

Phosphorylation of PhoP Protein Plays Direct Regulatory Role in Lipid Biosynthesis of *Mycobacterium tuberculosis**[§]

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Background: PhoP is global regulator of *Mycobacterium tuberculosis* physiology. However, the role of phosphorylation of PhoP remains unknown.

Results: PhoP activates complex lipid biosynthesis only upon phosphorylation.

Conclusion: PhoP regulates lipid biosynthesis by a phosphorylation-dependent mechanism to contribute to morphology of the bacilli.

Significance: This study sheds light on the unexplored role of phosphorylation of PhoP in regulating biosynthesis of lipids unique to *M. tuberculosis*.

Mycobacterium tuberculosis PhoP is essential for virulence and intracellular growth of the tubercle bacilli. Genetic evidence suggests that PhoP regulates complex lipid biosynthesis, and absence of some of these lipid molecules in a *phoP* mutant partly accounts for its attenuated growth in macrophages and/or mice. To investigate the mechanism of regulation, here we demonstrate the essentiality of phosphorylation of PhoP in the regulation of complex lipid biosynthesis. We show that phosphorylated PhoP activates transcription of *pk2* and *msl3*, gene(s) encoding polyketide β -ketoacyl synthases through direct DNA binding at the upstream regulatory region(s) of the target genes. Our results identify the genetic determinants recognized by PhoP and show that activation of target genes requires interaction(s) of the phosphorylated regulator at the cognate binding sites. The fact that these sites within the regulatory region of respective genes do not bind *in vitro* with either unphosphorylated or phosphorylation-deficient PhoP protein is consistent with phosphorylation-dependent assembly of the transcription initiation complex leading to *in vivo* transcriptional activation. Together, these results reveal so far unknown molecular mechanisms of how PhoP contributes to *M. tuberculosis* cell wall composition by regulating complex lipid biosynthesis.

Despite the apparent importance of two-component signaling (TCS)⁵ systems at various stages of the *Mycobacterium tuberculosis* life cycle (1–3), the functioning of TCS proteins is

still poorly characterized. The PhoP-PhoR system has been implicated in the *M. tuberculosis* growth and survival in animal and cellular models because a *phoP* knock-out mutant of *M. tuberculosis* showed significant growth attenuation in both macrophages and in mice (4–7). Growing evidence indicates that PhoP regulates biosynthesis of sulfolipids (SL), diacyltrehaloses (DATs), and polyacyltrehaloses (PATs), and the absence of these complex lipid molecules in the *phoP* mutant is a major reason for the attenuated growth of the bacilli in a mouse model (5, 6, 8, 9). More recent studies suggest that PhoP, in addition to complex lipid biosynthesis, impacts numerous aspects of *M. tuberculosis* physiology, including early and enduring hypoxic responses (10), regulation of ESAT-6 secretion, specific T-cell recognition during virulence regulation of the bacilli (10–12), and pH sensing during intracellular adaptation (13). However, biochemical evidence showing the role of PhoP in transcription activation has been lacking.

PhoP typically consists of two domains: although the N-terminal receiver domain exhibits (α/β)₅ topology with a conserved phosphorylation site and geometry (14), the C-terminal effector domain comprises a winged helix-turn-helix DNA-binding motif (14, 15) and is involved in transcriptional regulation of genes necessary to respond to environmental stimuli. Although *M. tuberculosis* PhoP regulates ~114 genes acting both as a transcriptional activator and repressor (5), the role of phosphorylation in transcription regulation by PhoP remains unknown. In agreement with what was evident from the structural data of *M. tuberculosis* PrrA (16), the closest homologue of *M. tuberculosis* PhoP, we recently reported inter-domain interactions in PhoP leading to phosphorylation-dependent high affinity DNA binding of the regulator (17). However, molecular mechanism of how PhoP functions as a transcriptional regulator of its target genes remains undefined.

M. tuberculosis PhoP has been shown to be a regulator of the synthesis of three classes of polyketide-derived acyltrehaloses known as SL, DAT, and PAT (5, 6). Recent studies establish that a single nucleotide polymorphism within the DNA binding effector domain of PhoP in the avirulent *M. tuberculosis* H37Ra accounts for part of its attenuation (18) and absence of polyketide-derived acyltrehaloses compared with the virulent

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⁵ The abbreviations used are: TCS, two-component system; ATc, anhydrotetracycline; DAT, diacyltrehalose; PAT, polyacyltrehalose; SL, sulfolipid; AcP, acetyl phosphate.

