and MPB83, it was reasoned that a second polymorphism in SigK-mediated control of MPB70/MPB83 expression might explain their differential production between *M. tuberculosis* and *M. bovis*.

Most ECF sigma factors and their cognate anti-sigma genes are adjacent to each other and are co-transcribed (Missiakas and Raina, 1998; Bashyam and Hasnain, 2004). The repressor of the ECF sigma factor (the anti-sigma) binds to it and inhibits its activity. Upon an extracellular stimulus, the repressor dissociates from the sigma factor, which now becomes available and binds to the promoters of the target genes to initiate their transcription (Missiakas and Raina, 1998; Bashyam and Hasnain, 2004). As SigK is implicated in the regulation of MPB70/MPB83 expression, we hypothesized that the phenotypic difference between *M. tuberculosis* and *M. bovis* may be due to a dysfunctional anti-SigK in *M. bovis*. To test this, we employed the recently derived phylogenetic scenario for MTC evolution to search candidate genes for polymorphisms that correlated with the expression phenotype, and then performed molecular studies to document that *Rv0444c* encodes the anti-SigK, and that mutations in this gene explain the variable expression of MPB70 and MPB83 across different MTC members.

Results

Differential expression of MPT70 and MPT83 expression across MTC members

Differential *in vitro* expression of MPB70 has been described in *M. tuberculosis* and *M. bovis* as well as other traditional MTC members (Liebana *et al*., 1996; Cousins *et al*., 2003). Using the revised MTC phylogeny, we documented MPT70 and MPT83 expression across a panel of MTC members to specifically determine which organisms manifest altered expression. By quantitative reverse transcription-PCR (qRT-PCR) coupled with a molecular beacon to analyse mRNA levels of *mpt70*, our results demonstrate that this gene is differentially expressed across MTC members *in vitro*. Specifically, *M. tuberculosis* H37Rv and H37Ra, *M. africanum* and *M. microti* had low expression whereas *M. caprae*, *M. bovis*, BCG Russia and the Oryx bacillus were high expressors of *mpt70* (Fig. 1A). To show that differential expression of *mpt70* (Fig. 1A) also affects protein levels, we performed a Western blot analysis on culture filtrates using a rabbit polyclonal antibody against MPB70. As shown in Fig. 1B, the results correlate perfectly with the qRT-PCR data. We previously noted that the expression of *mpb70* by BCG strains is coupled to *mpb83* expression *in vitro* (Charlet *et al*., 2005). To determine whether this is also the case across natural MTC members, we tested MPT83 expression by Western blot using a polyclonal rabbit antibody; the results parallel those of MPT70 (Fig. 1C). A polyclonal antibody raised against MPB64, a secreted antigenic protein whose expression is independent of SigK, was used as a loading control. As evidenced in Fig. 1D, all strains produced this protein. In addition, for each member of the MTC, we also compared the *in vitro* transcriptome against that of *M. tuberculosis* H37Rv, to screen for important transcriptional differences across organisms (data provided in Table S1). Microarray-based interrogation revealed that upregulation of *mpb70*, *mpb83* and neighbouring genes (*dipZ*, *Rv2876*) by *M. caprae*, *M. bovis*, BCG Russia and the Oryx bacillus figured prominently among their principal differences in expression compared with *M. tuberculosis*. Based on these results, MTC members analysed could be divided into two groups: *M. tuberculosis* H37Rv and H37Ra, *M. africanum* and *M. microti* had low *in vitro* production of MPT70 and MPT83, while high production was observed for *M. bovis*, BCG Russia (early BCG), *M. caprae* and the Oryx bacillus.

Expression of mpt70 *and* mpt83 *in* M. tuberculosis *is induced inside THP-1 macrophages*

The low levels of MPT70 and MPT83 in *M. tuberculosis* have been documented *in vitro* (Hewinson *et al*., 1996). However, during intracellular infection, the organism modulates its expression, and *mpt70/mpt83* figure among the genes reported to be strongly induced during adaptation to the phagosomal environment (Schnappinger *et al*., 2003). In this report, confirmation of microarray results by qRT-PCR was only performed for *mpt83*. To confirm that *mpt70* expression is also inducible in *M. tuberculosis*, THP-1 macrophages were infected with *M. tuberculosis* H37Ra and virulent H37Rv, and bacterial mRNA was isolated to study *mpt70* expression by qRT-PCR. As shown in Fig. 2, *mpt70* levels are significantly higher in both H37Ra and H37Rv after 24 h inside THP-1 cells pretreated with interferon-γ (IFN-γ) as compared with non-stimulated cells, indicating that *mpt70* is induced in stimulated macrophages (Fig. 2). Similar results were obtained for *mpb83* (data not shown). These data imply that the wild-type expression phenotype, as exemplified by *M. tuberculosis*, is low *in vitro* and inducible during infection.

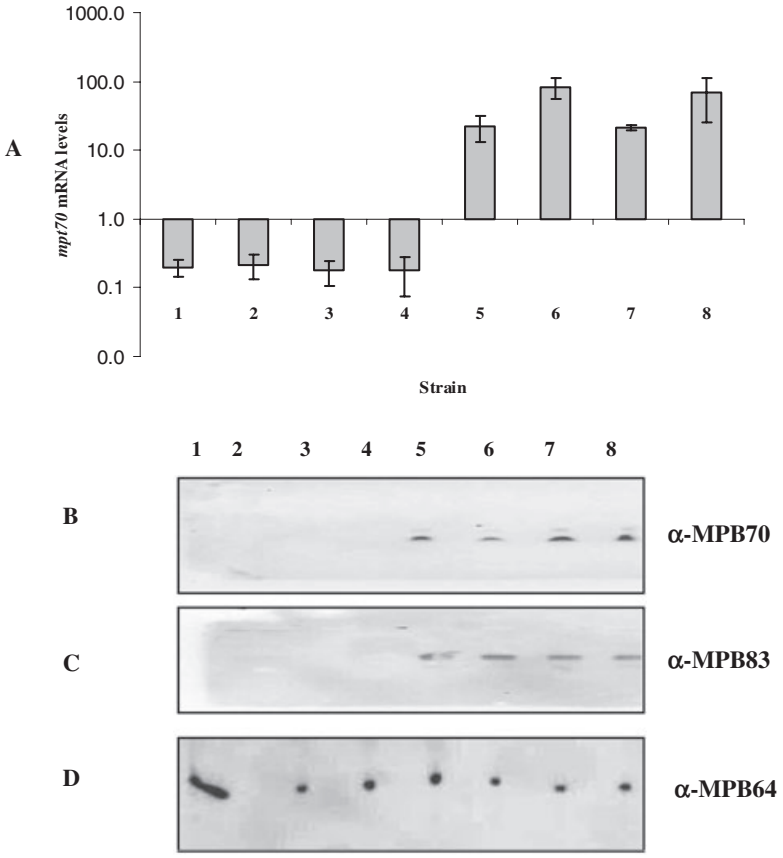

Fig. 1. A. *In vitro mpt70* mRNA levels across different MTC members. mRNA levels were measured by molecular beacon RT-PCR. Values represent the average of at least two independent RT-PCR reactions and are normalized to *sigA* mRNA.
B–D. Western blot analysis of MPT70 (B), MPT83 (C) and MPT64 (D) production across MTC members. MPT64, a secreted antigen not regulated by SigK, was used as loading control. (B) and (D) are SDS-PAGE of culture filtrate proteins probed with a rabbit polyclonal antibody against MPB70 and a mouse polyclonal antibody against MPB64 respectively. (C) represents SDS-PAGE of cell extracts immunoblotted with a rabbit polyclonal antibody against MPB83.
MTC members used are as follows: 1, *M. tuberculosis* H37Rv; 2, *M. tuberculosis* H37Ra; 3, *M. africanum*; 4, *M. microti*; 5, Oryx bacillus; 6, *M. caprae*; 7, *M. bovis*; 8, *M. bovis* BCG Russia.

