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Elizabeth A. García, Federico C. Blanco, Laura I. Klepp, Adriana Pazos, Michael R. McNeil, Mary Jackson, Fabiana Bigi

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Role of PhoPR in the response to stress of *Mycobacterium bovis*

Elizabeth A. García [1,2] garcia.elizabeth@inta.gob.ar, Federico C. Blanco [1,2] blanco.federico@inta.gob.ar, Laura I. Klepp [1,2] klepp.laura@inta.gob.ar, Adriana Pazos [3] pazos.adriana@inta.gob.ar, Michael R. McNeil [4] M.Mcneil@ColoState.EDU, Mary Jackson [4] Mary.Jackson@ColoState.EDU and Fabiana Bigi [1,2]* bigi.fabiana@inta.gob.ar
1Instituto de Agrobiotecnología y Biología Molecular, (IABIMO) INTA-CONICET
2Instituto de Biotecnología,CICVyA, Instituto Nacional de Tecnología Agropecuaria (Institute of Biotechnology, National Institute of Agricultural Technology, Argentina)
3Instituto Tecnología de Alimentos (ITA), INTA. Food Technology Institute, Argentina
4Colorado State University, Dept. of Microbiology, Immunology and Pathology, USA
*Corresponding author
correspondence and reprints.
N. Repetto and De los Reseros, Hurlingham, 1686; Buenos Aires, Argentina
Tel: +54 11 4621 1447 ext 3538
N. Repetto and De los Reseros, Hurlingham, 1686; Buenos Aires, Argentina
Tel: +54 11 4621 1447 ext 3538
MJ: 1682 Campus Delivery. Ft. Collins, CO 80523-1682, USA
Tel: + 970 491 2506

Highlights

- PhoP participates in the ammonia production in response to acidic stress in *M. bovis*
- Urea degradation seems to be a strategy of *M. bovis* to respond to acidic stress
- The lack of Phop negatively affects the *de novo* synthesis of PDIM in *Mycobacterium bovis*

Abstract

PhoP is part of the two-component PhoPR system that regulates the expression of virulence genes of Mycobacteria. The goal of this work was to elucidate the role of PhoP in the mechanism that *Mycobacterium bovis*, the causative agent of bovine tuberculosis, displays upon stress. An analysis of gene expression and acidic growth curves indicated that *M. bovis* neutralized the external acidic environment by inducing and secreting ammonia. We found that PhoP is essential for ammonia production/secretion and its role in this process seems to be the induction of asparaginase and urease expression. We also demonstrated that the lack of PhoP negatively affected the synthesis of phthiocerol dimycocerosates. This finding is consistent with the role of the lipid anabolism in maintaining the redox environment upon stress in mycobacteria. Altogether the results of this study indicate that PhoP plays an important role in the response mechanisms to stress of *M. bovis*.

Key words: *Mycobacterium bovis*, PhoP, acidic stress, PDIM

1. Introduction

Mycobacterium bovis and *Mycobacterium tuberculosis* are the causative agents of bovine and human tuberculosis, respectively. Although human and animal tuberculosis

are clinically indistinguishable and *M. bovis* and *M. tuberculosis* are phylogenetically related, the genomes of both species differ in at least 1200 single-sequence polymorphisms and seven regions of difference (RD), which are absent from *M. bovis* [1]. Among these differences, *M. bovis* and *M. tuberculosis* present non-synonymous mutations in 20 transcriptional regulators or two-component systems [2]. These polymorphic proteins regulate key metabolic pathways and stress response mechanisms. One of these polymorphic regulators is PhoR, a histidine kinase that phosphorylates the transcriptional regulator PhoP. PhoPR is a key two-component system that regulates different aspects of mycobacterial metabolism and with a central role in the interaction of pathogenic mycobacteria and their host [3,4]. Although PhoR carries non-synonymous mutations affecting its functionality in *M. bovis* [5], this regulator is essential for the full virulence of this species in mice [4]. Furthermore, this two-component system of *M. bovis* shares some functions with its orthologous in *M. tuberculosis* [6]. In accordance with previous research from our laboratory and others [7,8], PhoP plays a key role in the response of pathogenic mycobacteria to acidic stress. The mechanism by which PhoP responds to this stress, however, is not completely understood.

