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ORIGINAL ARTICLE

Polymorphisms of 20 regulatory proteins between *Mycobacterium tuberculosis* and *Mycobacterium bovis*

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ABSTRACT

***Mycobacterium tuberculosis* and *Mycobacterium bovis* are responsible for tuberculosis in humans and animals, respectively. Both species are closely related and belong to the *Mycobacterium tuberculosis* complex (MTC). *M. tuberculosis* is the most ancient species from which *M. bovis* and other members of the MTC evolved. The genome of *M. bovis* is over >99.95% identical to that of *M. tuberculosis* but with seven deletions ranging in size from 1 to 12.7 kb. In addition, 1200 single nucleotide mutations in coding regions distinguish *M. bovis* from *M. tuberculosis*. In the present study, we assessed 75 *M. tuberculosis* genomes and 23 *M. bovis* genomes to identify non-synonymous mutations in 202 coding sequences of regulatory genes between both species. We identified species-specific variants in 20 regulatory proteins and confirmed differential expression of hypoxia-related genes between *M. bovis* and *M. tuberculosis*.**

Key words *Mycobacterium bovis*, *Mycobacterium tuberculosis*, polymorphism, regulator.

The *Mycobacterium* genus includes pathogens responsible for serious diseases such as tuberculosis and leprosy in mammals. Within this genus, the MTC refers to a group of genetically related pathogenic species that can cause tuberculosis in several mammals, including humans. Indeed, *Mycobacterium bovis* is a MTC member of significant importance in livestock.

The complete genome sequence of *M. bovis* was published (1) 5 years after the publication of the genome sequence of *M. tuberculosis* (2). The genome of *M. bovis* is >99.95% identical to that of *M. tuberculosis* but has seven deletions. The region containing the deletions is called the region of difference (RD) and ranges from 1 to 12.7 kb. This finding, therefore, could suggest that, overall, the main evolutionary force shaping the genome of the *M. bovis* gene has been the deletion. Interestingly, many of the missing or altered genes in *M. bovis* are missing in *Mycobacterium leprae*, an obligate intracellular pathogen that suffered a considerable genome reduction (1).

Major advances have been achieved in the understanding of the evolutionary mechanisms that led to the emergence of the species of MTC and, particularly, *M. bovis*. Nevertheless, it is still difficult to explain how this bacterium, a microorganism whose genome has largely lost genomic regions and with no unique genes, inhabits a broader biological niche than its ancestor, *M. tuberculosis*. Probably, the presence of more than 1200 SNP in coding regions or genes that distinguish *M. bovis* from *M. tuberculosis* may, in part, explain this characteristic. Garnier *et al.* have found that the most variable genes between both species are those related to cell wall and secreted proteins. In particular, they described polymorphisms in several genes involved in the synthesis or transport of lipid complexes (1) as well as in genes encoding the families

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List of Abbreviations: MTC, *Mycobacterium tuberculosis* complex; SNP, single nucleotide polymorphism.

of PE-PGRS and PPE proteins. PE-PGRS and PPE proteins are expressed on the surface of mycobacteria and may provide different antigenic variations causing immune responses in *M. tuberculosis*, their corresponding genes represent >10% of the genome (3). In addition, *esxR* and *esxS* are genes encoding members of the Esx protein family and are absent from several *M. tuberculosis* strains (4). The Esx protein family consists of >20 proteins including antigens CFP7 and CFP10 (5) and many of them strongly stimulate T-cell responses.

A polymorphism in *pncA* between *M. bovis* and *M. tuberculosis* was identified previous to the sequencing of mycobacterial genomes. *pncA* encodes the enzyme pyrazinamidase (PncA) (6) that activates the first-line antituberculous drug pyrazinamide into pyrazinoic acid. In *M. bovis*, a point mutation produced the replacement of a histidine for an aspartate in a specific position with the consequent loss of enzyme activity. Although *M. tuberculosis* H37Rv carries an intact copy of *pncA*, some clinical isolates of *M. tuberculosis* show a variety of mutations in this gene (7).