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TABLE 1

Bacterial strains and plasmids used in this work

	Description	Source or Ref.
Strains		
<i>E. coli</i> DH5 α		Novagen
<i>E. coli</i> BL21(DE3)		56
<i>M. tuberculosis</i> H37Ra		ATCC25177
<i>M. tuberculosis</i> H37Rv		ATCC27294
<i>M. smegmatis</i> mc ² 155		57
Plasmids		
pET- <i>phoP</i>	PhoP residues 1–247 cloned in pET15b ^a	34
pET- <i>phoPD71N</i>	Asp ⁷¹ codon mutated to Asn in pET- <i>phoP</i>	17
pME1mL1	Mycobacterial protein expression vector ^b	27
pME1mL1- <i>phoP</i>	PhoP residues 1–247 cloned in pME1mL1	This work
pME1mL1- <i>phoPD71N</i>	Asp ⁷¹ mutated to Asn in pME1mL1-PhoP	This work
pSM128	Integrative promoter probe vector for mycobacteria ^c	26
pSM-pks2up1	pks2up1- <i>lacZ</i> fusion in pSM128	This work
pSM-pks2up1sDR2	Mutant pks2up1- <i>lacZ</i> fusion carrying changes in DR2 site	This work
pSM-msl3up1	msl3up1- <i>lacZ</i> fusion in pSM128	This work
pSM-msl3up1sDR2	Mutant msl3up1- <i>lacZ</i> fusion carrying changes in DR2 site	This work
pJEM15	Promoter-less <i>E. coli</i> -mycobacteria shuttle vector ^d	25
pJEM- <i>phoP</i>	Entire regulatory region along with <i>phoP</i> encoding gene (residues 1–247) cloned in pJEM15	This work
pJEM- <i>phoPD71N</i>	Asp ⁷¹ codon of <i>phoP</i> mutated to Asn in pJEM- <i>phoP</i>	This work

^a Amp^r indicates ampicillin resistance.

^b Hyg^r indicates hygromycin resistance.

^c Str^r indicates streptomycin resistance.

^d Kan^r indicates kanamycin resistance.

parent strain *M. tuberculosis* H37Rv (19). Together, these studies suggest that PhoP likely regulates expression of acyl-transferase, polyketide synthase (*pks*), or *pks*-associated genes involved in the synthesis or transfer of methyl branched fatty acyl substituents found in SL, DAT, and PAT (5, 6). Furthermore, genetic studies on complementation of 1237 Δ *phoPR*::hyg (a 774-bp fragment of *M. tuberculosis* genome encompassing part of the *phoP* and *phoR* coding sequences was replaced with a hygromycin resistance cassette; see Ref. 5) with *phoP* alone could restore lipid biosynthesis (5) suggesting that phosphorylation may not be essential for regulation of target genes as has been shown by PhoQ-independent activation of target genes of *Salmonella enterica* PhoP (20). However, there is evidence that response regulators often get phosphorylated by noncognate kinases belonging to different TCSs (for a review see Ref. 21). In contrast, Walters *et al.* (5) had clearly shown that the intracellular growth phenotype of *phoP* mutant in macrophages was complemented only in the presence of both *phoP* and *phoR* genes, and the *phoP* gene alone was unable to complement intracellular growth. Although recent studies strongly suggest a link between altered lipid compositions of the *phoP* mutant strain to its interaction with macrophages (22), the mechanism of how gene(s) involved in lipid biosynthesis are regulated by the important DNA binding transcription factor remains unknown.

Based on these studies, we predicted that PhoP functions as a regulator of genes involved in complex lipid biosynthesis. In this report, we demonstrate that only phosphorylated PhoP directly stimulates transcription of *pks2* and *msl3* by high affinity DNA binding to the upstream regulatory region of the target genes. Results reported here identify the genetic determinants recognized by PhoP in the effector regions of *pks2* and *msl3*, and evidence is provided that the regulator-promoter interaction(s) at the newly identified PhoP-binding sites lead to activation of the genes. The results have implications for the mechanism of PhoP-mediated regulation of lipid biosynthesis, which

is of critical importance to cell wall composition and morphology of the tubercle bacilli.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Media, and Growth Conditions—Growth of *Mycobacterium smegmatis*, *M. tuberculosis* H37Ra, and *M. tuberculosis* H37Rv and transformation by electroporation of test plasmids were as described previously (23). All *M. tuberculosis*-related work was carried out in a Biosafety Level 3 (BSL3) laboratory. Growth of *Escherichia coli* strains, cloning, and propagation of plasmids and overexpression of recombinant forms of PhoP proteins were as described (17). For mycobacterial strains, the antibiotics kanamycin and hygromycin were used at concentrations of 25 and 50 μ g/ml, respectively; streptomycin was used at 20 μ g/ml. For *E. coli*, kanamycin, hygromycin, and streptomycin were used at 50, 250, and 100 μ g/ml, respectively.

General DNA Techniques—Cloning, restriction digestion using endonucleases, and DNA manipulations were carried out by standard techniques (24). Plasmid pJEM-*phoP* and pJEM-*phoPD71N* were used to complement H37Ra with PhoP and phosphorylation-deficient PhoP-D71N proteins of *M. tuberculosis* H37Rv, respectively. In these plasmids, the *phoP* genes were expressed under the control of its own promoter. To this effect, FPphoPup and RPphoP (supplemental Table S1) amplified the entire regulatory region of *phoP* and the complete *phoP* coding sequence of H37Rv (from –350, relative to the translation start site of *phoP* to the end of the *phoP* open reading frame). The PCR-amplified region was cloned between BamHI and KpnI sites of the promoter-less pJEM15 (25) to construct pJEM-*phoP* (Table 1).