Genetic basis behind MPT70/MPT83 differential expression

To elucidate the genetic reason for the variable expression of MPT70 and MPT83 across MTC members, we first focused our attention on *M. bovis* (high producer) and *M. tuberculosis* H37Rv (low producer), whose whole genome sequences are publicly available (http://genolist.pasteur.fr/TubercuList, http://genolist.pasteur.fr/BoviList). The sequences of *M. bovis mpb70* and *mpb83* are identical to their *M. tuberculosis* counterparts *mpt70* and *mpt83*. As both organisms also have the identical sequence of *sigK*, the gene encoding the positive regulator of the two antigens (Charlet *et al*., 2005), we hypothesized that *M. bovis* may lack a functional anti-SigK. By analysing the sequence of the genes in the vicinity of *sigK* in *M. tuberculosis* H37Rv versus *M. bovis* 2122, we found that only one gene, *Rv0444c*, located immediately downstream of *sigK*, varies between the two organisms. In *M. bovis*, a high producer of MPB70 and MPB83, *Rv0444c* harbours two non-synonymous single nucleotide polymorphisms (SNPs): C320T and C551T, resulting in an amino acid change of glycine to aspartic acid and glycine to glutamic acid respectively.

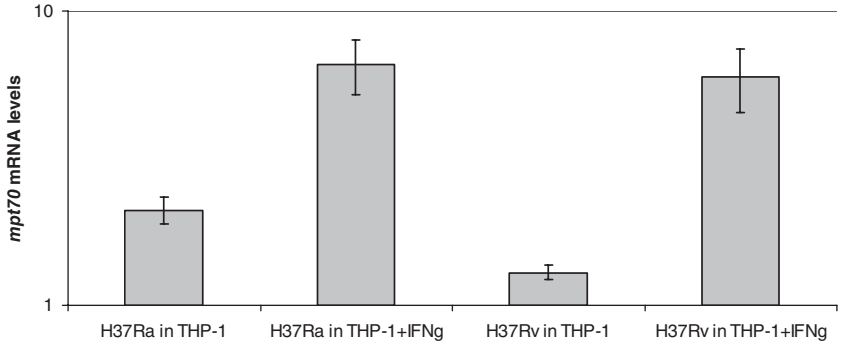

Fig. 2. Induction of *mpt70* expression in *M. tuberculosis* H37Rv and H37Ra inside THP-1 cells. mRNA levels were measured by molecular beacon RT-PCR. Data are expressed as the ratio between mRNA levels inside THP-1 versus mRNA levels of the same organism during extracellular growth *in vitro*. Values represent the average of at least two independent RT-PCR reactions and are normalized to *sigA* mRNA.

Table 1. *Rv0444c* SNPs across members of the MTC.

MTC member	NT@320	NT@551	NT@698	MPT70/MPT83 production
M. tuberculosis H37Rv	C	C	G	Low
M. tuberculosis H37Ra	C	C	G	Low
M. africanum	C	C	G	Low
M. microti	C	C	G	Low
Oryx bacillus	C	C	*C*	High
M. caprae	T	T	G	High
M. bovis	T	T	G	High
Early BCG (Russia)	T	T	G	High

The changes from C to T result in G320D and G551E in *M. caprae M. bovis,* and BCG Russia. Changes from G to C in the Oryx bacillus results in stop codon replaced by Serine. SNPs in *Rv0444c* correlate with high *mpb70* expression.

We then sequenced *Rv0444c* across different MTC members and found that members with low-level production of MPT70/MPT83 have the wild-type gene (H37Rv sequence) whereas members with high-level expression of these antigens have a mutated *Rv0444c* (Table 1). Of note, the Oryx bacillus, which produces MPT70/MPT83 at high levels *in vitro* (Fig. 1), has a different *Rv0444c* mutation than *M. bovis*; the stop codon has been substituted with a serine, resulting in a read-through mutation that may affect protein translation and/or stability. The observation that the gene immediately neighbouring *sigK* had suffered two independent mutations that were associated with the same expression phenotype strongly implicated *Rv0444c* as the gene encoding anti-SigK.

Complementation of BCG Russia with wild-type Rv0444c *results in a significant decrease in* mpb70/83 *expression*

To functionally prove that *Rv0444c* gene product is the anti-SigK, we complemented BCG Russia, which has a mutated *Rv0444c* and is a high producer of MPB70/83, with the wild-type *Rv0444c* amplified from *M. tuberculosis* H37Rv, expressed under the mycobacterial optimal promoter (MOP) which consists of the BCG*hsp*70 promoter and the *Escherichia coli* tac promoter (George *et al*., 1995). To control for gene copy, we separately complemented BCG Russia with an overexpressed mutant *Rv0444c* (amplified from BCG Russia itself). Overexpression of wild-type and mutant *Rv0444c* was verified by qRT-PCR with SYBR green (data not shown). Next, using qRT-PCR, coupled with molecular beacons, we observed that addition of wild-type *Rv0444c* resulted in a pronounced decrease in *mpb70/83* expression as compared with BCG Russia complemented with the empty vector, but complementation with the mutant *Rv0444c* had no effect in *mpb70/83* expression (Fig. 3). This indicated that the decrease in expression we observed was specifically due to the introduction of wild-type *Rv0444c*.

Lack of Rv0444c *expression results in overexpression of* mpt70 *and* mpt83

As a second means of proving that *Rv0444c* encodes the anti-SigK, we aimed to determine whether the lack of a functional *Rv0444c* in *M. tuberculosis* would result in an overexpression of *mpt70*/*mpt83*. To this end, we constructed a *M. tuberculosis* H37Rv mutant that expresses *sigK* but not *Rv0444c* in the following manner. A Δ*sigK* mutant was engineered by allelic exchange, replacing *sigK* with a kanamycin cassette using the pKO knock-out plasmid as described in Lewis *et al*. (2003). Deletion of *sigK* and its replacement by the kanamycin gene was confirmed by Southern blot analysis (Fig. 4A and B) and sequence analysis of the mutant indicated that the kanamycin cassette inserted between nucleotide positions 533 815 and 534 397 of the *M. tuberculosis* H37Rv genome resulting in the complete removal of *sigK* (Fig. 4C). As *sigK* and *Rv0444c* are in close proximity to each other (only 43 bp between them) and predicted to be co-transcribed, we analysed the expression of both *sigK* and *Rv0444c* in the deletion mutant by qRT-PCR. As