In this study we demonstrated that PhoP modulates the expression of genes involved in ammonia production, which is necessary to neutralize acidic pH. In addition, we demonstrated that in the absence of PhoP *de novo* synthesis of PDIM is negatively affected in *M. bovis*.

2. Materials and methods

2.1 Bacterial strains, media and growth conditions

The *M. bovis* strains were grown at 37 ºC on Middlebrook 7H9 medium supplemented with 0.5% albumin (A), 0.4% dextrose (D), 0.5% pyruvate (P) and 0.05% Tween 80 (T80) or 0.5 g/L KH2PO4, 0.5 g/L MgSO4, 2 g/L citric acid, 0.5% pyruvate (minimal medium), 0.05% Tween 80 and 50 mM asparagine at pH 5.6-5.7 adjusted with NaOH. The mutant strain Mb04-303ΔphoP and complemented strain Mb04-303ΔphoP::phoPR were obtained as previously described [7].

2.2 Growth under acid stress, ammonium and pH measurement

The bacterial cultures were grown in 7H9 medium supplemented with ADP-T80. The bacterial pellets were washed twice with PBS and resuspended in 1 ml PBS. The bacteria were used at an optical density of 0.05 at 600 nm (OD600) in minimal medium with 50 mM asparagine at pH 5.6- 5.7 or pH 7. The bacteria were grown in agitation at 37ºC and 2 ml of culture were removed at different time points. One millilitre was used to determine OD and the remaining aliquot was centrifuged at 1300 rpm for 2 min to collect the culture supernatants. Ammonium concentration was determined by diluting supernatants four times in PBS and 50 µl of diluted samples were mixed in a 96 plate with 50 µl of Nessler's reagent (Sigma Aldrich) and incubated for 20 min at room temperature. The data of OD at 520 nm were determined in Multiskan Microplate Photometer (Thermo Fisher Scientific). Indicator strips (MERCK) were used to determine pH.

2.3 qRT-PCR

Total RNA was extracted from mycobacterial cultures grown until an OD600 of 0.5 in 7H9 medium with ADP-T80 at pH7 or pH 5.65 (for 3 or 24 h) as previously described [7]. The synthesis of cDNA was performed using 1 μg DNA-free RNA, 50 ng of random primers (Invitrogen, Thermo Fisher) and M-MLV RT (Promega). Each real-time qPCR reaction contains 25 ng of cDNA as the template, Taq platinum (Invitrogen, Thermo Fisher Scientific), SYBR green I dye (Invitrogen, Thermo Fisher Scientific) and gene-specific primers (Supplementary table 1). The reactions of qPCR were performed in duplicate of three biological replicates and run on an Applied Biosystem Step One Plus instrument. Gene expression was analysed with LinReg software for curve analysis [9]. Ratio calculation was performed with Fg statistical software [10]. Differences on gene expression between groups were analysed using *sigA* as the control gene.

2.4 Mycobacterial lipid extraction

The *M. bovis* strains were grown to an OD600 of 0.5 in 7H9 medium with ADP at pH 7 followed by addition 1% of ^{14}C acetate and incubation for 24 h under agitation at 37ºC. Then, the bacteria were pelleted, washed in PBS and the lipids were extracted once overnight in 1:2 (chloroform: methanol) followed by two overnight extractions in 2:1 (chloroform: methanol). Upon Folch wash, 14C incorporation was determined in the total extractable lipids by scintillation counting. Thin layer chromatography (TLC) plates were used to analyse lipid samples, by spotting 10000-15000 counts per minute (CPM) of each lipid sample. The petroleum ether: acetone system (98:2) was used to resolve PDIMs [8]. Labelled lipids were detected using Typhoon fluorescent scanner (GE Healthcare). Lipid extractions and TLCs were repeated in four independent biological replicates with similar findings. Unlabelled lipids were obtained following the same extraction protocol described above from cultures grown until stationary phase at neutral pH. Unlabelled lipids were analysed by LC/MS as previously described [11].

3. Results

3. 1 PhoPR regulates the expression of genes involved in ammonia production

One strategy by which *M. tuberculosis* neutralizes acidic environments is through the production of ammonia [12]. The studies of Gouzy et al. (2014) [13] have demonstrated that ammonia can be produced in *M. tuberculosis* from the hydrolysis of asparagine. The proteins involved in this mechanism are the asparaginase AnsA (Mb1565c) and its transporter AnsP2 (Mb0354c) [13]. In addition to asparagine, bacteria can employ urea as a source of ammonia [14]. The enzyme that generates ammonia and carbon dioxide from urea is the urease, an enzyme consisting of six subunits (UreA-B-C-D-F-G) in *M. tuberculosis* and *M. bovis*.

