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Characterization of the two component regulatory system PhoPR in *Mycobacterium bovis*

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Highlights

- PhoPR of *M. bovis* regulates virulence genes associated with redox balance.

- PhoPR is required for resistance to acidic stress of *M. bovis.*

- PhoPR is associated to biofilm formation in *M. bovis.*

- The synthesis/secretion of ESAT6 is regulated by PhoPR.

Abstract

Mycobacterium bovis is the causative agent of bovine tuberculosis and is a member of *Mycobacterium tuberculosis* complex, which causes tuberculosis in a number of mammals including humans. Previous studies have shown that the genes encoding the two-component system PhoPR, which regulates several genes involved in the virulence of *M. tuberculosis*, are polymorphic in *M. bovis,* when compared to *M. tuberculosis,* which results in a dysfunctional two-component system. In this study we investigated the role of PhoPR in two *M. bovis* strains with differing degrees of virulence. We found that the deletion of *phoP* in an *M. bovis* isolate reduced its capacity of inducing phagosomal arrest in bovine macrophages. By gene expression analysis, we demonstrated that, in both *M. bovis* strains, PhoP regulates the expression of a putative lipid desaturase Mb1404-Mb1405, a protein involved in redox stress AhpC, the sulfolipid transporter Mmpl8 and the secreted antigen ESAT-6. Furthermore, the lack of PhoP increased the sensitivity to acidic stress and alteration of the biofilm/pellicle formation of *M. bovis*. Both these phenotypes are connected to bacterial redox homeostasis. Therefore, the results of this study suggest a role of PhoPR in *M. bovis* to be linked to the mechanisms that mycobacteria display to maintain their redox balance.

Introduction

Many pathogenic *Mycobacterium* species belonging to the *Mycobacterium tuberculosis* complex (MTBC) cause tuberculosis in some mammals. In this complex, *M. tuberculosis* is the most ancestral species, from which *Mycobacterium bovis* arose after successive genomic deletions. Thus, the *M. bovis* genome is more than 99.95% identical to that of *M. tuberculosis* but with seven deleted regions (RD) ranging from 1 kilobase (kb) to 12.7 kb. While *M. bovis* has a broader host range, *M. tuberculosis* mainly affects humans. Therefore, it is apparently contradictive that *M. bovis*, whose genome has underwent a substantial loss of genomic regions and has no unique genes or *loci*, resides in a wider ecological niche than *M. tuberculosis,* its ancestor. Indeed, the explanation lies, in part, in the single nucleotide mutations, more than 1200, in the coding regions of genes that differentiate *M. bovis* from *M. tuberculosis*. In particular, *M. bovis* and *M. tuberculosis* have non-synonymous mutations in 20 regulatory genes (Bigi et al., 2016). Among these polymorphic regulatory systems are PhoPR and DosR; both systems mediate hypoxia responses in *M. tuberculosis*. While the immune system of humans latently infected with *M. tuberculosis* recognized antigens encoded in DosR-regulon of these bacteria, *M. bovis*-infected cattle showed a poor immunological response towards DosR regulated proteins (Jones et al., 2011). This finding suggests that either *M. bovis* rarely enters in dormancy or DosR plays different roles between species.

Gonzalo–Asensio et al. (Gonzalo-Asensio et al., 2014) have reported differential functionality of PhoPR between *M. bovis* and *M. tuberculosis,* showing that, compared to *M. tuberculosis*, PhoPR of *M. bovis* plays a minor role in the bacterial virulence.

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Therefore, the potential differences in the regulatory mechanisms exerted by PhoPR and DosR could have emerged as an adaptation of *M. bovis* and *M. tuberculosis* to their respective biological niches.

The *phoP-phoR* operon encodes PhoR and PhoP, a histidine protein kinase and a transcriptional regulator that receives a phosphate from PhoR. This two-component system is a virulence factor that plays a role in diverse aspects of metabolic physiology, lipid metabolism regulation and respiration. PhoPR increases triacylated mannose-capped lipoarabinomannans (ManLAM) acyl forms and regulates many genes of lipid metabolism (Ludwiczak et al., 2002). Therefore, some *M. tuberculosis* lipid components such as diacyl trehalose (DAT), polyacyl trehalose (PAT) and sulfolipid (SL) are diminished in the *phoP* mutants (Gonzalo-Asensio et al., 2014). Importantly, PhoPR controls the secretion of antigens transported by the twin-arginine translocation pathway (TAT), such as Ag85A and Ag85C, and the secretion of CFP-10 and ESAT-6 (Solans et al., 2014a)(Solans et al., 2014b).

We have previously demonstrated that *phoP* elimination from a bovine tuberculosis vaccine candidate, *M. bovis* Δmce2 strain, resulted in a significantly more attenuated mutant strain, than its parental strain in mice (Garcia et al., 2015). This finding indicates that similarly to *M. tuberculosis*, *M. bovis* requires PhoPR for the full virulence in mice. Herein, we investigated the importance of PhoP in the interaction of *M. bovis* with the bovine macrophage. We also defined a set of genes regulated by PhoP in *M. bovis* and analysed the role of this two-component system in the secretion of ESAT-6 and Ag85A and in the resistance to acidic stress and biofilm formation.