Because of their amplifying effect, the finding of mutations in genes encoding transcriptional regulators between *M. tuberculosis* and *M. bovis* is remarkable. For example, Peirs *et al.* (8) reported a gene coding for *PknD* (Rv0931c) that is truncated in *M. bovis* and Gonzalo-Asensio *et al.* (9) identified a mutation in the *phoR* gene of *M. bovis* isolates, which could explain the low transmission of this species to humans. PhoR, together with PhoP, forms a two-component system that activates the expression of numerous proteins relevant for *M. tuberculosis* interaction with the host. The higher expression level of the humoral antigens MPB70 and MPB83 in *M. bovis* with respect to *M. tuberculosis* is also relevant. This difference is a result of a mutation in *Rv0444c,* which encodes an anti-sigma factor K (10). The sigma factor K (SigK) positively regulates the transcription of *mpb70* and *mpb83* and a mutation in RskA, its repressor, therefore produces upregulation of *mpb70* and *mpb83* in *M. bovis*. Besides these few previously described polymorphisms in regulatory genes, no compiled genomic data are available on potential variants in regulatory proteins between *M. bovis* and *M. tuberculosis*. To fill this gap, in the present study, we search for conserved non-synonymous mutations in coding sequences for transcriptional regulators and two component systems in *M. bovis* compared to *M. tuberculosis*.

From 202 potential transcriptional regulators and two-component systems, we identified 20 genes with either non-synonymous SNP or deletion/insertions (INDEL) between *M. bovis* and *M. tuberculosis*.

MATERIALS AND METHODS

Genomic analysis

A total of 202 MTC regulatory genes were selected and downloaded from the TubercuList database server (http://genolist.pasteur.fr/TubercuList) and BoviList database server (genolist.pasteur.fr/BoviList/). These genes were selected because they encode for transcriptional regulators, two-component systems or regulatory proteins. The sequence of each selected gene of *M. tuberculosis* H37Rv (Supporting Information, List S1) was manually compared to that of *M. bovis* AF2122/97 by using the commercial software DNA Strider 1.4f13. Genes with non-synonymous polymorphisms between *M. tuberculosis* H37Rv and *M. bovis* AF2122/97 (Supporting Information, List S2) were translated to protein, and the *M. bovis* variants of each protein were used as query sequences in a BlastX analysis (https://blast.ncbi.nlm.nih.gov). The query sequences (Supporting Information, List S2) were aligned to 23 *M. bovis* sequenced proteomes and a list of proteins that conserved the non-synonymous polymorphisms (SNP, INDEL) in all *M. bovis* protein sequences was generated (Supporting Information, List S3). The *M. tuberculosis* H37Rv orthologs of these conserved proteins (Supporting Information, List S3) were then used as query sequences for alignment to 75 *M. tuberculosis* sequenced proteomes (http://blast.ncbi.nlm.nih.gov/Blast.cgi#). The *M. tuberculosis* orthologs of the selected *M. bovis* proteins (Supporting Information, List S3) that conserved the species-specific polymorphism in all *M. tuberculosis* protein sequences were considered as species-specific regulatory genes (Table 1).

Table 1. Primer sequences of selected genes used in qRT-PCR experiments

Primer	Sequence
hptX	**F**: GACATTATGGTCCGCGATG
	R: GCCTTAATGTCGTCCTCGTC
pks2	**F**: ATCGGTGACCCCATTGAATA
	R: GACTGGGTGTGTCCGAAGTT
Rv3074	**F**: TGGTTTACGAGATGCCACAC
	R: ACATCCAGACATGCGCTTT
iclI	**F**: CCAAGTTCCAGAAGGAGCTG
	R: TTCCTGCAGTTCGACATACG
Rv1456c	**F:** TGTTGGTTGCCTACCTTGC
	R: CGGTGAAGTATTGCGTGGT
narK2	**F**: GTGACCTGGGAGATGTCGTT
	R: AGAACCCGTAGATCGTGGTG

sigA was the calibrator gene.