Since our previous results indicated that PhoP is essential for *M. bovis* to neutralize extracellular acidic pH [7], we investigated the role of PhoP in the expression of enzymes involved in asparagine (AnsA and AnsP2) and urea (UreC) degradation. A qRT-PCR analysis revealed that the *phoP* mutant (Mb04-303ΔphoP) displayed lower expression levels of *ansA*, *ansP2* and *ureC* at early exposure to low pH (Fig. 1A). We also observed lower expression of *ansA* and *ansP2* at neutral pH in the *phoP* mutant (Fig. 1A). In addition, while the expression of *ansP2* and *ureC* in the wild type strain significantly increased upon 3 h of acidic stress relative to neutral condition, there were no differences in the expression level of these genes in the mutant strain

between both culture conditions (Fig. 1B). The expression of *ansP* did not showed increase in acidic condition in both the mutant and wild type strains (Fig. 1B). These results demonstrate that PhoP positively modulates the expression of genes involved in two mechanisms of ammonia production in *M. bovis*. However, at 24 h of acidic stress, the expression of *ansA*, *ansP2* and *ureC* showed not statistical differences between the mutant and wild type strains (Supplementary fig. 1), indicating that at a longer low pH exposition time other mechanisms would compensate the absence of *phoP* in the mutant strain.

Because the expression of genes related to ammonia production was affected in the *phoP* mutant strain, we next determined ammonia production by the mutant strain under acidic condition of growth. According to a previous study [7], the *phoP* mutant strain grew normally at neutral pH and showed a retarded growth at low pH (Fig. 1C). Importantly, the accumulation of ammonia in the extracellular environment was significantly impaired in the *phoP* mutant strain compared to the wild type and complemented strains (Fig. 1D). This reduction in the ammonia content in the culture supernatant of the *phoP* mutant strain was consistent with a lack of pH neutralization indeed observed in the cultures of the wild strain and the complementary strain (Fig. 1E). As shown in Figure 1E, while cultures of the wild type and complemented strain neutralized the pH on the sixth day of culture, cultures of the mutant strain did not reach a neutral pH up to the seven day of culture. Importantly, all strains, including the *phoP* mutant, maintained their viability after exposition to low pH as demonstrated by plating in neutral solid medium and colony forming units (CFU) counts (data not shown).

These results indicate that PhoP is directly or indirectly involved in the secretion of ammonia for neutralizing the extracellular pH by *M. bovis*.

3. 2 PhoP negatively regulates the expression of whiB3 under acidic condition

In *M. tuberculosis*, WhiB3 regulates the lipid anabolism in response to redox stress [15]. The genes upregulated by WhiB3 under acidic pH overlaps with the genes regulated by PhoP, such as genes are related to SL-1 (*pks2*, *mmpL8*), diacyltrehaloses (DAT) and polyacyltrehaloses (PAT) (*pks3*), cysteine metabolism (*cysA*), among others [8,16]. To investigate a possible connection between PhoP and WhiB3 in the response of *M. bovis* to stress, we analysed the transcription of *whiB3* in neutral and acidic conditions in the *phoP* mutant and wild type *M. bovis* strains. The expression of *whiB3* increased in the *phoP* mutant relative to wild type upon 3 and 24 h of acidic stress (Fig. 2). Therefore, these results showed that at low pH, in the absence of PhoP the expression of *whiB3* was upregulated and this increased expression of *whiB3* was more pronounced in longer times of low pH exposition (Fig. 2).

3.3 PhoP is relevant for de novo *synthesis of PDIM in* M. bovis

Several publications reported the relevance of phthiocerol dimycocerosate (PDIM) in the stress response of mycobacteria [17–19]. In order to determine whether PDIM synthesis and the PhoPR regulatory network are connected, we evaluated the impact of *phoP* mutation in *de novo* synthesis of PDIM. Mycobacterial cultures were incubated for 24 h with ^{14}C to analyse lipid metabolisms through the identification of extractable lipids resolved in thin layer chromatography (TLC). Bands corresponding to PDIM-related lipids showed a decreased level in *phoP* mutant in comparison to the wild type

and complemented strains (Fig. 3A and B). This impaired synthesis of PDIM in *phoP* mutant was reproducible in three independent experiments and was also observed in an independent *M. bovis phoP* mutant (MbNCTC10772ΔphoP [7]) (Supplementary fig. 2). However, at low pH the results of ^{14}C lipid labelling and TLC were inconsistent between the biological replicates for all *M. bovis* strains (data not shown).