Methods, techniques

Bacterial strains and culture media

Escherichia coli DH5α was used as host strain for all cloning steps. *Escherichia coli* were grown either in Luria-Bertani (LB) broth or on LB agar. *M. bovis* strains were grown in Middlebrook 7H9 medium (*Difco Laboratories*. Ref: 271310) supplemented with albumin (A) 0.5 %, dextrose (D) 0.4 %, and pyruvate (P) 0.5 % (M7H9-AD-P) and either Tween 80 (T80) 0.05 % or Middlebrook 7H10 (*Difco Laboratories*. Ref: 262710) supplemented with AD-P. When necessary, either 50 µg/ml hygromycin or 20 µg/ml kanamycin was added to the media.

Acid stress

For acid stress experiments, the bacterial pellets were washed with PBS and resuspended in 1 ml PBS. Then, the resuspended bacteria were diluted at 0.1 OD_{600} in 7H9 medium supplemented with AD-P-T80 in which 100mM MOPS (pH 6.6-7) or HCl (pH 5.3-5.6) were added. *M. bovis* strains were grown in agitation at 37 °C. The data of optical density at 600 nm were taken every 3 or 5 days.

The quantification of biofilm was carried out by staining with crystal violet in 24 well-plates. Once the biofilms of the *M. bovis* wild-type strains completely covered the surfaces of the cultures, the liquid medium of all cultures (including the mutant and complemented strains) was removed and the biofilms adhered to the plate were incubated with 2 ml of 0.5% crystal violet solution for 10-30 min. Three washes were made with water and then the crystal violet was extracted with 2 ml of ethanol by incubation for 10-30 min. The absorbance of extracted crystal violet was measured at 570nm.

To determine the number of bacteria in the biofilms, the biofilms were dissolved with PBS containing 0.5% T80 and the solution was homogenized by pipetting. After 15 min

of incubation at room temperature, the bacterial solution was transferred to a tube and centrifuged at 2500g for 10 min at room temperature. The pellets were resuspended in buffer (PBS with 0.05 % T80). After three washes, the pellets were resuspended in 1ml buffer and the optical density was measured at 600nm. To convert the OD values into bacteria/ml, a OD value = 1 was established for $10.e^7$ bacteria/ml.

Construction of *Mycobacterium bovis* mutant strain

The *MbΔphoP* mutant strain was previously obtained (Garcia et al., 2015) and *Mb04-303ΔphoP* was constructed using similar procedures. The selection of the *Mb04-303ΔphoP* mutant strain was performed as previously described (Santangelo et al., 2008). The absence of *phoP* in the candidate mutant clones was confirmed by PCR (Supplementary Material S1).

The mutant strains were complemented with the entire *phoPR* operon and the promotor using the integrative plasmid pWM221 (Gonzalo-Asensio et al., 2014).

Microarray assay

Genomic DNA of *M. bovis* AF2122/97 (*M. bovis* reference strain) was kindly provided by BEIResourse. DNA-free RNA was extracted from 50 ml mid-exponential-phase cultures of *M. bovis* strains as described by Santangelo et al. (Santangelo et al., 2009). Complementary DNA (cDNA) synthesized from RNA was fluorescently labelled in a reverse transcription reaction using SuperScript II reverse transcriptase (*Invitrogen, Thermo Fisher Scientific.* Ref: 18064-022) in the presence of Cy5-CTP (Amersham Biosciences) as previously described (Golby et al., 2007). cDNA synthesized using DNA as template was fluorescently labelled using Cy3-dCTP (GE Healthcare. Ref:

PA53021 and PA55021) and Klenow DNA polymerase (*New England Biolabs* MO210L) as previously described (Golby et al., 2007).

The microarrays were developed by the Bacterial Microarray Group (St Georges, University of London) in collaboration with the Veterinary Laboratories Agency (Weybridge). Microarray prehybridization, hybridisation, and washing steps were performed as previously described (Lopez et al., 2010)(Santangelo et al., 2009). Microarrays were hybridised with a combination of Cy3-cDNA generated from genomic DNA and Cy5-cDNA obtained from total RNA of either *M. bovis* NCTC10772 or *M. bovis* ΔphoP.

Ten sets of microarray data were produced in total, consisting of five biological replicates (bacterial cells from independent culture batches) for each *M. bovis* strain.

The microarrays were scanned using an Affymetrix 428 scanner and fluorescent spot intensities were quantified using BlueFuse for Microarrays v3.2 (*BlueGnome Limited*, http://www.cambridgebluegnome.com) as described previously (Santangelo et al., 2009). The histograms of all microarrays were brought to the same scale by applying the median absolute deviation using Mathematica 5.2 (*Wolfram Research Inc*.). Technical replicates were averaged. Differentially expressed genes between the strains were detected by using t-tests with a Benjamini and Hochberg adjusted p-value correction.

RT-qPCR

DNA-free RNA (1 µg) was reverse transcribed to total cDNA by using 50 ng of random primers (*Invitrogen*, *Thermo Fisher Scientific.* Ref: 48190-011) and SuperScript II reverse transcriptase (*Invitrogen, Thermo Fisher Scientific.* Ref: 18064-022) according to the manufacturer's instructions (20 µl final volume). cDNA concentration was

determined in a spectrophotometer (ND-1000 Nano Drop). Approximately 25 ng of the cDNA was used as the template for each real-time qPCR reaction. qPCR reactions, containing Taq platinum (*Invitrogen, Thermo Fisher Scientific.* Ref: 10966-030), SYBR green I dye (*Invitrogen, Thermo Fisher Scientific.* Ref: S7567), and specific primers (Supplementary Table S2), were run on an Applied Biosystem Step One Plus instrument under standard cycling conditions. qPCR reactions were performed in duplicate of three biological replicates and data were analysed with LinReg (Ramakers et al., 2003) software. Curve analysis and ratio calculation were determined using Fg statistical software (Di Rienzo, 2002) The *sigA* gene was used as the control gene to assess differences on gene expression between groups.