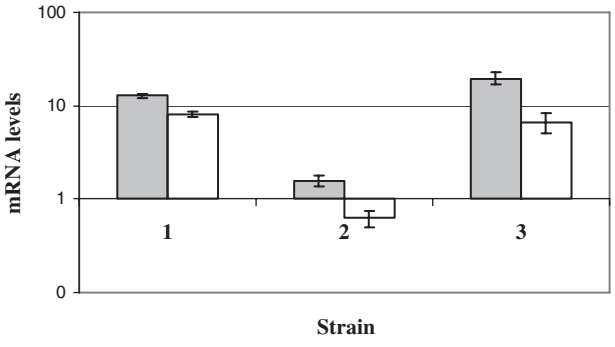

Fig. 3. Expression of *mpb70* and *mpb83* in Russia complements. Values represent the average of at least two independent cDNA samples and are normalized to *sigA* mRNA. 1, Russia-PMH (Russia complemented with empty vector); 2, Russia::wt*Rv0444c* (Russia complemented with wild-type *Rv0444c* amplified from *M. tuberculosis* H37Rv); 3, Russia::*mutRv0444c* (Russia complemented with mutant *Rv0444c* amplified from BCG Russia). Grey bars, *mpb70;* white bars, *mpb83*.

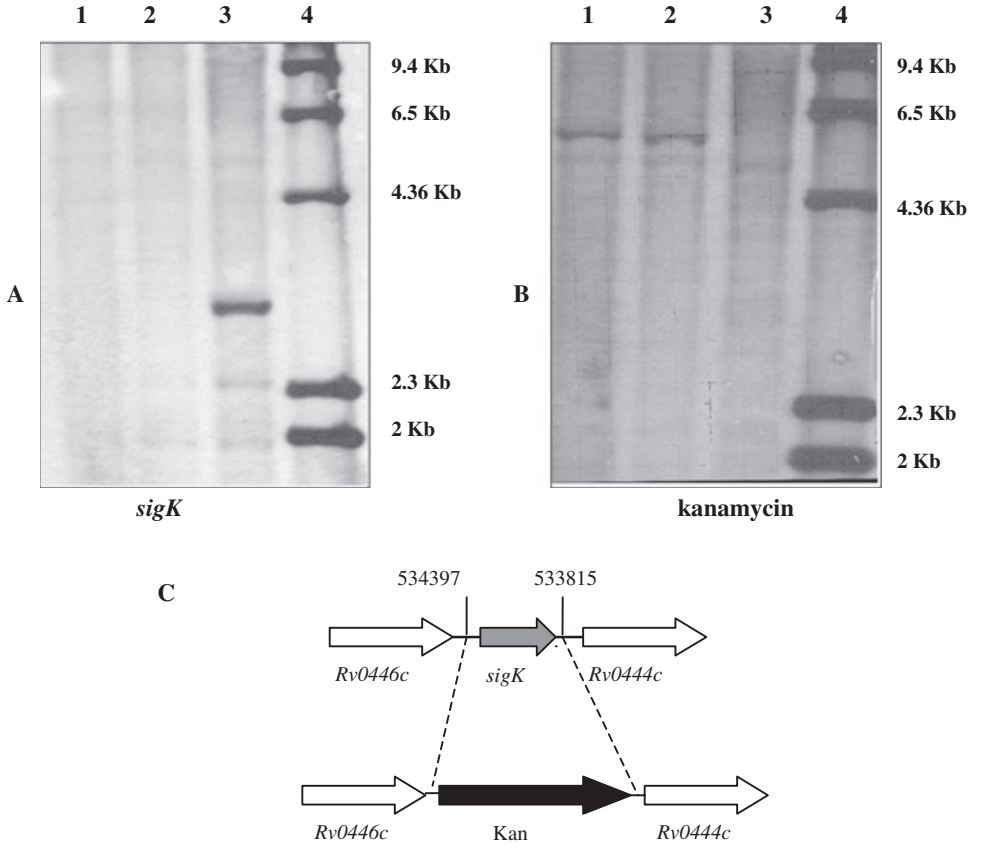

Fig. 4. Confirmation of *sigK* deletion. Southern blot analysis of *sigK* (A) and kanamycin (B) in *M. tuberculosis* H37Rv and two independent clones of *sigK* deletion mutants. Genomic DNA was digested with PvuII, and probed with the specified probe. 1, Δ*sigK* clone 2-2; 2, Δ*sigK* clone 2-5; 3, H37Rv; 4, ladder with sizes of the bands marked on the right. (C) *sigK* locus and schematics of the kanamycin cassette insertion in the mutant. Numbers refer to the nucleotide position in the H37Rv genome sequence.

expected, neither gene was expressed in the mutant, and hence the *sigK* deletion created a polar mutation affecting *Rv0444c* expression (Fig. 5A). Next, we separately complemented this strain with *sigK* alone (single gene complement) and with both *sigK* and *Rv0444c* gene (dyad complement). As shown in Fig. 5A, the single gene complement only expressed *sigK* whereas the dyad complement expressed both *sigK* and *Rv0444c*. These data were confirmed by a Western blot analysis of the *Rv0444c* gene product (Fig. 5B). We then analysed the expression of *mpt70* and *mpt83* in these clones by qRT-PCR. As shown in Fig. 6A, the single gene complement, which expressed *sigK* but not *Rv0444c*, expressed high levels of *mpt70*/*mpt83 in vitro*. The expression of *mpt70*/*mpt83* in the Δ*sigK*::dyad strain was at low levels, similar to H37Rv (Fig. 6A). In perfect agreement with the qRT-PCR data, Western blot analysis of the same strains demonstrated that the *sigK* complement (Δ*sigK*::*sigK*) became a high producer of MPT70 and MPT83 proteins, whereas the dyad complement expressed MPT70 and MPT83 at levels similar to H37Rv (Fig. 6B and C). These results once again showed that *Rv0444c* encodes a protein with anti-SigK activity.

Co-transcription of sigK *and* Rv0444c *and physical interaction of their gene products*

For other anti-sigma factors, the genes encoding the anti-sigma factor and the sigma factor are co-transcribed, and there is physical interaction between the anti-sigma factors N-terminus and its cognate sigma factor (Yoshimura *et al*., 2004; Hahn *et al*., 2005). To determine whether these properties could be observed for *Rv0444c* and *sigK*, first RT-PCR was performed using *sigK* and *Rv0444c* primers, providing the expected product (data not shown). Next, analysis of the membrane topology of *Rv0444c* gene product *in silico* (http://bioinf.cs.ucl.ac.uk/psipred/) predicted that the *Rv0444c*-encoded protein would be a transmembrane protein with an intracellular N-terminal and an extracellular C-terminal, consistent with other anti-sigma factors of *M. tuberculosis* (Hahn *et al*., 2005). We therefore tested for direct interaction between