Importantly, the accumulation of PDIMs in the *phoP* mutant along 30 days of culturing was equivalent to that of the wild type and complemented strains, according to TLC (Fig. 3C) and LC-MS (data not shown) analyses.

Altogether these results suggest that although PhoP participates in the PDIM synthesis, other metabolic lipid pathways and regulatory mechanisms may compensate the lack of PhoP in *M. bovis*.

In addition, the expression level of *ppsA*, a gene involved in the synthesis of PDIMs [20,21], showed no statistical differences between the mutant and wild type strains (Fig. 3D). This finding indicates that PhoP plays a role in the synthesis of PDIMs by a mechanism that does not directly involve the upregulation of PDIM-related gene transcription.

4. Discussion

In this study we demonstrated that PhoP regulates genes involved in the neutralization of acidic environments in *M. bovis*. This finding is consistent with previous research indicating that the *phoP* gene is required for phagosome maturation arrest by *M. bovis* and *M. tuberculosis* in bovine and human macrophages, respectively [7,22]. Thus, one of the ways by which PhoP participates in the maturation arrest of phagosome containing *M. bovis* could be through the neutralization of acidic intraphagosomal pH. Indeed, the exclusion of proton ATPase in phagosome containing *M. tuberculosis* avoids the intraphagosomal acidification and blocks the phagosome maturation [23]. In addition, another way of inhibiting the phagosomal acidification is through the production and secretion of ammonia [24]. Moreover, *M. bovis*, as well as *M. tuberculosis*, responds to acidic stress by reprograming its metabolism from replicative to non-replicative states [25].

respond to acidic stress. Furthermore, Clemens et al., [14] reported that urease expression contributes to the inhibition of phagosome-lysosome fusion and phagosome acidification and that bacteria use the available nitrogen from the generated ammonia for the biosynthesis of biomolecules.

The lack of PhoP binding box upstream of *ansA*, *ansP2* and urease operon suggests that PhoP does not regulate the transcription of these genes by binding to their promoter regions.

M. tuberculosis H37Ra strain carries a mutation in *phoP* that produces an amino acid change. H37Ra does not express sulfolipids (SL), DAT and PAT or PDIM [26]. It has been reported that Here we provided new insights into the mechanism of response to acidic pH of *M. bovis*. In wild type *M. bovis* the expression of *ansP2* and *ureC* was upregulated after 3h of low pH treatment, indicating that at least these genes are involved in the early response to acidic stress. A previous study has shown that in response to acidic stress, *M. tuberculosis* employed AnsA and AnsP2 to produce and export ammonia, respectively, through asparagine degradation [13] and here we found that this role for AnsA is conserved in *M. bovis*. However, our findings

demonstrated that the *ureC* of urease operon also participates in acid stress response and that the expression of this gene together with that of *ansA* and *ansP2* is dependent on PhoP in the early stage of the stress response. Thus, urea degradation is an additional strategy of *M. bovis* to complementation of H37Ra with *phoP* from *M. tuberculosis* H37Rv restored *de novo* synthesis of SL-1, PAT and DAT but not PDIM [27]. This result could indicate that PhoP is not required for *de novo* PDIM synthesis. However, H37Ra carries numerous mutations in the genes encoding for the proteins involved in PDIM synthesis, thus, it is likely that the sole complementation with a functional copy of *phoP* was not enough to revert the PDIM synthesis defect of H37Ra.