PBMC isolation and BMDM differentiation.

Blood samples were obtained from cattle maintained in the National Institute of Agricultural Technology (INTA)'s experimental herd. All selected animals were negative for bovine tuberculosis infection test. The blood samples (60 ml) were taken from each animal following the instructions of the Committee for Institutional Care and Use of Animal Experimentation (CICUAE-CICVyA) of INTA. Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by centrifugation over Ficoll-Paque plus (*GE Healthcare*. Ref: 17-1440-03) according to the manufacturer's protocol. Monocyte-derived macrophages (BMDMs) were obtained following a previously described protocol (Vázquez et al., 2017).

Indirect immunofluorescence and confocal microscopy.

The *M. bovis* strains were cultured until exponential growth phase, harvested, washed and resuspended in PBS 1X. The bacterial suspensions were passed through a syringe

needle (25 gauge) 30 times to disaggregate bacterial clumps. The remaining clumps were separated from individual bacteria by a centrifugation at 900g for 8 min. The bacteria were covalently stained with fluorescein isothiocyanate (FITC) (*Sigma-Aldrich.* Ref: 3326-32-7) as previously described (Vázquez et al., 2017). The stained bacteria were used to infect BMDMs at an MOI of 5 for 1 h at 37 °C and 5% CO_2 (uptake). Infected BMDMs were washed to eliminate the extracellular bacteria and incubated again for 2 h (chase). Indirect immunofluorescence was performed on the infected cells as previously described (Vázquez et al., 2017). Briefly, infected BMDMs were fixed with 4% paraformaldehyde solution in PBS (PFA), permeabilized with 0.05% saponin and incubated with antibodies against, LAMP-3 (*Santa Cruz Biotechnology*. Ref: SC-25184) or EEA1 (*Santa Cruz Biotechnology*. Ref: SC137130). Anti-goat or anti-mouse antibodies conjugated to Cy5 (Indodicarbocyanine. *Jackson ImmunoResearch Inc.*) were used as secondary antibodies. The cells were treated with mounting medium (*Dako*, *Agilent*. Ref: 53023) and analysed by confocal microscopy using a Leica TCS-SP5 spectral confocal microscope (Leica Microsystems, Germany) at the integrated Microscopy laboratory (LIM), CICVyA, INTA or Olympus FV300/BX61 at FCEyN-UBA. Mycobacterial internalization was analysed and quantified using Fiji software (U.S. National Institutes of Health, Bethesda, MD) as described previously (Vázquez et al., 2017). Each experiment was performed in duplicates. Statistical analysis was performed using analysis of variance (ANOVA) and Bonferroni's post-tests. Three independent infections were performed for each assay.

Image analysis.

For image acquisitions a single focal plane was monitored over time (xyt scanning mode) using a 40× or 63× (according to the microscope used) oil objective lens, an

argon laser (488 nm), and a DPSS laser (561 nm), when applicable; a scanner frequency of 200 to 400 Hz; and line averaging 6, using PMT detectors at a scanning resolution of 1,024 by 1,024 pixels or 512 by 512 pixels (zoom of 2.5). The same settings for laser powers, gain, and offset were maintained for the different experiments. The association of bacterial particles to the cell markers was determined as previously described (Vázquez et al., 2017). Fiji software was used for images analysis (http://fiji.sc. Fiji is an image processing software based on ImageJ (National Institutes of Health).

Fluorescence intensity values were plotted and analysed using Microsoft Excel 2011 (Microsoft) and GraphPad Prism 5 (GraphPad Software, Inc., USA).

SDS-PAGE and Western blot analysis.

After three weeks of incubation, bacterial cells were resuspended in PBS 1X, harvested by centrifugation, disrupted with glass beads in a homogenizer, centrifuged at 12000g for 10 min at 4 °C and supernatants were collected for protein quantification. To obtain secreted proteins from the bacterial culture supernatants, we omitted the addition of albumin to the culture medium and added 0.4% glucose. The supernatants containing secreted proteins were precipitated with 10% trichloroacetic acid overnight and centrifuged at 7000g for 30 min at 4 °C. The proteins from culture supernatant and bacterial cells were resuspended in cracking buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 0.01% bromophenol blue). For Western blot analyses, the samples were subjected to electrophoresis in 15% SDS-PAGE gels and then transferred to nitrocellulose membranes and stained with Rouge Ponceau solution (*Sigma-Aldrich* Ref: P7170). The membranes were blocked with TBS (10 mM tris-HCl pH 7,5, 150 mM NaCl) supplemented with 5% skim milk for 30 min before overnight incubation with primary antibodies (monoclonal anti-ESAT-6 or polyclonal Ag85A).

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The antibodies were used at dilutions of 1:50 and 1:100 for ESAT-6 and anti-Ag85A respectively. Then, the nitrocellulose membranes were washed in TBS thrice, and incubated with a secondary antibody alkaline phosphatase-conjugated (*Sigma-Aldrich.* Ref: A9316/A3687) at a 1:10000 dilution for 2h. Western blots were revealed by incubation with BCIP/NTP solution (*Promega Corporation.* Ref: S3771).