Transcriptional fusions to *lacZ* were obtained by cloning PCR-amplified fragments of the *M. tuberculosis* *pks2* (*pks2up1*) and *msl3* (*msl3up1*) regulatory regions into the *ScaI* site of pSM128 (a promoter-less integrative *lacZ* reporter vector (26) with a streptomycin resistance gene) using primer pairs

FPpks2up1/RPpks2up1 and FPmsl3up1/RPmsl3up1, respectively (supplemental Table S1). pks2up1 and msl3up1 DNA fragments comprised upstream of the coding region included first 40 and 60 coding bases of the *pks2* and *msl3* genes, respectively. pSM128 was a kind gift by Dr. Issar Smith, Public Health Research Institute, University of Medicine and Dentistry of New Jersey. To examine the importance of PhoP-binding direct-repeat motif(s) within the *pks2* and *msl3* promoters, sequence variants that were altered in the downstream repeat unit were generated using the two-stage overlap extension method by interchanging all the As with Cs and all the Gs with Ts, and vice versa, keeping the other repeat unit unaltered (see supplemental Table S1 for mutagenic primers). All of the *lacZ* transcriptional fusion constructs were verified by restriction analysis and DNA sequencing.

To express *M. tuberculosis* PhoP in *M. smegmatis* under the control of TetR, *lacZ* of pME1mL1 (27) was replaced with *phoP* open reading frame. To this effect, the *phoP* gene was amplified from the genomic DNA using primers that introduced an NdeI site (phoPstart) at the start codon and a PstI site (MphoPstop) 3' of the stop codon (supplemental Table S1). Prior to ligation of the *phoP* gene in pME1mL1, the 920-bp *tetR* gene with flanking NotI sites was excised out of the plasmid pME1mL1 by NotI restriction digestion as it included an NdeI site. Subsequently, the NdeI/PstI double-digested backbone fragment of pME1mL1 without the *tetR* gene was gel-purified and ligated to *M. tuberculosis phoP* using NdeI and PstI sites. Finally, *tetR* open reading frame was inserted back using NotI restriction sites to construct pME1mL1-*phoP*.

Promoter fragments of varying size were PCR-amplified from the full-length promoter templates as indicated under "Results" (see Fig. 1), purified by gel extraction (Qiagen), and end-labeled by T4 polynucleotide kinase (New England Biolabs) and [γ - 32 P]ATP (BRIT, India). Unincorporated free nucleotides were separated from labeled DNA fragments using a Sephadex G-50 quick spin column (GE Healthcare). When appropriate, PCR-amplified DNA probes were end-labeled as indicated above and digested with BamHI or KpnI to remove the radiolabel at one end. Finally, the end-labeled fragments were extracted with phenol/chloroform/isoamyl alcohol (25:24:1 (v/v/v)) and ethanol-precipitated.

Analysis of Complex Lipids of *M. tuberculosis* Strains—Extraction of lipids from *M. tuberculosis* strains, fractionation, and analysis were performed as described (28). The different organic phases were combined, washed extensively, and dried. The crude lipid extracts were subsequently analyzed by thin layer chromatography using chloroform/methanol/water (60:16:2, v/v/v) as described (5). Glycolipids were detected by spraying α -naphthol (in sulfuric acid), followed by baking. The identity of lipid moieties was confirmed by comparing previously reported relative migration of lipids under identical solvent conditions (5, 19). To detect and probe PATs in *M. tuberculosis* H37Ra, H37Rv, and *phoP*-complemented strains of H37Ra, total lipids were analyzed by two-dimensional TLC as described (19).

RNA Preparation and Primer Extension—Total RNA from *M. tuberculosis* grown in 7H9 was isolated and purified with some modifications of a previously reported protocol (29) as

described elsewhere (30). Briefly, 25 ml of culture of each bacterial strain was grown in 7H9 media to mid-log phase (an optical density at 600 nm of \approx 0.6). Cells were transferred to 30-ml conical tubes, pelleted down by centrifugation, and lysed by resuspending the cells in acetate/EDTA buffer containing glass beads and water-saturated phenol (Ambion). Following incubation at 65 °C for 30 min with severe vortexing after each 10 min, total RNA was chloroform-extracted and ethanol-precipitated. Resulting RNA was incubated with RNase-free DNase I (Promega) for 2 h at 37 °C to remove contaminating genomic DNA and purified by RNeasy column (Qiagen). The integrity of the RNA samples was checked by formaldehyde-agarose gel electrophoresis.

For primer extension, complementary strand oligonucleotides pks2PEx and msl3PEx (supplemental Table S1) that anneal 60 nucleotides (nt) downstream of *pks2* and 120 nucleotides downstream of *msl3*, respectively, with respect to the corresponding translational start sites were end-labeled with [γ - 32 P]ATP as described above. The labeled primers were annealed to 1 μ g of RNA and then extended with 1 unit of Moloney murine leukemia virus reverse transcriptase (Ambion) at 50 °C for 1 h in accordance with the manufacturer's recommendation. After heat inactivation at 92 °C for 10 min, primer extension products were extracted with phenol/chloroform/isoamyl alcohol (25:24:1 (v/v/v)). The nucleic acid was precipitated with 95% chilled ethanol, and the dried pellet was resuspended in formamide loading buffer. To determine the size of the extended product, both pks2up1 and msl3up1 DNA templates were sequenced using the respective primers (as used in the reverse transcription reactions, see supplemental Table S1) to generate a sequencing ladder by the dideoxy chain termination method using SequiTherm Excell II DNA sequencing kit (Epicenter). Primer extension products were loaded onto an 8% polyacrylamide sequencing gel along with the sequencing ladder and run for 2–3 h at 1800 V. After electrophoresis, the gel was dried and exposed to a phosphorimager screen overnight.