Bacterial growth conditions

Mycobacterium bovis isolates (*M. bovis* 04-303 and *M. bovis* 534) were grown under shaking conditions in 7H9 medium supplemented with 0.05% Tween 80, 0.5% albumin, 0.4% dextrose and 0.4% pyruvate. *M. tuberculosis* H37Rv and *M. tuberculosis* CDC1551 strains were grown under shaking conditions in 7H9 medium supplemented with 0.05% Tween 80, albumin/dextrose and 0.4% glucose.

RNA extraction

Bacterial cultures (50 mL, in duplicates) of *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551, *M. bovis* 04-303 and *M. bovis* 534 were harvested at the exponential phase of growth (optical density 600_{nm}: 0.3–0.4). Cell pellets were immediately resuspended in 1 mL Trizol (Sigma-Aldrich, St Louis, MO, USA) and transferred to a 2-mL screw-cap microcentrifuge tube containing 0.1-mm-diameter zirconium beads. Cells were disrupted with a Fastprep FP120 bead-beater (MP Biomedicals, Santa Ana, CA) for 20 s at a speed of 6 m/s. The samples were treated twice with 200 μL chloroform, centrifuged at 9.000 *g* for 5 min and the nucleic acids present in the upper phases (aqueous phases) were precipitated with isopropanol. The RNA/DNA pellets were washed up with ethanol 70% and resuspended in RNase-free water. Finally, the samples were cleaned up with RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany) and treated with DNaseI Ambion (Thermo Fisher Scientific, Waltham, MA) following the manufacture's specifications.

qRT-PCR

qRT-PCR reactions were carried out as previously described (11) using specific primers (Table 1) and DNA-free RNA (1 μg) extracted from mid-exponential growth-phase cultures of *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551, *M. bovis* 04-303 and *M. bovis* 534. Briefly, RNA (1 μg) was mixed with 50 ng random primers (Invitrogen, Life Technologies, Carlsbad, CA) in 20 μL final volume and reverse-transcribed to total cDNA with SuperScript II reverse transcriptase (Invitrogen, Life Technologies) following the manufacturer's instructions. Control reactions without reverse transcriptase were included.

The cDNA (0.5 μL) was used as a template for each qRT-PCR reaction. All primers were designed using Primer 3 Software (bioinfo.ut.ee/primer3-0.4.0/) (Table 2). The qPCR reactions were carried out with Taq Platinum DNA polymerase (Invitrogen, Life Technologies) and SYBR reagent (Thermo Fisher Scientific) following the manufacturer's instructions.

All reactions were carried out in duplicate and the qPCR data were analyzed using LinRegPCR software (12). Default settings were used for the LinRegPCR software. All samples without plateau or amplification and with very low Cq values were excluded for mean efficiency calculation. Strictly continuous log-linear setting was used for baseline estimation and the excluded samples were analyzed individually and corrected with the manual correction baseline option. Fold change was calculated using *sigA* as the reference gene. Final results and permutation statistical analysis were assessed with fg statistic software (13), which is part of the Infostat software package. For the statistical test, the parameters were set to defaults with 5000 permutations at random.

RESULTS

In silico analysis of 202 regulatory genes of *Mycobacterium* spp.