As mentioned above, WhiB3 is a sensor and regulator protein involved in maintaining redox balance through the anabolism of complex lipids such as PDIMs and methyl-branched fatty acid [15]. The findings of our study indicate that PhoP represses the transcription of *whiB3* under acid stress, probably through the binding to PhoP box upstream of *whiB3* in *M. bovis*. This result together with the fact that PhoP is necessary for PDIM *de novo* synthesis, as we indeed observed in this work, is consistent with the results of Singh et al. (2009), who have shown that in the absence of WhiB3, de novo synthesis of PDIMs increased in *M. tuberculosis* [15]. This last study [15] also has suggested that the lack of PhoP does not affect the accumulation of PDIM (reported as unpublished results); which is also consistent with the results of this study. Therefore, the fact that the mutation in *phoP* affects the PDIM synthesis, although it has not significant impact in the PDIM accumulation throughout time, suggests compensatory mechanism operating in the absence of PhoP. In this regard, Lsr2 and EspR, two small nucleoid associated proteins (NAP) that modulate of the accessibility of free DNA to the transcription machinery at the promoter regions [28,29], regulate the PDIM biosynthesis [30,29]. Mycobacterial NAPs have been suggested to influence gene expression in relation to environmental stresses [29]. Likely, under stress condition, such as environmental low pH, there is a reprograming of the mycobacterial regulatory network to overcome the absence of PhoP. Therefore, the role of WhiB3 and PhoP and other regulatory proteins in PDIM metabolism is not completely understood and needs further research. Moreover, Feng et al have demonstrated that, contrary to our findings, PhoP positively regulates the *whiB3* expression in response to low pH in *Mycobacterium marinum* [31]. These contrasting results between PhoP-Whib3 cross talk in both mycobacterial species highlights the complexity of the stress response machinery in *Mycobacterium* genera.

5. Conclusions

In conclusion, we present data indicating that PhoP is relevant for *M. bovis* to produce molecules or compounds involved in the response mechanisms to stress.

9. Author contributions

EAG performed most of the experiments and wrote the manuscript, FCB collaborated with qRT-PCR analysis, LIK and AP collaborated with ammonia detection experiments, MJ and MMN collaborated with lipid analysis and corrected the manuscript, FB conceived the study and wrote the manuscript.

Conflict of interest

Authors have no conflict of interest.

6. Acknowledgements

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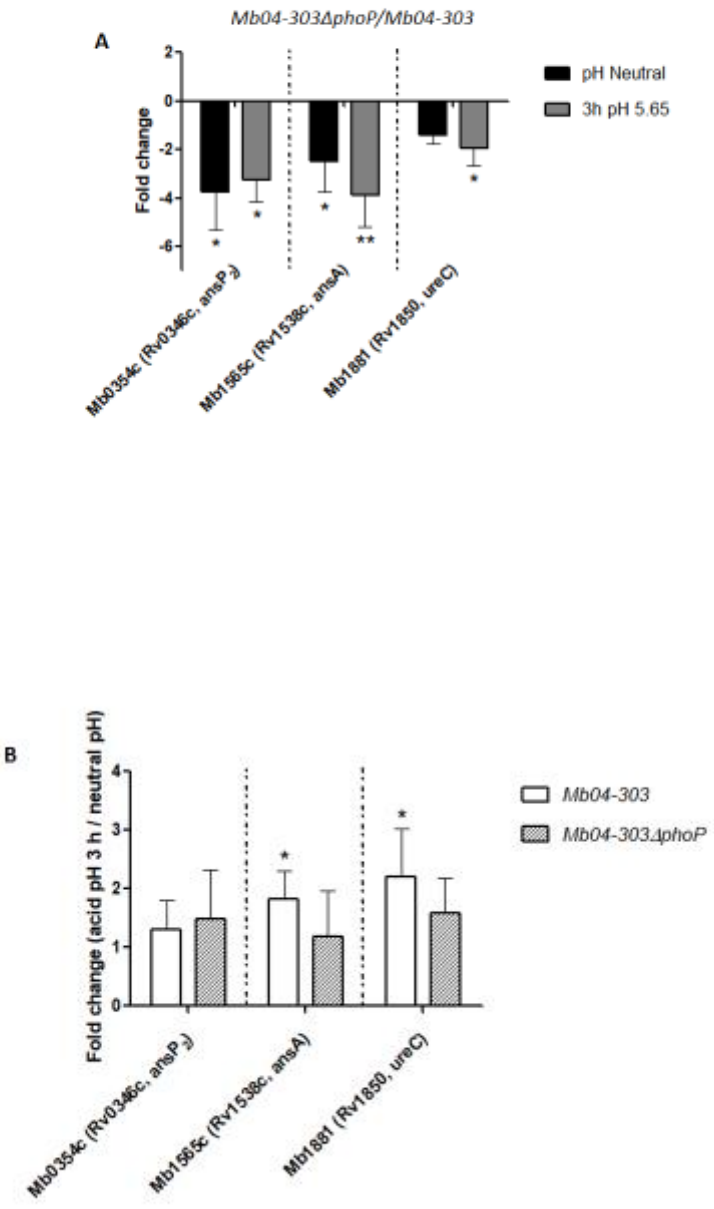
Figure 1