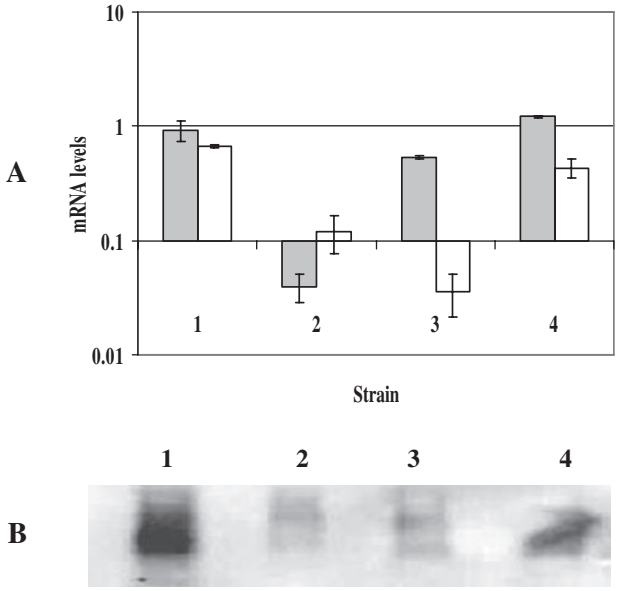

Fig. 5. Expression of *sigK* and *Rv0444c* in Δ*sigK* mutant, *sigK* complement and dyad complement in comparison with the wild-type H37Rv.
A. Relative amounts of *sigK* and *Rv0444c* mRNA. mRNA levels were measured by SYBR green RT-PCR. Values represent the average of two independent RNA samples and are normalized to *sigA* mRNA. Grey bars, *sigK*; white bars, *Rv0444c*.
B. Western blot analysis of the production of *Rv0444c*-encoded protein in the mutant and complements as compared with H37Rv, using polyclonal rabbit serum raised against recombinant *Rv0444c*-encoded protein. 1, H37Rv; 2, Δ*sigK*; 3, Δ*sigK*::*sigK* (*sigK* complement); 4, Δ*sigK*::dyad (dyad complement).

SigK and the N-terminal part of *Rv0444c* gene product using the yeast two-hybrid system. As shown in Fig. 7A, the yeast β-galactosidase reporter was activated upon coexpression of SigK and the N-terminal region of *Rv0444c*, indicating that the proteins can physically interact. Additionally, this transformant grew on media lacking uracil (Fig. 7B), indicating activation of the URA3 reporter gene. Consistent with induction of URA3, the same transformant was unable to grow in the presence of 5-fluoroorotic acid (5FOA), a compound converted to the toxic 5-fluorouracil by uracil (Fig. 7C). In sum, our data indicate that *sigK* and *Rv0444c* are co-transcribed, that the N-terminal (cytoplasmic) part of the *Rv0444c* gene product physically interacts with SigK, and that this interaction results in decreased expression of the SigK-regulated genes (*mpt70*/*mpb83*). We therefore refer to the protein encoded by *Rv0444c* as RskA (regulator of SigK).

Defining the transcriptional impact of RskA by microarray

To better define the regulon of the SigK-RskA system, we performed two different whole genome microarray analyses. In the first set of microarray experiments, we compared the transcriptional profile of Russia::pMH416 (complement with empty vector) with Russia::*Rv0444c* (complement with wild-type *Rv0444c* under the mop promoter). Genes most significantly downregulated upon overexpression of *Rv0444c* were *Rv2873* (*mpb83*), *Rv2875* (*mpb70*) and *Rv2874* (*dipZ*) (data provided in Table S2). In a second set of microarray experiments, we analysed the transcriptional profile of the Δ*sigK* mutant against Δ*sigK*::*sigK* (single gene complement). In these microarrays, the addition of *sigK* significantly upregulated expression of *Rv0446c* and *Rv0449c* (both part of the *sigK* locus), and *Rv2873* (*mpb83*), *Rv2875* (*mpb70*) and *Rv2876* (Fig. 8). Of note, the only discordance between these two sets of transcriptome analyses was the apparent downregulation of *lipP* in the *sigK* complement arrays. As *lipP* is next to the *attB* site (where pMV306 integrates), and complementation of BCG Russia employed an extrachromosomal plasmid, we postulate that this result is an artefact of the complementation process, and not that *lipP* is a SigK-dependent gene. Taken together, these results revealed that the SigK-RskA regulon is small and restricted to two regions: the *sigK* and the *mpt70*/*mpt83* regions. These results are in agreement with our previous study in which the addition of *sigK* in a BCG background affected only the expression of the *sigK* and *mpb70*/*mpb83* regions (Charlet *et al*., 2005).

Discussion

Although *M. tuberculosis* and *M. bovis* share remarkable genomic similarity, phenotypic differences between these two organisms have long been remarked. One of these differences is in the production of the two antigenic proteins MPB70 and MPB83, which are highly expressed in *M. bovis* and minimally expressed *in vitro* by *M. tuberculosis* (Hewinson *et al*., 1996; Wiker *et al*., 1996). In this study, we provide evidence that the genetic basis behind this difference is due to variable sequences of a regulatory gene, *Rv0444c*. We extended this phenotype to other members of the MTC whose levels of MPT70 and MPT83 expression were previously unknown and showed a tight genotype–phenotype correlation between *Rv0444c* sequence and MPT70/MPT83 expression levels. As in most cases, activity of mycobacterial sigma factors is induced in the presence of a specific signal (Manganelli *et al*., 2004) and phylognetically, *M. tuberculosis* presents as ancestral to modern *M. bovis* (Brosch *et al*., 2002; Mostowy *et al*., 2002; 2005), it can be inferred that the inducible phenotype seen in *M. tuberculosis* is the wild-type scenario whereas the constitutive phenotype in *M. bovis* is the result of mutations in *Rv0444c*. It is interesting to note that two different sets of mutations in two different lineages (C320T and C551T in *M. bovis* and G698C in the Oryx bacillus) led to the same