We first searched for total mutations in regulatory genes between the reference strains *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv. The criterion to define a regulatory gene was that on the TubercuList database (http://tuberculist.epfl.ch/), by combining genes classified as transcriptional regulator, two-component systems and regulatory proteins. As a result, we obtained 202 genes (Supporting Information, List S1). We then downloaded the 202 gene sequences from the TubercuList and BoviList databases and carried out the pairwise comparisons (*M. bovis* AF122/97 *vs M. tuberculosis* H37Rv) with the DNA Strider program. A total of 80 genes had nucleotide mutations between *M. bovis* AF2122/97 and *M. tuberculosis* H37Rv. From these genes, 25 were synonymous mutations (Supporting Information, List S2) and 55 were non-synonymous (Supporting Information, List S2). Some polymorphisms that emerged during the divergence of *M. bovis* and *M. tuberculosis* should have had an impact in the niche adaptation of these species and therefore they should be conserved in all strains of the same species. With this in mind, we looked for the species-specific variants of regulatory genes among the 55 genes with non-synonymous mutations. For these purposes, we assessed 75 *M. tuberculosis* genomes (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch; Taxid 1773) and 27 *M. bovis* genomes. The *M. bovis* genomes corresponded to nine strains isolated from Brazil, seven from Argentina (http://www.ncbi.nlm.nih.gov/bioproject/214551) and 11 from USA (Thacker T, unpublished results). Only 20 regulatory genes conserved the species-specific polymorphisms (SNP or INDEL) in the 23 *M. bovis* and 75 *M. tuberculosis* genomes (Table 2). Eight polymorphisms mapped in conserved predicted

Table 2. Genes with conserved non-synonymous mutation between *M. tuberculosis* H37Rv and *M. bovis* AF2122/97

Rv no./Mb no.	Gene	Description	INDEL/SNP†	AA change
Rv0018c/ Mb0018c	*pstP*	Phosphoserine/threonine phosphatase PstP	g1363t c1387t	S455A P463S
Rv0078/Mb0080		Probable transcriptional regulatory protein	a337g	I113V
Rv0153c/ Mb0158c	*ptbB*	Phosphotyrosine protein phosphatase PTPB (protein-tyrosine-phosphatase) (PTPase)	a314g	D105G
Rv0465c/ Mb0474c		Probable transcriptional regulatory protein	g977a	R326Q
Rv0602c/ Mb0618c	*tcrA*	Two-component DNA-binding transcriptional regulatory protein TcrA	g82a	V28I
Rv0758/Mb0781	*phoR*	Possible two-component system response sensor kinase membrane-associated PhoR	g211a g212t	G71I
Rv0823c/ Mb0846c		Possible transcriptional regulatory protein	g388t t988c	G130C F330L
Rv0931c/ Mb0955c	*pknD*	Transmembrane serine/threonine-protein kinase D PknD (protein kinase D) (STPK D)	a828ins	*T277fsTer14*
Rv1358/Mb1393		Probable transcriptional regulatory protein	a1325c	D442A
Rv1460/Mb1495		Probable transcriptional regulatory protein	c797t	A266V
Rv1746/Mb1775	*pknF*	Anchored-membrane serine/threonine-protein kinase PknF (protein kinase F) (STPK F)	g973a	A325T
Rv1846c/ Mb1877c	*blaI*	Transcriptional repressor BlaI	g274t	D92Y
Rv2027c/ Mb2052c	*dosT*	Two-component sensor histidine kinase DosT	t46g	L16V
Rv2175c/ Mb2197c		Conserved regulatory protein	c50t	P17L
Rv2176/Mb2198	*pknL*	Probable transmembrane serine/threonine-protein kinase L PknL (protein kinase L) (STPK L)	t154g	S52A
Rv2621c/ Mb2654c		Possible transcriptional regulatory protein	g581_g583del	G195del
Rv2720/Mb2739	*lexA*	Repressor LexA	t350c	V117A
Rv2779c/ Mb2801c		Possible transcriptional regulatory protein (probably Lrp/AsnC-family)	t368_g391del	V123_A130del
Rv3220c/ Mb3246c		Probable two-component sensor kinase	g139a	D47N
Rv3291c/ Mb3319c	*lrpA*	Probable transcriptional regulatory protein LrpA (Lrp/AsnC-family)	c65t	A22V