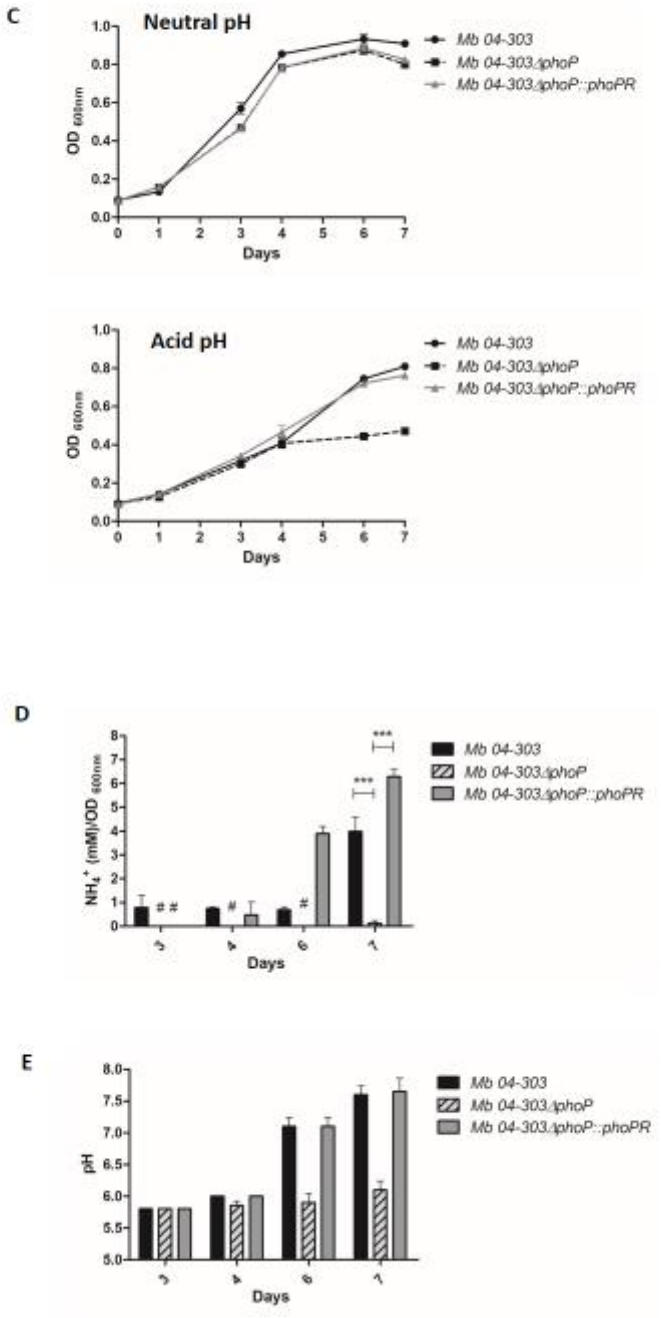


Figure 1: PhoP is required for *M. bovis* resistance mechanisms to acid stress.
A) Gene expression in Mb04-303ΔphoP (mutant) relative to Mb04-303 (wild type) after 3 h of acidic stress (pH 5.75) or in neutral condition (pH 7). The relative quantification of each gene was performed using the ΔΔCt method using *sigA* as the reference gene. The bars represent average expression ratios of triplicates ± SD. The asterisk indicates differentially expressed genes with significant differences among the studied conditioned (* $p<0.05$ **$p<0.01$). B) Relative expression of genes in acid pH (pH 5.75)/neutral pH (pH 7). The relative quantification of each gene was performed as described in (A). C) Bacterial growth of *M. bovis* strains in minimal medium supplemented with 50 mM asparagine at pH 5.6- 5.7 or pH 7. D, E) NH4+

concentrations relative to OD 600nm (D) and pH determinations (E) in *M. bovis* strain culture supernatants. *M. bovis* strains were grown in minimal medium with 50 mM asparagine. Data were analysed using one-way ANOVA analysis and Bonferroni's post-test (*** p< 0.001). Data are representative of at least two independent experiments. #, not detected.