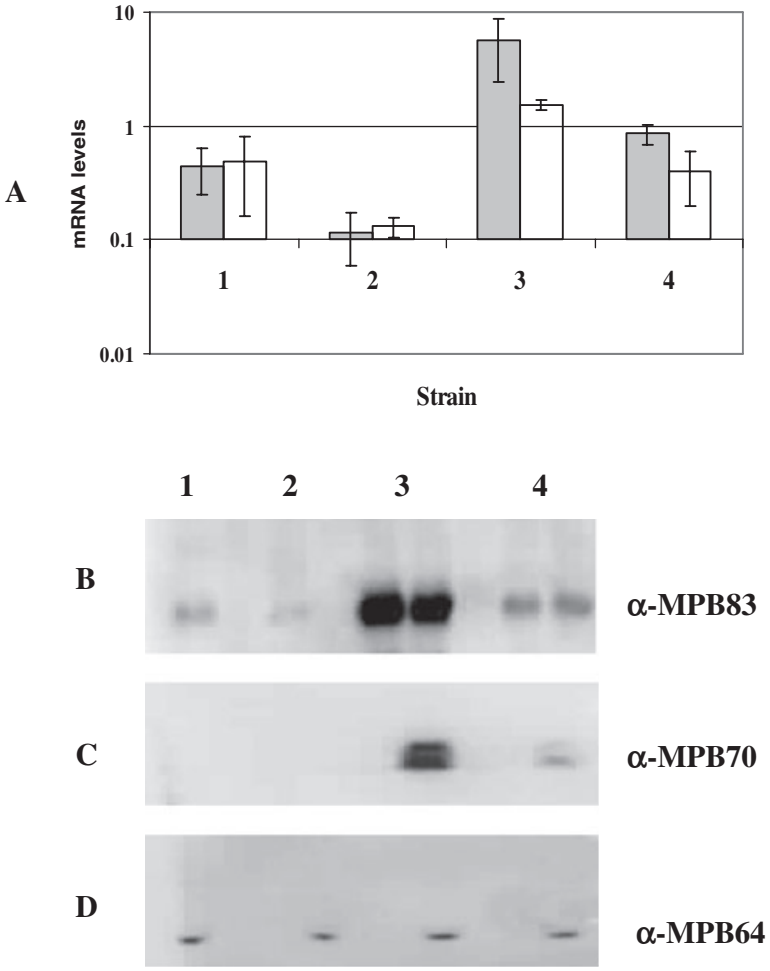

Fig. 6. Expression of *mpt70* and *mpt83* in Δ*sigK* and Δ*sigK* complements as compared with the wild-type strain H37Rv.
A. mRNA levels as assayed by RT-PCR coupled with molecular beacon. Grey bars, *mpt70*; white bars, *mpt83*.
B–D. Western blot analysis of MPT83 (B), MPT70 (C) and MPT64 (D) in Δ*sigK* and Δ*sigK* complements. Expression of MPT83 was tested in two clones of Δ*sigK::sigK*, and Δ*sigK::*dyad and similar results were obtained for each clone (B). For MPT70 and MPT64 (loading control) expression (C and D respectively), only one clone of Δ*sigK::sigK* and Δ*sigK::*dyad was tested. 1, H37Rv; 2, Δ*sigK*; 3, Δ*sigK*::*sigK*; 4, Δ*sigK*::dyad.

functional consequence, i.e. overproduction of MPT70/MPT83 *in vitro.* One wonders if these different bacterial lineages have gained a biological advantage by becoming high producers of these two antigenic proteins.

While large genomic deletions initially presented as the most evident form of genetic variability between *M. tuberculosis* and *M. bovis* (Behr *et al*., 1999; Gordon *et al*., 1999), a number of well-characterized phenotypic differences between these organisms have instead been attributed to SNPs. For instance, unlike *M. tuberculosis*, *M. bovis* requires the addition of pyruvate in the culture media for optimal growth, due to a SNP in the pyruvate kinase gene, *pykA*, that has resulted in an inactive pyruvate kinase in *M. bovis* (Keating *et al*., 2005). In another study, the natural resistance of *M. bovis* to pyrazinamide was shown to be due to a single non-synonymous SNP (C169G) in *pncA*, resulting in a non-functional pyrazinamidase (Scorpio and Zhang, 1996). By altering the expression profile of two important antigens, this study adds *Rv0444c* SNPs to the list of *M. bovis* SNPs that have led to phenotypic differences between this organism and *M. tuberculosis.*

When compared with the *M. tuberculosis* lineage, the genome sequence of *M. bovis* revealed that no genes are unique to *M. bovis*, leading to the prescient suggestion that differential gene expression due to SNPs in regulatory genes could explain differences in the biology and host preference displayed by these two organisms (Garnier *et al*., 2003). Additionally, Sreevatsan *et al*. documented a remarkably restricted polymorphism in structural genes among MTC members and suggested that the rare SNPs observed are generally in regulatory genes and may lead to functional consequences (Sreevatsan *et al*., 1997). Our laboratory has previously shown that a SNP in the start codon of *sigK*, a gene encoding one of *M. tuberculosis* ECF sigma factors, SigK, causes a decrease in the levels of two antigenic proteins MPB70 and MPB83 in BCG strains obtained after 1927 (Charlet *et al*., 2005). The current study extends this theme by presenting another regulatory gene, *Rv0444c*, in which SNPs are shown to be functionally significant.

On the basis of these findings and accepted models of Sigma factor regulation, we propose the following model for the regulation of *mpt70* and *mpt83*. In *M. tuberculosis*

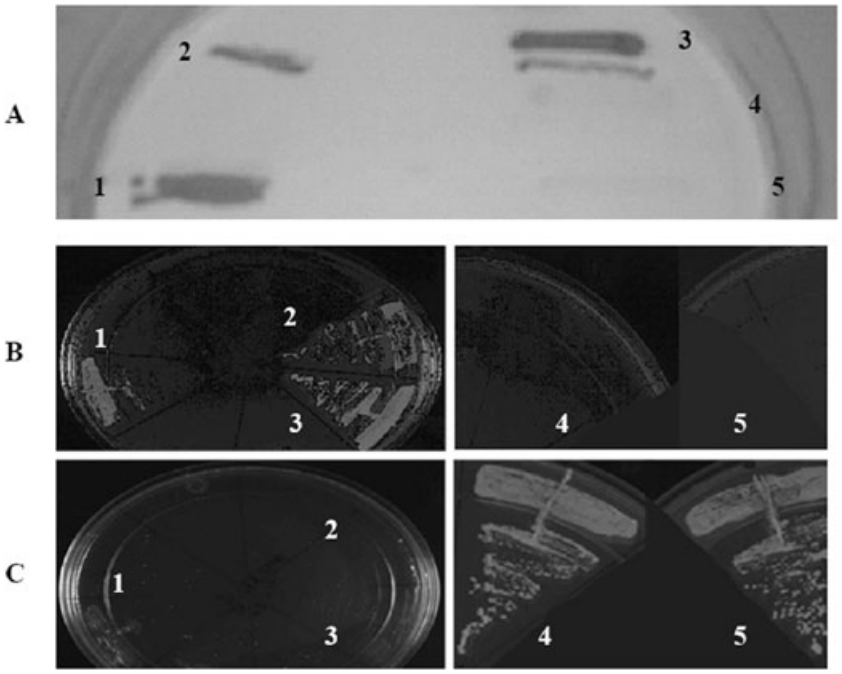

Fig. 7. Interaction of SigK and the N-terminal domain of *Rv0444c*-encoded protein by the yeast two-hybrid system. Interaction is confirmed by increased level of β-galactosidase activity (4A) and growth in the absence of uracil (4B) but not 5FOA (4C). 1, test (transformant with pDEST32 harbouring SigK and pDEST22 harbouring N-terminus of *Rv0444c* gene product); 2, positive control 1 (control D, ProQuest series 10835); 3, positive control 2 (PQ10001-01); 4, negative control 1 (transformant with bait plasmid harbouring SigK coexpressed with empty prey vector); 5, negative control 2 (transformant with empty bait vector coexpressed with pDEST22 harbouring N-terminus of *Rv0444c* gene product).

H37Rv, the referent wild-type strain, RskA, through its N-terminal domain, binds to SigK and inhibits its activity *in vitro*. Upon sensing an *in vivo* stimulus through its extracellular C-terminal domain, RskA releases SigK, freeing it to bind to the promoter of its target genes (*mpt70* and *mpt83*) and initiate their transcription. In *M. bovis*, RskA is dysfunctional due to at least one of the two SNPs in its encoding gene, *Rv0444c*, with further site-specific study required to resolve the functional consequence of each mutation. Consequently, in *M. bovis*, SigK is always 'on' and directing transcription of *mpt70* and *mpt83* in the absence of a stimulus. Anti-sigma factors such as *Streptomyces coelicolor* RsrA, and *M. tuberculosis* RshA have been demonstrated to sense and respond to oxidative stresses through regulation of their cognate sigma factors (Kang *et al*., 1999; Raman *et al*., 2001). For others, like RslA, the signal and the response are not known but the role of SigL and its regulon *in vivo* is clearly demonstrated by the significant attenuation of a *sigL* mutant in mice (Hahn *et al*., 2005). Similar to RslA, the signal to which RskA senses and responds to is yet to be determined, but it is expected that defining the biological role of the SigK-RskA target genes will provide insight into the *in vivo* stimuli that activate this system.