†Nucleotide positions in *M. tuberculosis* H37Rv.

domains (Fig. 1). Two polymorphisms were located in the protein kinase domains of PknL and Mb3246c (Fig. 1a) and one in the helix-turn-helix DNA-binding domains (HTH) Mb3319c (Fig. 1b). Several amino acid changes and a deletion of eight amino acids mapped in the region encompassing amino acid position 124 to 136 of Mb2801c, compared to its orthologous Rv2779c of *M. tuberculosis*. This highly polymorphic region was localized in a domain conserved in a bacterial transcriptional regulatory protein, AsnC. Finally, TcrA showed a polymorphism in a glycoside hydrolase family RRRD domain.

Experimental study of polymorphism consequences

To gain an insight into the potential impact of the observed polymorphisms on the function of regulatory proteins, we compared the transcription of some selected genes between *M. bovis* and *M. tuberculosis* strains. We chose one or two genes among those transcriptionally regulated by each of the five selected polymorphic regulatory proteins and assessed gene expression by quantitative PCR using cDNA obtained from total RNA of *M. tuberculosis* and *M. bovis*, as template. Expression levels of *Rv3074* and *Rv1456c*, the expressions of which are regulated by LexA and BlaI (14–16), respectively, were similar in both mycobacterial species (Table 3). Remarkably, the expression of *hspX* and *narK2*, which is regulated by DosT/R and the expression of *pks2* regulated by PhoP (17, 18), was higher in *M. tuberculosis* strains than in *M. bovis* isolates (Table 3), whereas the expression of *icl1*, which is regulated by Rv0465, was downregulated in *M. tuberculosis*.