Real Time Quantitative PCR—Specific primer sets for target genes were synthesized by Sigma and are listed in supplemental Table S1. Both cDNA synthesis and PCRs were performed by using 0.2 μ g of DNase-treated RNA in a reaction buffer containing 2 units of Superscript III Platinum-SYBR Green one-step quantitative RT-PCR kit (Invitrogen) with gene-specific primer pairs (final concentration of 0.2 μ M) according to the manufacturer's recommendation. Control reaction mixtures containing identical reactions with 2 units of Platinum TaqDNA polymerase (Invitrogen) instead of reverse transcriptase were also carried out to confirm the absence of genomic DNA in all of the samples. All PCRs were performed using the iCycler real time PCR detection system (Bio-Rad), and melting curve analysis was carried out to confirm amplification of a single product. To compensate for variations in transcript numbers, *pks2* and *msl3* expression level between strains were normalized to that of *sigA* (31), expression of which is known to be constitutive in *M. tuberculosis* (32).

Promoter Regulation by *M. tuberculosis* PhoP in *M. smegmatis*—To express PhoP in *M. smegmatis*, electrocompetent cells were transformed with pME1mL1-*phoP* expressing

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wild type PhoP with the $P_{myc1tetO}$ promoter under the control of TetR repressor. Cultures of *M. smegmatis* strains harboring different *lacZ* fusions and transformed with PhoP expression construct (or no expression plasmid as control) were grown to an absorbance at 600 nm ≈ 1.5 as described earlier (27). Cultures were inoculated in fresh medium (1:100) either in the absence or in presence of 50 ng/ml of anhydrotetracycline (ATc) and allowed to grow. Aliquots were withdrawn from this culture at indicated time points and centrifuged, and cell pellets were washed with phosphate-buffered saline. Cells were resuspended in 0.5 ml of phosphate buffer (pH 7.2) containing 75 mM NaCl, and cell suspensions were sonicated. β -Galactosidase activity of the cell extracts were determined by using chromogenic substrate 2-nitrophenyl- β -D-galactoside at a final concentration of 1 mg/ml. The reaction mixtures were incubated at 37 °C for 10 min, and the reactions were terminated by adding 0.2 M Na_2CO_3 . The absorbance at 420 nm (A_{420}) values of the supernatant was determined, and β -galactosidase activity was calculated in Miller units as described (33).

To examine TetR-controlled PhoP expression, crude lysates from *M. smegmatis* ($\approx 5 \mu\text{g}$ of protein) expressing PhoP were resolved by 12% SDS-PAGE and transferred to PVDF membrane for Western blot analysis. The blots were probed with anti-PhoP primary and HRP-conjugated anti-rabbit IgG secondary antibodies (Abxome Biosciences, India) and developed with Luminata Forte Chemiluminescence reagent (Millipore).

Gel Mobility Shift and DNase I Protection Assays—DNA binding by PhoP proteins, expressed and purified as described earlier (17, 34), were evaluated by gel shift assays as described (17, 34). For DNase I footprint mapping, binding reaction mixtures contained ~ 50 fmol of ^{32}P -labeled PCR-amplified DNA fragment and the indicated amounts of purified PhoP in 50 μl of reaction buffer as used in gel shift assays. Reaction mixtures were incubated for 20 min at 15 °C. Following incubation, 50 μl of a solution containing 5 mM CaCl_2 and 10 mM MgCl_2 was added to the reaction mixtures at 15 °C, and DNase I digestion was carried out with 0.75 units of DNase I for 1 min. The digestion reactions were terminated by addition of 100 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS) and extracted with phenol/chloroform/isoamyl alcohol (25:24:1 (v/v/v)). The nucleic acids were ethanol-precipitated with 95% chilled ethanol, and the dried pellets were resuspended in 5 μl of formamide loading buffer (1% bromophenol blue, 1% xylene cyanol, 10 mM EDTA in 98% formamide). After electrophoresis on an 8% denaturing polyacrylamide sequencing gel, the gel was dried, and the footprinting reactions were analyzed by autoradiography. To identify the protected sequence on each strand, footprinting reactions were analyzed alongside a DNA sequencing ladder generated by SequiTherm Excell II DNA sequencing kit (Epicenter) on both ^{32}P -labeled DNA fragments. Primers used to generate DNA fragments are described in [supplemental Table S1](#).

RESULTS

Phosphorylation of PhoP Regulates Cell Wall Composition by Controlling Lipid Biosynthesis in *M. tuberculosis*—Studies aimed at investigating phenotypic differences between *M. tuberculosis* H37Ra and the pathogenic H37Rv showed striking