Figure 2

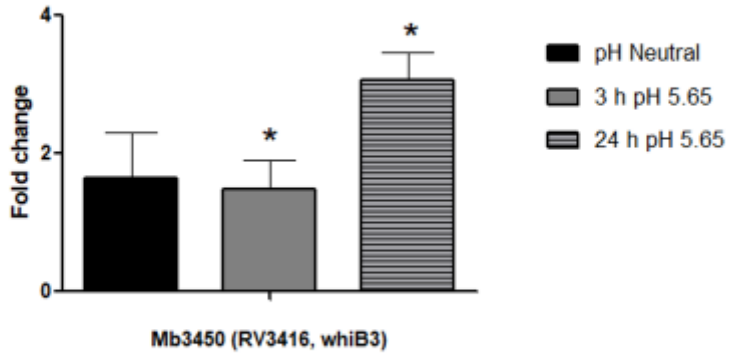


Figure 2. The expression of *whiB3* is increased in the absence of PhoP in *M. bovis*
Relative expression (Mb04-303ΔphoP/wild type) of genes related to ammonia production under acid stress (pH 5.75) or neutral condition. The relative quantification of each gene was performed using the ΔΔCt method using *sigA* as the reference gene. The bars represent average expression ratios of triplicates ± SD. The asterisk indicates differentially expressed genes with significant differences among the studied conditioned (* p<0.05).

Figure 3

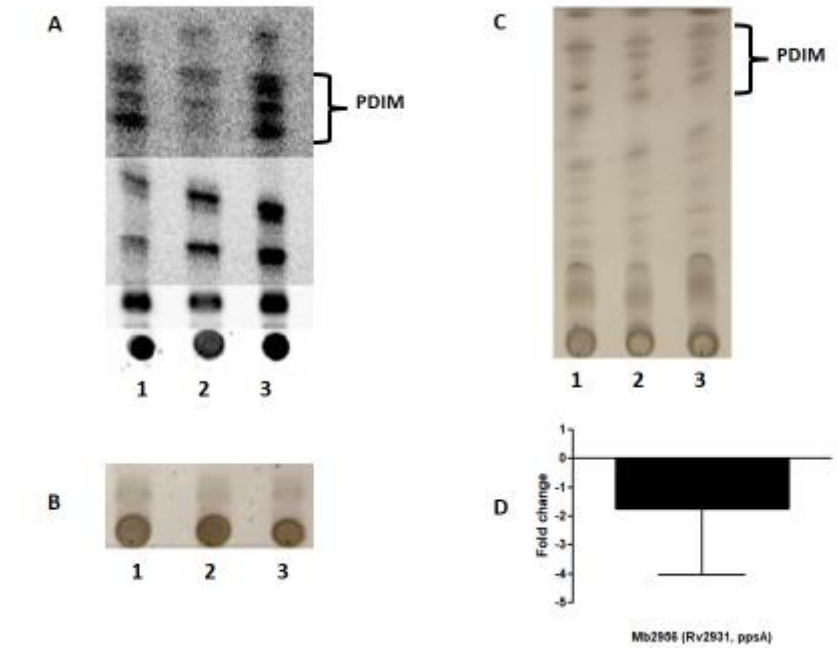


Figure 3. The lack of PhoP affects the synthesis of PDIMs in *M. bovis*
A) *De novo* synthesis of PDIMs in *M. bovis* strains. Total extractable lipids were labelled for 24 h with 14C acetate, purified as described in Material and Methods and resolved in TLC using petroleum ether: acetone system (98:2) as solvent system. 1: Mb04-303, 2: Mb04-303ΔphoP, 3: Mb04-303ΔphoP::phoPR. B) Total lipids of A revealed with copper sulphate. C) Accumulation of PDIMs in *M. bovis* strains in neutral pH condition. Total unlabelled extractable lipids were purified from cultures at stationary phase resolved in solvent system of A and revealed as in B. D) Relative expression (Mb04-303ΔphoP/wild type) of *ppsA*. The relative quantification of each gene was performed and analysed as described in Figure 1.