Results

The *phoP* gene is required for *M. bovis* phagosome maturation arrest in bovine macrophages

A previous study has shown that the elimination of *phoP* gene from *M. tuberculosis* facilitates the maturation of phagosomes containing mycobacteria to late compartments (Ferrer et al., 2010). To determine the role of PhoP in the phagosomal arresting mechanisms exerted by *M. bovis*, we evaluated the trafficking of *M. bovis* strains inside bovine macrophages by using immunofluorescence and confocal microscopy. Bovine macrophages were infected with *M. bovis* strains for 3 h, as described in Methods, techniques section. The *M. bovis phoP* mutant strain (*MbΔphoP*) was significantly more associated (P ≤ 0.05) with LAMP-3 than its wild-type strain and its complemented strain (Figure 1). This result indicates that the *phoP* participates in the phagosomal arrest induced by *M. bovis* during its intracellular replication inside macrophages.

Consistent with the above results, the association of *MbΔphoP* with the early endosomal antigen 1 (EEA1) in bovine macrophages was lower than that of the wild-type strain (Supplementary material S3).

Altogether these results indicate that at early times of infection *M. bovis* requires PhoP to induce phagosomal arrest in bovine macrophages.

Genes regulated by PhoP in *M. bovis*

To define the PhoP regulon, we performed a whole-genome *in vitro* expression profiling on the *MbΔphoP* and wild-type parental *M. bovis* strains. Only 70 genes presented differential expression between the strains (p <0.005) with fold changes equal or higher than 1.5 (absolute value) (Supplementary Table S4). This finding indicates that most of the genes were similarly expressed in the mutant and the wild-type strains.

By RT-qPCR, we assessed a subset of the differentially expressed genes and validated 9/14 genes with differential expression (*Mb0750, Mb0780, Mb1404-Mb1405, Mb2454, Mb2455, Mb2966, Mb3372, Mb3708, Mb3853c*). The rest of the genes showed concordant results to those of the microarrays (*Mb0834, Mb1284, Mb3262c, Mb3401, Mb3406*) but the values between strains were not significantly different (Figure 2).

In addition, we evaluated the expression of *pks2 (Mb3855)* and *lipF (Mb3517c)*, which are regulated by PhoP in *M. tuberculosis.* While *pks2* and one of its co-transcribed genes, *mmpL8*, are significantly downregulated in the mutant strain, *lipF* did not show significantly different expression between the mutant and wild-type strains (Figure 2).

Thus, four genes involved in lipid metabolism (*Mb1404/Mb1405*, *fadD28*, *pks2* and *mmpL8*) and two genes associated with stress response (*ahpC* and *ahpD*) were downregulated in the absence of PhoP in *M. bovis*. Remarkably, five downregulated genes in the mutant strain are related to mycobacterial virulence (*ahpC, fadD28, Mb3708*, *mmpL8* and *pks2*) (Forrellad et al., 2013).

We also assessed the expression of these PhoP-regulated genes in another *M. bovis* strain. In this mutant, *phoP* was deleted from the *M. bovis* 04-303 strain. Unlike *M. bovis* NCTC10772, which is attenuated in cattle (Meikle et al., 2011), *M. bovis* 04-303 strain is highly virulent in cattle (Meikle et al., 2011) and mice (Aguilar León et al., 2009).

The expression of *Mb1404/Mb1405*, *ahpC*, *mmpL8* and *esat-6* was significantly downregulated in strain *Mb04-303ΔphoP*. The expression of *ahpD*, *fadD28*, *metC*, *lipF*, *Mb3708* and *espR* was also downregulated, although not significantly, in this mutant strain. Therefore, the expression of *Mb1404/Mb1405*, *ahpC*, *mmpL8* and *esat-6* in *M. bovis* depends on PhoP (Figure 2).

Solans (Solans et al., 2014b) and He (He and Wang, 2014) et al. have previously defined the PhoP binding sites in the *M. tuberculosis* chromosome. In this study, we observed that the upstream regions of *Mb1404/Mb1405* (*Rv1371*), *pks2* (*Mb3855*) and *ahpC/ahpD* have some similarity with the consensus sequences defined as targets for PhoP binding in *M. tuberculosis* (Figure 3). The rest of the promoters do not carry any similarity with these consensus sequences, suggesting either a higher divergence of the sequences or an indirect regulation by PhoP, i.e. mediated by other proteins.

PhoP is required for *M. bovis* growth under acid stress

Bansal et al. (Bansal et al., 2017) have demonstrated that, in conjunction with sigma factor E, PhoP induces a transcriptional program in *M. tuberculosis* in response to acidic stress. In order to determine the relevance of PhoP to the growth of *M. bovis* under acidic stress, we compared the in vitro replication of the Δ*phoP* and wild-type strains under normal or acid-stress conditions.

Figure 4 shows that the mutant strains were unable to growth at low pH, while the wild-type and complemented strains grew normally at that condition. At neutral pH all *M. bovis* strains grew normally (Figure 4). These results suggest that PhoP is essential for *M. bovis* when facing acidic stress.

PhoP is required by *M. bovis* strains to produce pellicle/biofilm in vitro

Trivedi et al. (Trivedi et al., 2016) identified *ahpCD* and *metC* as a subset of genes that changed their expression in response to stress and biofilm formation in *M. tuberculosis*. Because PhoP regulates these genes in *M. bovis*, as demonstrated in this study, we decided to quantify the effect of PhoPR in the biofilm/pellicle formation. For this purpose, we grew the *M. bovis* strains in sealed 24-wells plates and, after 28 days of growth, measured the bacteria attached to the well walls.