morphological differences displaying smaller colony size and lesser wrinkling on the colony surface for H37Ra compared with H37Rv. Later, these observations were extended to show that a similar and distinct difference exists between H37Rv and a *phoP* knock-out mutant of H37Rv when cultured on 7H10 Middlebrook agar plates (4, 18, 35). More interestingly, two independent studies show that much of the difference was significantly reduced when PhoP from H37Rv was expressed in H37Ra (18, 19). We extended these results to gain insight into the mechanism of how PhoP contributes to *M. tuberculosis* morphology. In agreement with previous studies, morphological differences in H37Ra and H37Rv disappeared significantly when H37Ra was transformed with pJEM15 (25) expressing PhoP of H37Rv (pJEM-phoP) from its endogenous promoter (see under “Experimental Procedures” for details) (Fig. 1A). In striking contrast, H37Ra-expressing phosphorylation-deficient PhoP-D71N of H37Rv failed to show morphological properties like H37Rv. It should be noted that the *M. tuberculosis* PhoP and PhoR proteins form a functional TCS, which in their simplest form utilize a histidine-aspartate phosphorelay between two modular proteins (for reviews, see Refs 36, 37), and the primary site of covalent phosphorylation of PhoP has been mapped to Asp⁷¹ (38, 39). Substitution of Asp⁷¹ to Asn⁷¹ alters the side chain functional group from carboxylic acid to carboxamide moiety, thus rendering PhoP phosphorylation-deficient (39). As expected, H37Ra transformed with the empty vector pJEM15 displayed H37Ra-like morphology. Together, these observations clearly suggest that expression of PhoP and its phosphorylation is necessary and sufficient to restore H37Rv-like morphology of H37Ra.

The fact that a single nucleotide polymorphism of PhoP in the avirulent H37Ra explains the absence of polyketide-derived acyltrehaloses compared with the parent strain H37Rv (19) prompted us to investigate and compare glycolipid moieties of H37Ra and H37Rv. As expected, H37Rv showed the presence of SL1, DAT, and PAT (as indicated on the figure), which were completely absent in H37Ra (compare *lane 1* with *lane 4*, Fig. 1B). However, in agreement with a previous report (19), H37Ra-expressing PhoP of H37Rv showed the presence of SL1, DAT, and PAT in the thin layer chromatography experiments (*lane 2*, Fig. 1B). Strikingly, expression of PhoP-D71N was unable to restore SL1, DAT, and PAT biosynthesis in H37Ra (Fig. 1B, compare *lane 2* and *lane 3*). The presence of PATs in H37Rv PhoP-complemented strain of H37Ra, but not that of PhoP-D71N, was further confirmed by analysis of total lipids using two-dimensional thin layer chromatography (Fig. 1C). Thus, we conclude that PhoP regulates complex lipid biosynthesis of *M. tuberculosis*, and phosphorylation of PhoP appears to be essential for its regulatory role in lipid biosynthesis.

PhoP Regulates Expression of *pks2* and *msl3* in Vivo in a Phosphorylation-dependent Manner—*pks2* and *msl3* gene(s) of *M. tuberculosis* encode enzymes that have been implicated in the synthesis of multiple methyl branched fatty acids essential for biosynthesis of cell wall lipids, like SL (40), DAT, and PAT (41). Furthermore, genetic data coupled with biochemical evidence show essential regulatory role of PhoP in the biosynthesis of SL, DAT, and PAT (5, 6). To this end, we examined relative expression of *pks2* and *msl3* *in vivo* by real time reverse transcription-