Previous data presented by Charlet *et al*. (2005), and further corroborated in the current study, indicate that

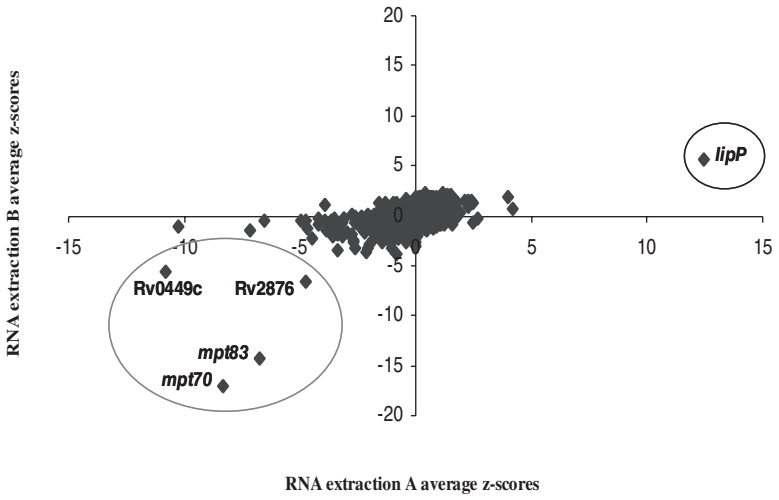

Fig. 8. Transcriptional profile of Δ*sigK* (mutant), and Δ*sigK::sigK* (*sigK* complement) by microarray analysis. Genes for which expression was decreased in the Δ*sigK* mutant as compared with the *sigK* complement are indicated by a negative Z score and genes with increased expression in the mutant are reported by a positive Z score. Genes whose expression is significantly upregulated by the addition of *sigK* complement are parts of the *sigK* and *mpt70/83* loci. The only probe presenting downregulation in the *sigK* complement was for the *lipP* gene (see text).

SigK-RskA target genes are restricted to just the *sigK* and *mpt70/mpt83* regions. Although it has been reported that ECF sigma factors control a small regulon (Bashyam and Hasnain, 2004), the restriction of the SigK regulon to the *mpt70/mpt83* region (besides the *sigK* locus) is atypical and noteworthy. The regulons of other reported *M. tuberculosis* ECFs are quite extensive, for instance, microarray analysis indicated that *M. tuberculosis* SigH positively regulates the expression of 31 genes (Kaushal *et al*., 2002), SigE controls the expression of about 100 genes, including *sigB* (Manganelli *et al*., 2001), and the regulon for SigD includes 47 upregulated genes (Raman *et al*., 2001). The biological function of MPT70 and MPT83 is still unknown but several lines of evidence strongly suggest their possible role *in vivo.* First, although *in vitro* MPT70 and MPT83 protein levels are low in *M. tuberculosis*, serum from mice infected with live *M. tuberculosis* react strongly to these antigens (Hewinson *et al*., 1996). Second, Schnappinger *et al*. described the transcriptional profile of *M. tuberculosis* inside stimulated macrophages, and found that *mpt70* and *mpt83* are both significantly induced in macrophages, with *mpt83* also confirmed in infected mice (Schnappinger *et al*., 2003). Finally, the shut-down in expression of MPB70 and MPB83 by BCG Pasteur argues that there was a cost to producing these proteins in high levels during prolonged *in vitro* passage in the absence of host pressures. The observation that this regulon has been upregulated in two independent lineages of the MTC further argues towards a yet-to-be determined role of these genes and their protein products *in vivo.*

By complementing the Δ*sigK* mutant with *sigK* alone, we have constructed a *M. tuberculosis* strain with the *M. bovis* phenotype for MPB70/MPB83 production. It has been previously reported that *M. bovis* is more virulent than *M. tuberculosis* in mice, as demonstrated by increased pathology and disseminated infection induced in the case of *M. bovis* but not *M. tuberculosis* (Medina *et al*., 2006). Additionally, the rabbit model of tuberculosis has historically been used to differentiate between *M. bovis* and *M. tuberculosis*, as first described by Theobald Smith, who wrote: 'The gross results show a sharp demarcation between the bovine and the human cultures. While all the rabbits inoculated with the former [bovine] succumbed in from 17 to 21 days, of the rabbits inoculated with the latter [human] only one succumbed in 35 days' (Smith, 1898). With this in mind, it shall be interesting to test the mutants generated in these experiments in animal models, to challenge a phenotype first described more than a century ago.

Experimental procedures

Bacterial strains and culture conditions

Mycobacterium africanum 60914 (previously characterized in Mostowy *et al*., 2004b), the Oryx bacillus 51145, *M. caprae* 60312 (previously characterized in Mostowy *et al*., 2005) and *M. bovis* 62389 are generous gifts from Louise Thibert. *M. microti* and *M. tuberculosis* H37Rv are gifts from Dr David Sherman. Mycobacteria and BCG strains were grown at 37°C in Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI) containing 0.05% Tween 80 (Sigma-Aldrich, St Louis, MO) and 10% albumin-dextrose-catalase (Becton Dickinson and Co., Sparks, MD) supplement on a rotating platform (Wheaton). For solid media, Middlebrook 7H10 (without Tween) supplemented with OADC was used. *E. coli* DH5-α used for cloning purposes was cultured at 37°C in Luria broth (Difco). Antibiotics were added as needed at the following concentrations: kanamycin: 50 μg ml^{-1} for *E. coli* and 25 μg ml^{-1} for mycobacteria; hygromycin: 100 μg ml^{-1} for *E. coli* and 50 μg ml^{-1} for mycobacteria; apramycin: 30 μg ml^{-1} for both *E. coli* and mycobacteria.

DNA extraction and sequence analysis

DNA from MTC members was extracted using a protocol based upon lysozyme and proteinase K (Van Soolingen *et al*., 1991), where samples were used as template for targeted sequence analysis. Primers were designed to amplify *Rv0444c* and flanking DNA (Rv0444cL: 5′-GGCGC TCATGACTGAACATA-3′ and Rv0444cR: 5′-CTAGTGGTA CCCGGCGTGTT-3′) where amplicons were subject to direct dideoxy terminal sequencing at the McGill University and Genome Quebec Innovation Center (http://genomequebec.mcgill.ca/). Sequenced products were analysed by alignment searches against published genome sequences, namely *M. tuberculosis* H37Rv using Tuberculist (http://genolist.pasteur.fr/TubercuList/), *M. tuberculosis* 210 and CDC1551 using the sequences provided at NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), *M. bovis* AF2122/97 using Bovilist (http://genolist.pasteur.fr/BoviList/), and the assembly sequence of BCG Pasteur (http://www.sanger.ac.uk/cgi-bin/blast/submitblast/m_bovis).