Using equivalent numbers of bacteria, the mass of cell growth as pellicle/biofilm was significantly lower in the mutant strains than in other strains (Figure 5). This result indicates that PhoP participates directly or indirectly in the biofilm formation of *M. bovis*.

The lack of PhoP impairs the synthesis/secretion of ESAT-6

PhoP controls the secretion of ESAT-6 and antigen 85 complex (Ag85A, B and C) in *M. tuberculosis* through different regulatory mechanisms (Solans et al., 2014a)(Solans et al., 2014b). To evaluate if these mechanisms are conserved in *M. bovis*, we analysed the content of ESAT-6 and Ag85A in culture supernatant of the *M. bovis* strains. While ESAT-6 secretion was significant impaired in the mutant strains compared to the wild-type and complemented strains (Figure 6), the amount of Ag85A was barely reduced in MbΔphoP and Mb04-303ΔphoP (Figure 6). This slight lower accumulation of Ag85A in the supernatant of the mutant strain culture was constant throughout three independent experiments (data not shown). However, the low quantitative power of the western blot assays does not allow us to reach an accurate conclusion about the role of PhoP in the secretion of this antigen.

The accumulation of both ESAT-6 and Ag85A in bacterial cells did not shown substantial differences among all *M. bovis* strains (Supplementary material S5). These

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results indicate that some features of PhoP regulation are not completely conserved between *M. bovis* and *M. tuberculosis*.

Solans et al. (Solans et al., 2014a) have reported that in *M. tuberculosis* H37Rv PhoP regulates the transcription of operon *espACD* and its regulator, *espR*. The operon *espACD* encodes proteins that, together with ESAT-6 and CFP10, are secreted by ESX-1 transporter (Fortune et al., 2005). In this study we demonstrated that while the transcription of *espC* and *esat-6* is impaired in the absence of PhoP, *espR* transcription remains unaltered (Figure 6, B). Altogether these results indicate that in *M. bovis*, PhoP regulates the synthesis and perhaps the secretion of ESAT-6 and EspC.

Discussion

The findings of this study reinforce existing evidence that the PhoPR regulon is not completely conserved between *M. bovis* and *M. tuberculosis*. In fact, the *phoPR* allele from *M. bovis* AN5 strain cannot restore the wild-type phenotype of an *M. tuberculosis* H37Rv mutant strain in the *phoP* gene (Gonzalo-Asensio et al., 2014). According to the researchers who reported this finding, the PhoPR regulation system may be deficient in *M. africanum* and animal-adapted MTC strains primarily because of a non-synonymous single nucleotide polymorphism (SNP) in *phoR* (Gonzalo-Asensio et al., 2014). Remarkably, this non-synonymous SNP is conserved in all *M. bovis* isolates sequenced to date (Bigi et al., 2016).

Here, we have demonstrated that PhoP plays a role in phagosome arrest induced by *M. bovis* inside bovine macrophages, thus indicating that, even deficiently, PhoP of *M. bovis* is still functional. This result is consistent with our previous study, in which the

deletion of *phoP* from the vaccine *M. bovis* strain, *MbΔmce2*, significantly reduced its virulence in mice (Garcia et al., 2015).

In *M. tuberculosis,* PhoP contributes to ESX-1-dependent ESAT-6 secretion by favouring the transcription of *whiB6*, which encodes a regulator required for ESAT-6/CFP10 secretion (Solans et al., 2014a), and by activating *espACD* expression (Fortune et al., 2005). The *espACD* operon is located outside RD1 and encodes proteins also required for ESAT-6/CFP10 secretion (MacGurn et al., 2005). Through a microarray experiment, we detected that *whiB6* was downregulated in *MbΔphoP* compared to the wild-type strain. However, in the RT-qPCR assay the expression values were not significantly different.

In *M. tuberculosis* the expression of *espACD* operon is regulated by EspR (Forrellad et al., 2012) and MprAB (Pang et al., 2013). On the other hand, PhoP regulates the expression of EspR in this mycobacterial species (Cao et al., 2015). The binding sites required for MprAB and EspR regulation map in the RD8 region that is absent from *M. bovis* and *M. africanum* L6 (Gonzalo-Asensio et al., 2014). Therefore, it is expected that the expression of *espACD* escape the PhoPR/EspR/MprAB control in these species. In contrast to these previous findings, in this study we found that in the absence of PhoP the expression of *espC*, a member of *espACD* operon, is downregulated, which indicates that at least in *M. bovis* NCTC10772 PhoP has a role in the transcription of *espC*. One possible explanation to these inconsistent results is that compensatory mechanisms have emerged during the evolution of *M. bovis* to correct the dysregulation of *espACD* (owing to RD8 deletion). However, further experimental evidence in more *M. bovis* isolates is necessary to establish whether or not PhoPR control *espACD* transcription.

Our results also demonstrate for the first time that at least in two *M. bovis* isolates the production/secretion of ESAT-6 is dependent on PhoP. Thus, the localization of the *M.*

bovis ΔphoP strain in late phagolysosomes or phagosomes may be because the mutant has an affected ESAT-6 production/secretion. Although is still unclear whether ESAT-6 plays a direct role in the phagosomal rupture or it is required to facilitate the secretion of other Esx-1 effectors with membrane rupture functions (Augenstreich et al., 2017)(Conrad et al., 2017)(Simeone et al., 2012), it is highly probable that this phagosomal escape mechanism has been conserved in all species of the *M. tuberculosis* complex because of their close evolutionary relationship.