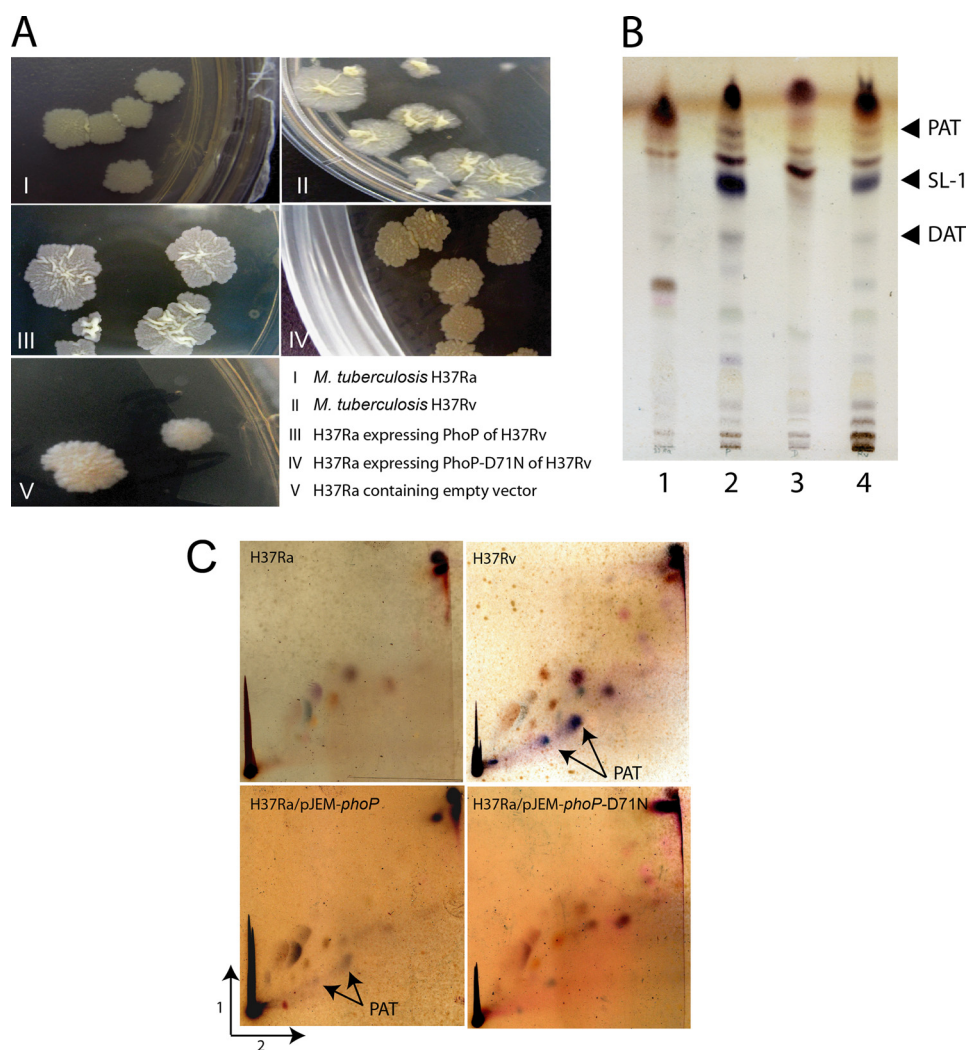


FIGURE 1. Role of *M. tuberculosis* PhoP in cell wall composition and complex lipid biosynthesis. *A*, complementation of *M. tuberculosis* H37Ra with the *phoP* gene of H37Rv reveals colony morphology similar to H37Rv. However, the *phoPD71N* gene of H37Rv fails to complement H37Ra. As a control, H37Ra carrying the empty vector (pJEM15) displays H37Ra-like colony morphology. *B*, TLC of indicated polar lipids of *M. tuberculosis* H37Ra (lane 1), H37Ra expressing PhoP or PhoP-D71N from H37Rv (lanes 2 and 3, respectively), and the *M. tuberculosis* H37Rv (lane 4). *C*, two-dimensional TLC of polar lipids of *M. tuberculosis* H37Ra, H37Ra expressing PhoP from H37Rv, or PhoP-D71N from H37Rv and the *M. tuberculosis* H37Rv. Note that glycolipids of *M. tuberculosis* strains displayed purple color on α -naphthol staining. Cell growth, lipid extraction, and analyses of lipid moieties were as described under "Experimental Procedures."

PCR from the *M. tuberculosis* H37Rv and H37Ra. Interestingly, H37Rv showed a significant up-regulation of *pks2* and *msh3* expression of 25.9 ± 5.9 - and 45 ± 4.9 -fold, respectively, relative to H37Ra cultures (Fig. 2). To study if PhoP from H37Rv contributes to the variation of expression, *pks2* and *msh3* expression was quantified in the H37Ra complemented with wild type H37Rv *phoP* allele (pJEM-*phoP*). Strikingly, both *pks2* and *msh3* expressions could be restored to wild-type levels when H37Ra expressed *phoP* from H37Rv. In fact, consistent with enhanced production of PATs, DATs, and SLs (Fig. 1B), H37Ra expressing PhoP from H37Rv displayed a higher level of *pks2* expression (≈ 1.7 -fold) and comparable *msh3* expression (≈ 0.9 -fold) with respect to wild-type H37Rv. Together, these *in vivo* results are in agreement with previous microarray data suggesting effect of PhoP on regulation of *pks2* and *msh3* expression (5). In sharp contrast, H37Ra transformed with pJEM-*phoPD71N* plasmid was completely ineffective in complementing *pks2* and *msh3* expression. From these results we conclude that phosphor-

ylation of PhoP is essential for regulation of expression of both *pks2* and *msh3* *in vivo*.

To investigate if PhoP functions as a direct regulator of *M. tuberculosis* *pks2* and *msh3* genes *in vivo*, we next constructed transcriptional fusions to *lacZ* by cloning PCR-amplified fragments of the *pks2* and *msh3* regulatory regions at the *ScaI* site of pSM128, an integrative promoter probe vector for mycobacteria (26). To this end, PCR-amplified DNA fragments, *pks2*up1 and *msh3*up1, comprising -206 to $+40$ and -350 to $+60$, respectively, with respect to their corresponding translational start sites were considered as regulatory region(s) of *pks2* and *msh3* genes. To express PhoP in *M. smegmatis*, strains harboring the transcriptional fusions were transformed with pME1mL1-*phoP*, an inducible expression system (27) expressing wild-type PhoP from the P_{myc1} promoter under the control of TetR repressor. Transformed cells were then grown in 7H9 medium containing appropriate antibiotics in the absence or presence of 50 ng/ml ATc as inducer of PhoP

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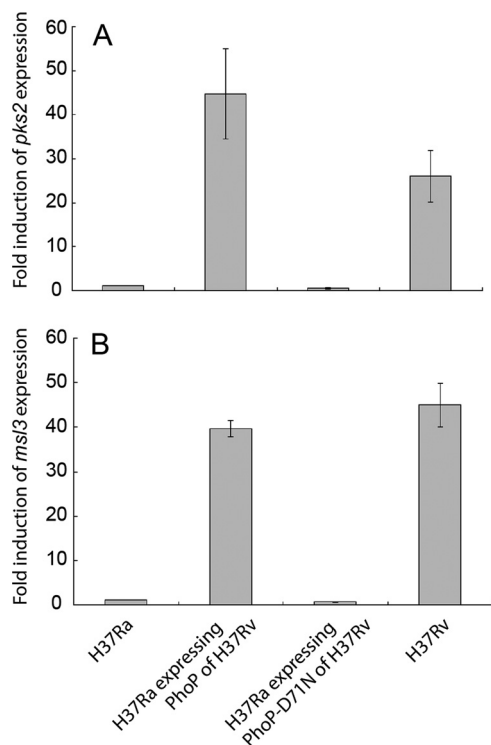