Preparation and infection of THP-1 cells

To prepare the human macrophage-derived THP-1 cell line for infection as previously described (Lewis *et al*., 2003), cells were pelleted by centrifugation, resuspended in RPMI 1640 medium plus 10% fetal calf serum (FCS) with 100 nM phorbol 12-myristate 13-acetate (PMA; Sigma), and delivered ($1–10 \times 10^7$ cells in 40 ml) into 175 cm^2 tissue flasks (Falcon). Stimulated THP-1 cells were additionally provided with human IFN-γ from BioDesign International at this time (100 U ml^{-1}). Cells were incubated at 37°C with 5% CO_2 for 24 h. PMA (with or without IFN-γ)-containing medium then was removed from the flasks, cells were washed with warm RPMI 1640 medium, given fresh RPMI 1640 medium plus FCS, and re-incubated for 24 h during the infection process. For each infection, bacteria stocks (*M. tuberculosis* H37Rv and H37Ra) were grown to mid-log phase in 7H9 media, diluted in warm RPMI 1640 medium plus 10% FCS (THP-1 complete media), and added to each flask containing $1–10 \times 10^7$ THP-1 cells at a multiplicity of infection of 5–10. Cells were incubated at 37°C in 5% CO_2. After 24 h, extracellular bacteria were removed, and RNA was isolated from internalized bacteria (see below).

Ex vivo *RNA isolation*

Based on published RNA extraction protocols (Mangan *et al*., 2002; Schnappinger *et al*., 2003), RNA was isolated from bacteria within infected macrophages, yielding approximately 1 μg from 10^7 host cells. Specifically, 24 h after infection, macrophages were lysed in low detergent concentrations in presence of guanidinium isothiocyanate, followed by phagosome isolation and bacterial harvest by ultracentrifugation. Bacterial RNA was subsequently extracted and analysed by qRT-PCR as described below.

Complementation assays

To complement BCG Russia, a 1068 bp DNA fragment - containing the *Rv0444c* gene plus 292 bp upstream was PCR amplified from *M. tuberculosis* H37Rv or from BCG Russia using the following primers: Rv0444F-XbaI: 5′-ATA AAATCTAGAGGTGCGGCCAACGTCGATC-3′; Rv0444R-HindIII: 5′-ATAAAAGCTTCCGGCGTGTTCGTCGCGATGC-3′. The PCR amplicon was first cloned into pCR 4-TOPO cloning vector (Invitrogen, Carlsbad, CA). The fragment was then removed from the TOPO vector with the restriction enzymes EcoRI (found in the TOPO vector) and HindIII and cloned into pMH416 digested with the same enzymes. pMH416 (a kind gift of D.R. Sherman and M.J. Hickey) is a derivative of pMH29 (George *et al*., 1995) that has the apramycin-resistance gene (Cangelosi *et al*., 2006). The resulting plasmid pMH416::*Rv0444c* as well as the pMH416 empty vector were electroporated into BCG Russia. Complementation was PCR-confirmed and amplicons were sequence-confirmed for all transformants.

Construction of sigK *mutant and complemented strains*

Mycobacterium tuberculosis H37Rv *sigK* mutant was created by allelic exchange in a two-step selection process, replacing *sigK* with a kanamycin cassette. For this purpose, we used the plasmid pKO, which contains the *sacB* (conferring sucrose sensitivity) and a hygromycin cassette in its backbone as described in Lewis *et al*. (2003). pKO multiple cloning sites are also flanked by a kanamycin-resistance gene. DNA fragments of 1464 bp and 1666 bp spanning the regions proximal and distal of *sigK*, respectively, were PCR amplified from H37Rv genomic DNA using the following primers: sigKproxF: 5′-ATAAGCATGCAGCGATGCGTTGG GAGAG-3′; sigKproxR: 5′-ATAAAAGCTTGGATGCAGCT GAGGGTCTG-3′ and sigKdistF: 5′-ATAAGGTACCTA GCCGTGCACTATGACCTG-3′; sigKdistR: 5′-ATAAGGTA CCCGATGGGTAGCCTATCGCCA-3′. The PCR amplicons were independently cloned into pCR 4-TOPO cloning vector (Invitrogen, Carlsbad, CA). The distal fragment was digested from TOPO with KpnI and ligated to the pKO vector digested with the same enzyme, making the plasmid pKO-distal. PCR and restrictions digests were used to confirm the presence and the correct orientation of the insert. The proximal region was digested from TOPO with SpHI and HindIII and cloned into pKO-distal plasmid digested with the same enzymes. The final construct pKOSigK contains the regions proximal and distal to *sigK* on each side of the kanamycin-resistance gene. The construct was confirmed by amplifying and sequencing the two regions (proximal and distal). We then transformed the pKOSigK plasmid into *M. tuberculosis* H37Rv using the method previously described (Belley *et al*., 2004). Hygromycin-resistant colonies obtained from the first selection were selected and analysed for site-specific integration using the Expand Long Template PCR System (Roche). The clones in which the plasmids integrated at the right place were plated on 7H10 plus kanamycin and 2% sucrose. Kanamycin- and sucrose-resistant colonies would have lost the plasmid backbone containing *sacB* (Pelicic *et al*., 1996). These colonies were tested on hygromycin and kanamycin independently. Those colonies that grew on kanamycin but not on hygromycin were selected for further analysis. *sigK* deletion was first confirmed by PCR with primers flanking the *sigK* gene and sequencing, and also confirmed by Southern blot analysis.

To complement the Δ*sigK* mutant with *sigK* only, we used the plasmid pH37Rv, which is a derivative of pMV306 containing the *sigK* region (including the complete gene and 288 bp upstream) as previously described (Charlet *et al*., 2005). To complement the Δ*sigK* with the dyad (*sigK* and *Rv0444c*), we cloned a 1703 bp PCR fragment containing *sigK*, *Rv0444c* plus 288 bp upstream of *sigK* into pMV306, using the following primers: SigKF-NotI: 5′-ATAAGCGG CCGCACGCGTCACCCCAACTACT-3′; Rv0444R-EcoRV: 5′-ATAAGATATCCCGGCGTGTTCGTCGCGATGC-3′. NotI and EcoRV sites were added to facilitate the cloning of this fragment into pMV306. Because pMV306 originally has a kanamycin-resistance gene and the Δ*sigK* mutant generated was kanamycin-resistant, we genetically replaced the kanamycin cassette with a hygromycin-resistance marker. The presence of the correct insert was confirmed by restriction digest and PCR followed by sequencing. The resulting plasmids pMV306-hyg::*sigK* and pMV306-hyg::dyad were electroporated into the Δ*sigK* mutant. Complementation was PCR-confirmed by amplifying the inserts with primers specific for the regions of pMV306 (Charlet *et al*., 2005) flanking the insert and amplicons were sequence-confirmed for all transformants.