The transcriptomic comparison between the wild-type and *M. bovis ΔphoP* strains allowed us to shed light on the PhoP regulon in *M. bovis*. Among the PhoP regulated genes is *ahpCD*. The proteins encoded in this operon are involved in the response to oxidative stress. Oxidative stress is defined as an unbalance in the redox environment in favour of oxidizing species that are capable of damaging biological systems. The redox reactions are essential for life and play an important role in aerobic and anaerobic respiration.In aerobic microorganisms, such as *M. tuberculosis*, reactive oxidant species and reactive substances are in equilibrium by the action of antioxidants to get a suitable redox balance. During infection, *M. tuberculosis* is exposed to a complex environment that may influence its physiology, including its redox environment. As in other bacteria, pathogenic mycobacteria have developed various mechanisms that allow it to monitor and respond to different gaseous signals such as nitric oxide, carbon monoxide and oxygen, and variations of the intra- and extracellular redox state (Trivedi et al., 2012)(Singh et al., 2007). For example, peroxides may react with different cellular compounds, including lipids, and thus produce highly reactive acyl hydroperoxides. The toxic effect of acyl hydroperoxides in *M. tuberculosis* is countered by the peroxiredoxins AhpC that reduces organic peroxides (Master et al., 2002). In addition,

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AhpC confers protection against reactive nitrogen by reducing peroxynitrite to nitrite (Master et al., 2002).

Trivedi et al (Trivedi et al., 2016) have described a link between reductive stress and biofilm formation in *M. tuberculosis,* since reductive stress induced by dithiothreitol (DTT) led to biofilm formation in *M. tuberculosis* cultures. Moreover, these authors have shown that the expression of the PhoP-regulated genes *ahpCD* and *metC* were part of the transcriptional programs induced during the signalling and formation phases of biofilm creation, respectively. However, in that study biofilm generation was induced with the reductive agent DTT and therefore the upregulation of *ahpCD* and *metC* may has been a collateral effect of the DTT treatment with no connection with the biofilm generation pathway. In this study we showed that the expression of these genes was positively associated with the presence of biofilm in the absence of DTT, therefore, these findings suggest a potential role of AhpCD and MetC in biofilm biosynthesis.

Another outcome of our present study is that the lack of PhoP turned *M. bovis* strains more susceptible to acid stress, which is consistent with the previously reported role of PhoPR system in the intracellular pH homeostasis (Abramovitch et al., 2011)(Baker et al., 2014)(Bansal et al., 2017). Thus, given these findings, we consider that the reported impaired persistence and replication of *M. bovis ΔphoP* strains within the host (Garcia et al., 2015) is a consequence of their high susceptibility to both the low pH and the oxidative/nitrosative phagosomal environment.

Another gene regulated by PhoP in the two *M. bovis phoP* mutants is *Rv1371* (*Mb1404/Mb1405*). This gene encodes a probable conserved membrane protein similar to cytochrome b5 fusion desaturases. Although these enzymes play a central role in polyunsaturated fatty acids (PUFAs) synthesis (https://doi.org/10.1016/S0952-3278(02)00263-6) in higher eukaryotes, there is no demonstrated role of *Rv1371* in

Mycobacterium species. Probably, this enzyme is a member of desaturases family involved in the synthesis of unsaturated fatty acids.

Conclusion

In conclusion, in this study we found that in *M. bovis* PhoP controls of the expression of genes likely involved in phagosome arrest, resistance to oxidative and acid stresses and biofilm formation. All these mechanisms are used by pathogenic mycobacteria to evade the host immune attack (Brennan, 2017). These observations indicate that despite the deleterious effect of the non-synonymous SNP in its locus, PhoPR still retains some relevant functionality in *M. bovis*.

Conflict of interest statement

The authors have no competing interests to declare.

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Figure captions

Figure 1: Confocal microscopy analysis of bovine macrophages infected with *M. bovis* strains. A) Representative images of bovine macrophages derived from peripheral blood infected with the different strains labelled with FITC (green fluorescence) using an MOI= 5: 1. The cells were fixed after 3 h of infection and used to detect LAMP-3 by indirect immunofluorescence using a specific antibody against LAMP-3 (red fluorescence). Scale bars: 10 μm. Merge indicates the location of the different strains in the phagolysosome. B) Fluorescence intensity quantification of LAMP-3 associated with the phagosome of *M. bovis* (circles), *MbΔphoP* (squares) and *MbΔphoP::phoPR* (triangles). The data were expressed in arbitrary units (a.u.). Fiji software was used for quantification. The values are representative of three independent experiments with similar results. The data were analysed using one-way ANOVA analysis and Bonferroni's post-test. The asterisk represents significant differences (***p <0.001). NS: not significant.

Figure 2. Comparative gene expression between *M. bovis* strains obtained by RT-qPCR. Fold-changes were calculated using *sigA* mRNA expression as reference gene and *M. bovis* wild-type strains as calibrator. Data were analysed using a random permutation test (fg statistic software *p <0.05 **p <0.01). Bars represent average expression ratios of triplicates ± SD between mutant/wild-type strains.