FIGURE 2. PhoP regulates *pks2* (A) and *msl3* (B) expression in vivo in *M. tuberculosis* by a phosphorylation-dependent mechanism. H37Ra, H37Ra-expressing wild type *phoP* allele from H37Rv, H37Ra carrying the mutant *phoP-D71N* allele, and wild-type H37Rv were grown to mid-log phase. Following cell growth, total RNA was extracted from bacterial cells for quantitative real time reverse transcription PCR (see “Experimental Procedures” for details). The values shown here represent the indicated fold induction of expression of *pks2* and *msl3* with respect to their expression levels in H37Ra and are derived from at least three independent experiments using at least two different RNA preparations.

expression. Strikingly, the *pks2up1-lacZ* fusion was significantly activated with induction of PhoP at indicated time points, as the β -galactosidase levels obtained in the presence of ATc was ≈ 3 -fold higher than that obtained in absence of ATc (Fig. 3A). Similarly, cells carrying *msl3up1-lacZ* fusion showed an ≈ 2 -fold enhanced β -galactosidase activity when PhoP expression was induced compared with the uninduced sample (Fig. 3B). However, both reporter constructs, under identical experimental conditions, failed to show promoter activation when expression of phosphorylation-deficient PhoP-D71N was induced (1.04 ± 0.2 - and 0.98 ± 0.2 -fold change in β -galactosidase activity, Fig. 3, A and B, respectively). *Insets* in Fig. 3, A and B, show comparable expression of *M. tuberculosis* PhoP and its mutant in *M. smegmatis* in the presence of ATc, thus ruling out the possibility that altered expression of the mutant could account for the failure to activate transcription. From these results we conclude that phosphorylation of PhoP alone is essential to activate expression of *pks2* and *msl3* in vivo.

PhoP Recognizes Regulatory Regions of *pks2* and *msl3*—To precisely locate and identify regulatory regions of *pks2* and *msl3*, the origin(s) of transcription of *pks2* and *msl3* were determined by primer extension using total RNA from *M. tuberculosis* H37Rv. A 100-bp extension product was obtained, which localized the *pks2* transcription start site to a guanine 40 nucleotides upstream of the ATG start site (Fig. 4A). However, a primer extension experiment generated a 127-bp extension

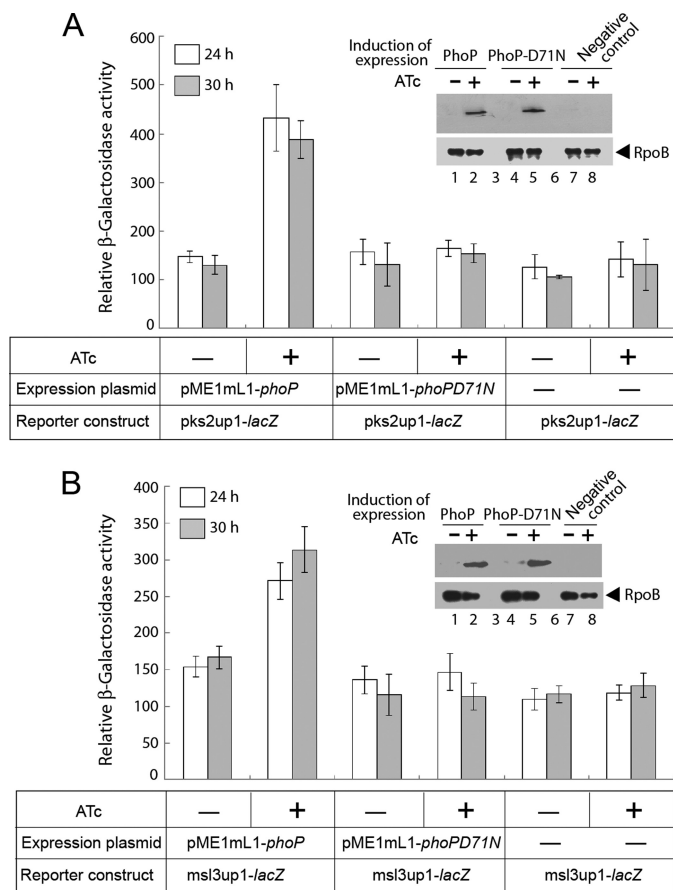


FIGURE 3. PhoP regulates expression of *pks2* (A) and *msl3* (B) in vivo in *M. smegmatis*. *M. smegmatis* strains harboring indicated constructs along with wild-type and mutant PhoP expression plasmids were grown in the absence or presence of ATc, and β -galactosidase activity from the transcription fusion was measured at indicated times. The values shown here are averages of at least three independent experiments. *Insets* compare expression of PhoP in crude extracts (24-h time point) containing equal amounts of total protein (as determined by Bradford assay) by Western blot using anti-PhoP antibody (Abxome Biosciences). As a loading control, the same crude extracts were probed with antibody against the β -subunit of RNA polymerase (Abcam).

product that localized the *msl3* transcription start site to the first guanine of the GTG start site (Fig. 4B).