RNA extraction and RT-PCR

Natural and genetically engineered strains were grown *in vitro* to an OD_{600} of 0.2–0.6, where they were pelleted by centrifugation, resuspended in 1 ml of wash buffer (0.5% Tween 80, 0.8% sodium chloride) and transferred to 2.0 ml screw-cap tubes. RNA was isolated by a modified phenol-chloroform extraction protocol as previously described (Charlet *et al*., 2005). Genomic DNA contamination was removed by RNAeasy on-column digestion, following the manufacturer's protocol (Qiagen, Mississauga, ON). Once the quality of RNA was confirmed by denaturing gel electrophoresis (formaldehyde), it was converted to cDNA using Fermentas cDNA kit.

To determine mRNA levels for *mpb70*, *mpb83* and *sigA*, we performed qRT-PCR with molecular beacons, applying *sigA* levels as a normalization control (Manganelli *et al*., 1999). The qRT-PCR method as well as primers and beacons for *sigA*, *mpb70* and *mpb83* used in this study are previously described in Charlet *et al*. (2005). To determine levels of *sigK*

and *Rv0444c* as function of our deletion and complementation mutants, we modified this protocol to test expression by qRT-PCR with SYBR green. To test for co-transcription of *sigK* and *Rv0444c*, primers within each gene (SigK-CTXL: 5′-GGGCTGACGTATGTCGAAGT-3′ and *Rv0444*-CTXR: 5′-CTCGTTCATCGTCGGACAC-3′) were employed in PCR analysis of mRNA with and without reverse transcriptase, to demonstrate message bridging the genes.

Protein preparation and immunoblot analysis

For secreted proteins, culture supernatants were filtered with a 0.22 μm membrane filter and concentrated with an Millipore Ultra-10 Centrifugal Filter Unit, 10 000 MW cut-off. For membrane-associated proteins, culture pellet was resuspended in 1 ml PBS and samples were subjected to the Fast Prep (BIO 101 Savant) for 15 s at 6.0 rpm. Samples were incubated on ice for 10 min. Cell debris were removed by spinning down the samples and the supernatant was boiled for 20 min. SDS-PAGE was performed under reducing conditions using the Mini-PROTEAN 3 electrophoresis system (Bio-Rad) with 12% polyacrylamide gels. Proteins were transferred to a polyvinylidene difluoride membrane. Rabbit polyclonal antibodies against MPB70 and MPB83 (gifts of Harald Wiker) were blotted onto the membrane and the ECL Western kit (Millipore, Billerica, MA) was used for protein detection. As internal loading control, the same samples were transferred to a membrane and blotted with a mouse polyclonal antibody against MPT64 (gift of Mark Doherty), a secreted antigen whose expression is not thought to be affected by SigK. To detect levels of *Rv0444c*-encoded protein, we used rabbit serum raised against recombinant *Rv0444c* (Chemicon International, Temecula, CA) obtained as follows. *Rv0444c* was amplified from H37Rv and cloned into the pET21 vector. After sequence confirmation, the plasmid was transformed into *E. coli* BL21 (DE3) pLysS (Novagen) and the protein expressed through overnight induction with 0.5 mM IPTG. Cells were harvested by centrifugation (5000 *g*, 10 min and 4°C) and disrupted by sonication at 4°C. Cell debris was removed by centrifugation, and the supernatant was loaded onto a 1 ml HiTrap chelating column (Amersham Biosciences). The column was extensively washed with loading buffer and eluted using a 0–600 mM imidazole gradient in loading buffer to reveal a single band of the expected length on SDS-PAGE gel. This product was then sent for preparation of serum.

Yeast two-hybrid analysis

Protein interaction was assayed using the ProQuest™ two-hybrid system (Invitrogen, Carlsbad, CA). By recombination cloning, we engineered a bait plasmid designed to overexpress recombinant SigK linked at its N-terminus to the Gal4 DNA-binding domain (pDEST32). Similarly, the prey plasmid expressed the N-terminal part of *Rv0444c* (amino acid residues 1–92) fused to GAL4 activation domain (pDEST22). pDEST32 and pDEST22 contain TRP1 and LEU2, respectively, permitting selection on media lacking tryptophan or leucine (synthetic complete media leu–trp–). Bait and prey plasmids were transformed into the mating-type yeast strain MAV203 using the method essentially described by Gietz *et al*. (1992). Briefly, yeast strains were grown in YPAD medium to an OD_{600} of ~1. Competent cells were prepared with 1× TE/LiAc solution and transformation was carried out by mixing yeast cell suspension with 50 μg of single stranded salmon sperm DNA, plasmid DNA (and 0.3–1 μg) and 40% PEG. Cells were incubated at 30°C with agitation (roller drum) for 30 min, followed by a heat shock at 42°C for 15 min. The MAV203 yeast strain contains integrated reporter genes such as *ura3* and *lac*Z and positive interactions are detected when the GAL4 binding and activation domains are bridged via a direct protein–protein interaction, and activation of the reporter genes. Transformants were plated on SC-L-T-Ura and 5FOA plates to test for growth. For the β-galactosidase assay, transformants were grown overnight on nitrocellulose filter paper placed on YPAD agar plate as described in the invitrogen ProQuest Two-hybrid system manual. The filter was soaked in Z-buffer containing β-ME and Xgal after rapid exposure to liquid nitrogen and incubated overnight on Whatman paper.

Microarray analysis

Microarray hybridization and analysis were performed as previously described (Charlet *et al*., 2005). In brief, mRNA from BCG strains and complemented strains was extracted during log-phase *in vitro* growth and labelled with Cy3 or Cy5 dUTP by reverse transcriptase (Amersham Biosciences). Labelled cDNA was hybridized to microarrays composed of oligonucleotide probes from the TB Array-Ready Oligo Set™ (Operon) printed onto Sigmascreen™ microarray slides (Sigma). Initial comparisons were *M. tuberculosis* H37Rv versus select MTC members (Table S1). After complementing BCG Russia with wild-type *Rv0444c*, comparisons of Russia::*pMV306* versus Russia::*Rv0444c* were also performed (Table S2). Hybridized arrays were scanned with ScanArray 5000XL and hybridization results were quantified with ScanArray software (PerkinElmer, Freemont, CA). Array analysis was performed as previously described (Mostowy *et al*., 2004c; Charlet *et al*., 2005) and can be summarized as such. Subtracting total spot intensity minus the surrounding background produced a corrected spot intensity. Negative corrected spot intensities were set to +1. All spots flagged as misrepresentative (array artefacts) or unrecognizable (quiescent in both channels) by ScanArray were analytically ignored. Intensity ratios (Cy3/Cy5 or Cy5/Cy3) were determined using corrected spot intensities and log10 transformed. Values for each gene were obtained in duplicate for each array (inherent to array design) and averaged. For each array, a representative *Z*-score, indicative of how many standard deviations a data point lies from the population mean, was calculated for each gene. *Z*-scores for each gene were determined across replicates within each experiment to minimize the probability of observing such variation by chance alone. Genes with Z-scores of two or greater across arrays are presented.

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Supplementary material

The following supplementary material is available for this article online:

Table S1. *In vitro* RNA expression profiles for members of the *Mycobacterium tuberculosis* complex.

Table S2. Global transcriptional profiling *in vitro* to determine genes whose expression was changed upon complementation of *Mycobacterium bovis* BCG Russia with wild-type *Rv0444c*.

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