Figure 3: Comparative analysis of PhoP binding sites between *M. tuberculosis* and *M. bovis*. The PhoP binding consensus sequences described in *M. tuberculosis* are

identified in bold. In *M. bovis*, similar and different bases of the consensus sequence are identified in bold and in red, respectively. ORF: open reading frame.

Figure 4: Growth of *M. bovis* strains at acidic pH. Growth of different *M. bovis* strains in 7H9 medium at acidic or neutral pH. The OD_{600} was measured at different days. Error bars represent the standard deviation and the data are representative of three independent experiments.

Figure 5: Biofilm formation in *M. bovis* strains. A, B Quantification of *M. bovis* biofilms by crystal violet staining. The data were analysed using one-way ANOVA analysis and Bonferroni's post-test (*p <0.05, ** <0.01). C) Growth of *M. bovis* strains in the biofilms. It was determined at OD_{600nm}. The data are representative of two independent experiments performed in duplicate.

Figure 6: Comparative analysis of secreted proteins in *M. bovis* strains. A) Detection of ESAT-6 and Ag85A in culture supernatant proteins of *M. bovis* strains by western blot. Protein bands were detected by using a monoclonal anti-ESAT-6 or a polyclonal anti-Ag85A antibody. Equivalent amount of total proteins of each strain was loaded in each lane as shown in the gels stained with Coomassie blue (bottom). WT: wild-type strains, Mut: mutant strains in *phoP* gene (MbΔphoP-left and Mb04-303ΔphoP-right), Comp: mutant strains complemented with *phoP* and *phoR* genes (MbΔphoP::phoPR-left and Mb04-303ΔphoP::phoPR-right). These images are representative of three independent experiments with similar results. B) Comparative gene expression of between *M. bovis* strains obtained by RT-qPCR. Fold-changes were calculated using *sigA* mRNA expression as reference gene and *M. bovis* wild-type