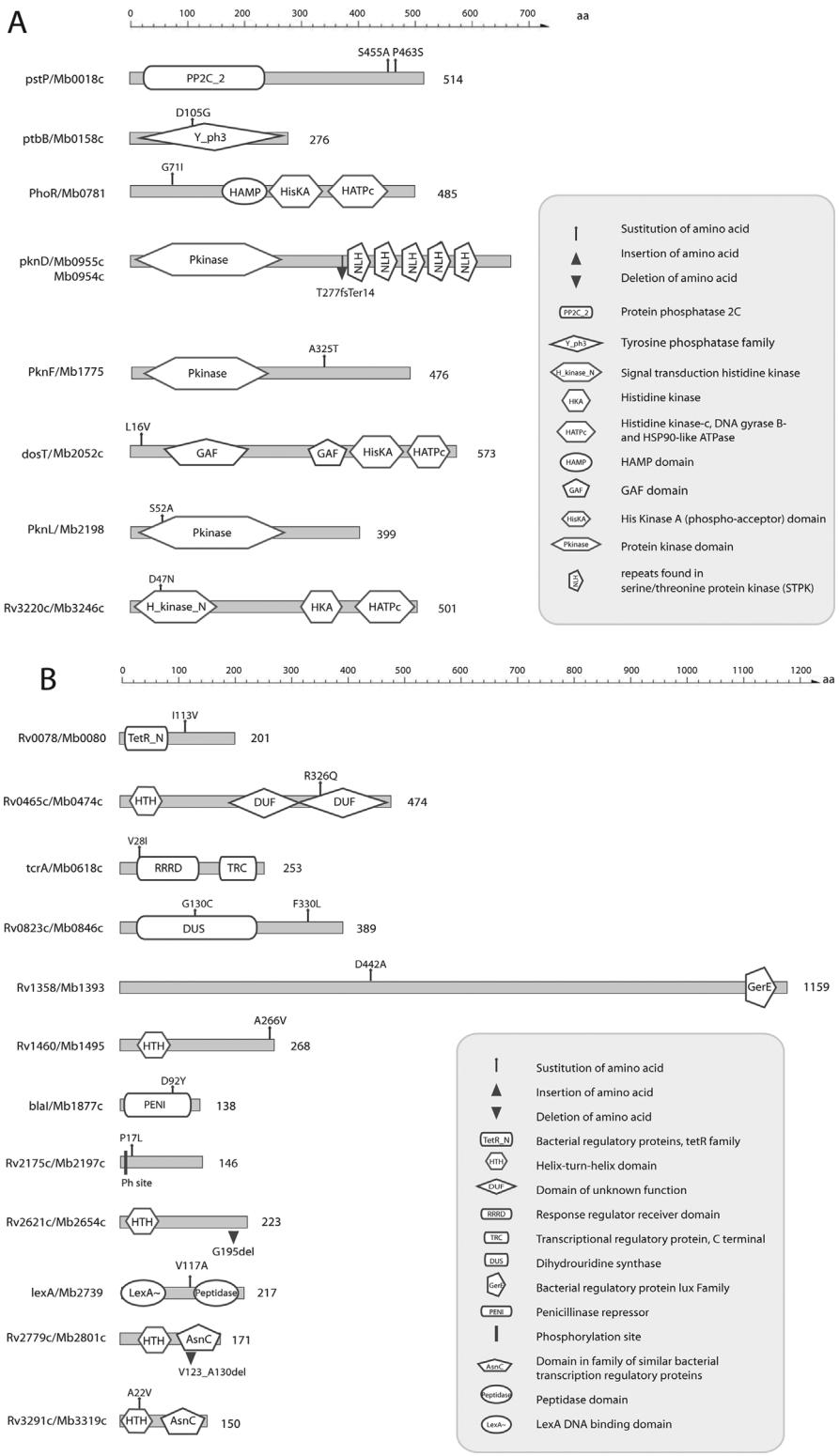

Fig. 1. Schematic representation of regulatory proteins. (a) Two-component systems, protein kinases and protein phosphatases. (b) Other regulatory proteins. Amino acid changes (*M. tuberculosis*/*M. bovis*) are indicated.

Table 3. Transcription of selected genes in *M. tuberculosis* and *M. bovis*

Gene	Fold change†	SD	*P*-value	Transcriptional regulator
pks2	11.79	1.20	0.0319	PhoP/R
Rv1456	3.71	2.42	0.1156	BlaI
hspX	27.98	2.96	0.0050	DosR/T
narK2	54.34	0.0031	0.0072	DosR/T
Rv3074c	2.05	1.35	0.1067	LexA
icl1	0.13	0.06	0.0082	Rv0465

†Expression ratio of *M. tuberculosis*/*M. bovis*.

DISCUSSION

Reversible protein phosphorylation is one of the main signal transduction pathways by which both eukaryotic and prokaryotic cells regulate metabolism in response to external stimuli. In bacteria, signal transduction events are carried out by two-component regulatory systems and by specific protein kinases and protein phosphatases.

The *M. tuberculosis* genome encodes 11 eukaryotic-like serine/threonine protein kinases (PknA to PknL, except for PknC). All of these *pkn* genes encode functional serine/threonine kinases and some of them participate in the modulation of different cellular events such as environmental adaptation, differentiation and cell division (19).

Interestingly, in the present study, we found conserved polymorphisms in three protein kinases, PknD, PknL, and PknF. We also identified polymorphisms in three two-component systems (DosT, PhoR, and Mb3246c) and in phosphatases Mb0018 and Mb0158c|*ptbB*; particularly, Mb0158c|*ptbB* participates in host infection of *M. tuberculosis* (20). These findings suggest a relevant role of the signal transduction pathways mediated by protein phosphorylation in the adaptation of *M. bovis* to its animal hosts.