strains as calibrator. Data were analysed using a random permutation test (fg statistic software *p <0.05). Bars represent average expression ratios of triplicates ± SD between mutant/wild type strains.
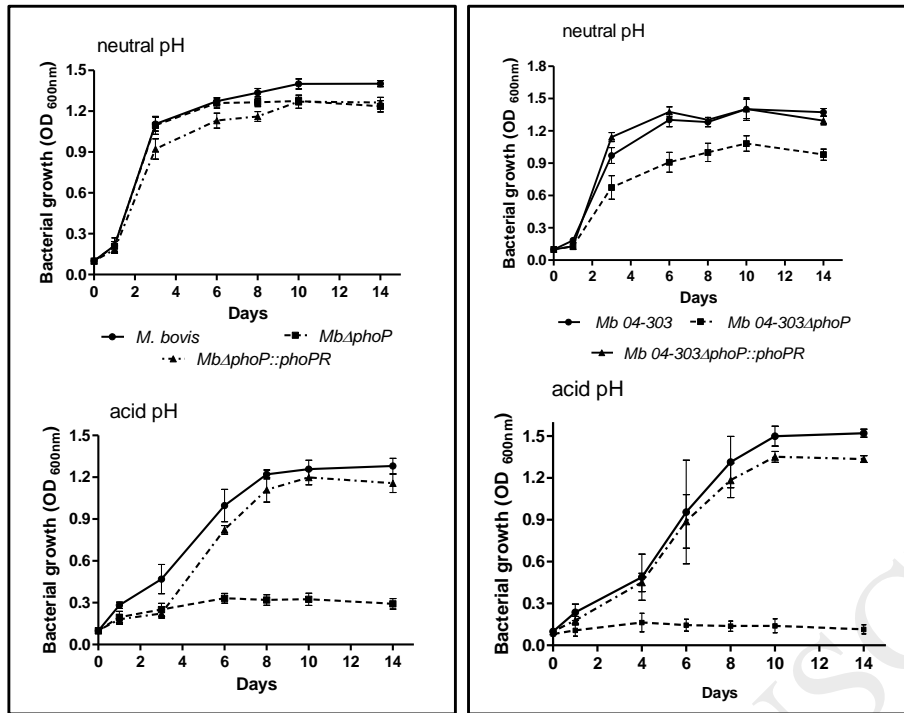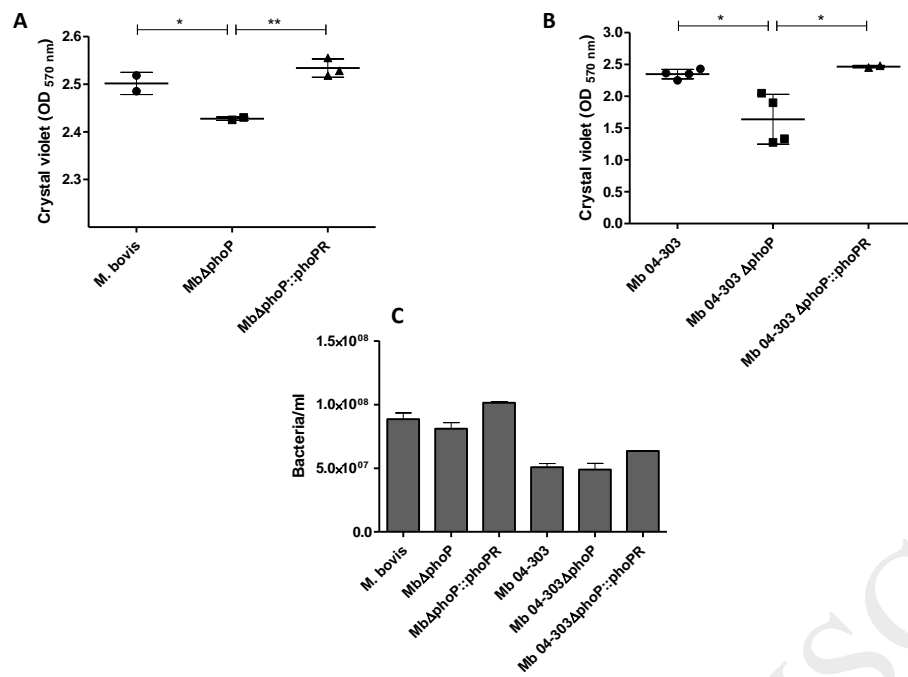
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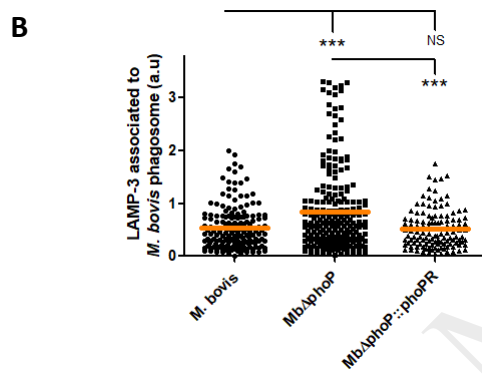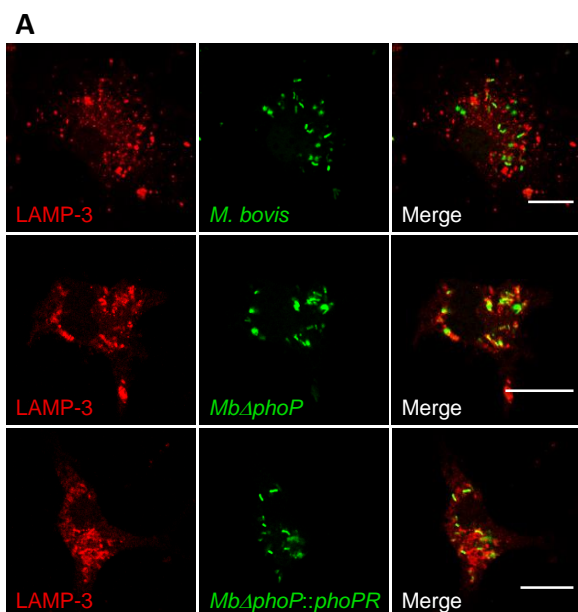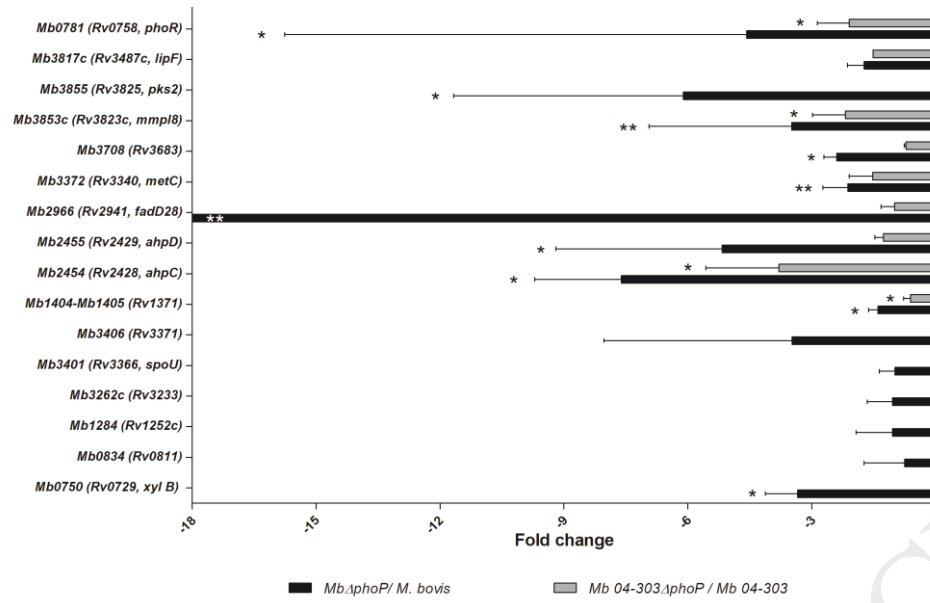
	Phop binding region	Distance to ORF start	Gene
M. tuberculosis	**AGCCAC** CAAAG **ACACAG** CTA	40	*pks2*
	| | |||| | |||| | || || | |||		
M. bovis	**AGCCAC** CAAAG **ACACAG** CTA	40	*pks2*
M. tuberculosis	**ACACAG** CTGAT **TAACAG** GAT	109	*whiB6*
	| || || || ||| ||		
M. bovis	**ACACAC** TCTAT **CAACGC** CAT	16	*Mb1404/Mb1405*
M. tuberculosis	**TCACAG** CAACT **TCACGG** TTG	47	*mcr7*
	| |||| | ||| |||||		
M. bovis	**TGACAG** CGACT **TCACGG** CAC	51	*ahpC/ahpD*

A

M. bovis NCTC10772

WT   Mut   Comp

α **Ag85A**

α **ESAT-6**

Total Proteins

M. bovis 04-303

WT   Mut   Comp

α **Ag85A**

α **ESAT-6**

Total Proteins

32