Particularly, *pknD* of *M. bovis* carries a nucleotide deletion that splits the gene in two parts, *pknDA* and *pknDB*. Greenstein *et al.* (21) proposed a model by which PknD alters the transcriptional program of *M. tuberculosis* by stimulating phosphorylation of a sigma factor regulator. Furthermore, Vanzembergh *et al.* (22) suggested that *pknD* is necessary for growing of *M. tuberculosis* in phosphate poor conditions. Indeed, *M. tuberculosis* requires *pknD* to invade brain endothelia but not macrophages, lung epithelia, or other endothelia, thus highlighting a role of *PknD* in *M. tuberculosis*'s host cell specificity (23).

Interestingly, we found polymorphisms in PknL and its substrate, Mb2197c (Table 1). PknL is a protein of unknown function that possesses an original winged helix-turn-helix motif, which is indicative of a transcriptional regulator (Fig. 1b) (24), whereas Mb2197c is a conserved regulatory protein of unknown function. This finding suggests that both polymorphisms have cosegregated in the adaptation of *M. bovis* to the animal hosts.

Structural or biochemical data are available for Mb0618c/TcrA (25) Mb2801c/Rv2779c (26), Mb2197c/Rv2175c (24), Mb2198|pknL (27), Mb3319c/Rv3291c (28) and PknD (29). In addition, a function has been demonstrated or suggested for the *M. tuberculosis* homologs of Mb0474c (15), Mb1775|PknF (30), Mb1877c|BlaI (16), Mb0018c|PstP (31), Mb0158c|mPtbB (32), PhoPR (33–35), Mb2052c|DosT (36–38, 17) and Mb2739|LexA (39, 14). There is no available information on the probable two-component sensor kinase Rv3220c|Mb3246c or on the putative transcriptional regulators Rv0078|Mb0080, Rv0823|Mb0846c, Rv1358|Mb1393, Rv1460|Mb1495 and Rv2621c|Mb2654c.

PtbB is a virulence factor that participates in the mechanism of *M. tuberculosis* immune evasion (40), whereas BlaI regulates the responses and resistance to beta-lactam antibiotics and ATP synthesis (24).

Remarkably, two of the polymorphic regulator genes described in this study, Mb0018c and PknF, participate in the cell division process (41, 30). However, PknF may have other functions. For example, in *M. tuberculosis*, PknF phosphorylates GroEL1 (42), Rv1747 (43) and mtFabH (44). Rv1747 is an *in vivo* essential ABC transporter, whereas mtFabH is a key component of the mycolic acid pathway. In contrast, GroEL1 is a conserved chaperone required for the folding of proteins in several microorganisms but with an imprecise role in pathogenic mycobacteria. GroEL1 seems to participate in the cytokine-dependent granulomatous response during *M. tuberculosis* infection and interacts with the β-ketoacyl-AcpM synthase KasA (42), which is another key component of the type II fatty acid synthase involved in mycolic acid biosynthesis. Therefore, PknF has multiples roles in the regulation of mycolic acid synthesis and also seems to control pathways of glucose utilization (30).

In contrast to *M. tuberculosis*, *M. bovis* does not grow in glucose or glycerol as unique carbon sources because of the inactive pyruvate kinase, PykA. Thus, *M. bovis* metabolism seems to be highly dependent on fatty acids for energy production. The utilization of fatty acids requires a functional glyoxylate cycle with the key enzymes malate synthase and isocitrate lyase (Icl). As Rv0465/Mb0474 represses the expression of *icl1* (45), mutations on Mb0474 and *pknF* may have allowed the adaptation of *M. bovis* to its biological niches.

Consistently with this presumption, in this work, we detected a non-synonymous polymorphism in Rv0465/Mb0474 and higher expression of *icl1* in *M. bovis* compared to *M. tuberculosis*.

Mb2801 encodes a possible transcriptional regulatory protein belonging to the leucine-responsive regulatory protein/asparagine synthase C products (Lrp/AsnC-family). This protein has a deletion of eight amino acids that maps in a conserved and predicted AsnC family domain. This class of regulators, which is widespread among prokaryotes, is involved in the regulation of amino-acid metabolism and related cellular processes. A preliminary X-ray analysis of Rv2779c, the *M. tuberculosis* homolog (26) of Mb2801, has recently been carried out, but no further information on the role of this protein in bacilli is available. Another polymorphic Lrp/AsnC family member is Mb3319c or LprA with a SNP in the helix-turn-helix protein domain. This regulator is among the most connected regulatory hubs in the *M. tuberculosis* transcriptional regulatory network (46).

Gonzalo-Asensio *et al.* have previously reported low expression of *pks2* in *M. bovis,* compared to *M. tuberculosis* (9). Both DosT and Phop mediate the hypoxia response in *M. tuberculosis*; furthermore, Gonzalo-Asensio *et al.* have suggested that PhoP regulates the dormancy/hypoxia regulon through cross-talking with DosR/T (47). Previous evidence has indicated differential behavior between *M. tuberculosis* and *M. bovis* under hypoxic conditions and one illustrative example of these differences is the unique capacity of *M. bovis* to disseminate extrapulmonary tuberculosis (48). In this study, the reduced *hspX* expression in *M. bovis* compared to *M. tuberculosis* may support these previous findings. However, to confirm this presumption, it is required to compare the expression of *hspX* in both species under hypoxic conditions of growth.

DosR/T also regulates the expression of *narK2*, which encodes a nitrate transporter, and the transcription of *narK2* is induced by hypoxia in *M. tuberculosis* but not in *M. bovis* (49, 50). Chauhan and collaborators have demonstrated that the deficient induction of *narK2X* operon in *M. bovis* under hypoxic conditions is a result of a t6c SNP in the −10 promoter element of the *narK2X* operon (51). However, we cannot conclude that the low expression of *narK2* here detected in *M. bovis* is a consequence of the reported promoter's mutation because the growth conditions assayed were different between Chauhan's study and the present study. The study of Chauhan *et al.* has also demonstrated that complementation of *M. bovis* with both *narGHJI* and *narK2X* genes from *M. tuberculosis* failed to restore nitrate reductase activity in *M. bovis* in both aerobic and hypoxic conditions (51), suggesting that additional regulatory mechanisms for nitrate reduction are altered in *M. bovis*. From these previous findings and the results of the present study, we hypothesize that the mutation in DosT might contribute to the failure of nitrate reductase activity in *M. bovis*.

Rehren *et al.* defined a set of genes that are differentially expressed between *M. bovis* and *M. tuberculosis* under standard conditions of growth (52). Interestingly, in their study, the transcription of *Rv0465c* and *Rv2779c*, which both encode polymorphic transcriptional regulators, was upregulated in *M. bovis*. Based on the premise that most of the transcriptional regulators regulate their own synthesis, we speculate that the regulatory capacities of Rv0465c and Rv2779c are impaired in *M. bovis* because of the mutations in their genes. In addition, in the study of Rehren *et al.*, the expression of *Rv1588c*, a member of the LexA regulon, was upregulated in *M. bovis*.

The finding of non-synonymous mutations in 20 regulatory genes of *M. bovis* strains compared to *M. tuberculosis* strains suggests that punctual polymorphisms may explain or contribute to the phenotypic differences between *M. bovis* and *M. tuberculosis*. In addition, herein we found evidence that can explain the differential behavior between *M. bovis* and *M. tuberculosis* under hypoxic environments. However, further studies need to be done to determine the impact of these polymorphisms in the niche specialization of both pathogens.

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DISCLOSURE

Authors declare no conflicts of interest for this article.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

List S1. Genes of transcriptional regulators and regulatory proteins of *M. tuberculosis* H37Rv.
List S2. Genes with non-synonymous and synonymous SNP/INDEL between *M. tuberculosis* H37Rv and *M. bovis* AF2121/97.
List S3. Polymorphic proteins conserved in *M. bovis* strains.