To study whether PhoP acts as a regulator by direct binding to the regulatory region(s) of target genes, we next examined the ability of PhoP to recognize *pks2up1* by EMSA. Although purified PhoP was unable to generate a complex stable to gel electrophoresis, PhoP preincubated in a phosphorylation mixture containing acetyl phosphate (AcP) as the phospho-donor displayed efficient DNA binding with end-labeled *pks2up1* by forming a complex of reduced electrophoretic mobility (compare lanes 3–5 with lanes 6–8, Fig. 4C). Previously, we provided a direct demonstration of phosphorylation of PhoP at Asp⁷¹ using AcP as the phospho-donor (39). A quantitative analysis suggests at least 20-fold stimulation of DNA binding by phospho-PhoP with the *pks2up1* compared with the unphosphorylated protein (based on the limits of detection in this assay). As expected, the PhoP-D71N mutant (with impaired phosphorylation) preincubated in the phosphorylation mixture with or without AcP, failed to generate a PhoP-DNA complex with identical DNA substrate (lanes 10–15, Fig. 4C). Thus, in conjunction with previous results (17) and the results shown here,

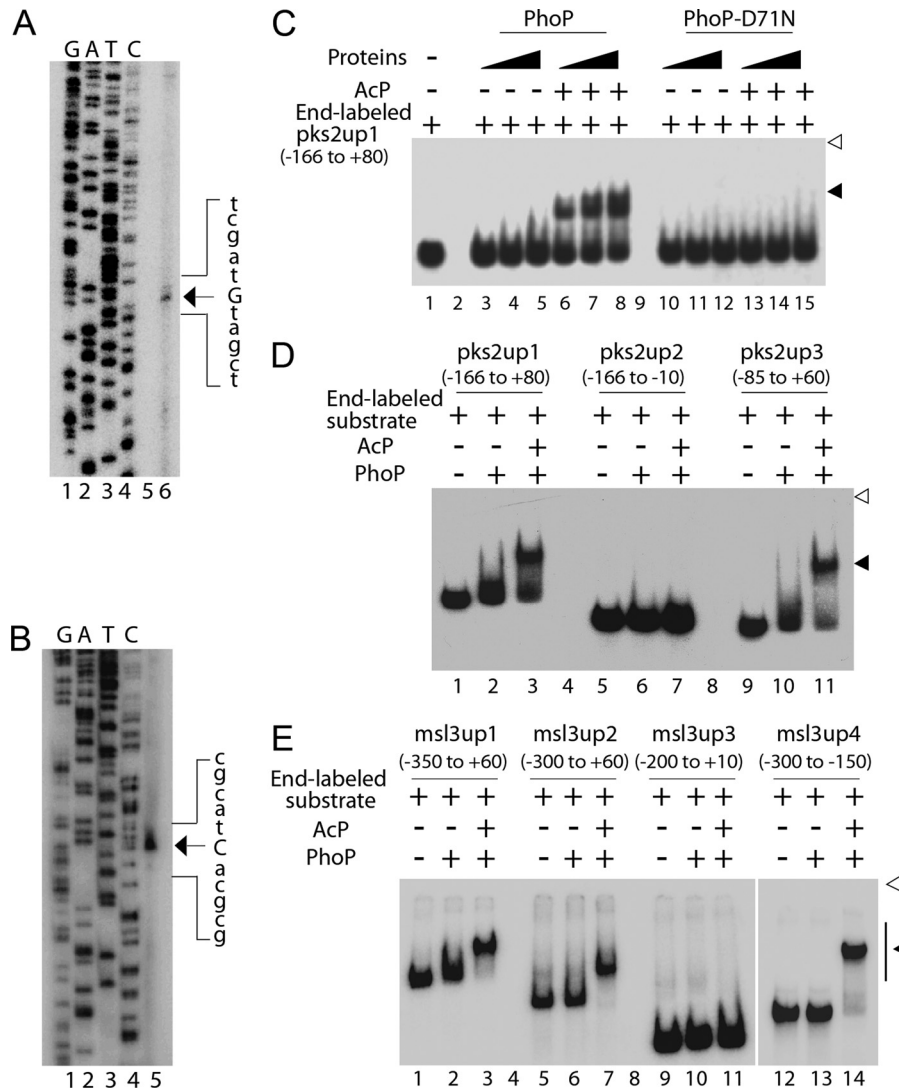


FIGURE 4. Mapping transcription start sites (A and B) and core PhoP-binding sites (C–E) within the regulatory regions of *pks2* and *msl3*. To determine transcription start sites, total RNA isolated from *M. tuberculosis* H37Rv was used in primer extension experiments using antisense oligonucleotide primers (see supplemental Table S1) located within *pks2* (A) and *msl3* (B) genes as described under “Experimental Procedures.” Sequencing samples as markers were prepared with the same labeled primer as used for the reverse transcription and resolved in lanes 1–4, adjacent to the primer extension product (lane 6 for A and lane 5 for B). The unique band identified in each primer extension reaction is indicated on the figure. C, EMSA of radiolabeled pks2up1 for binding of increasing concentrations of PhoP (lanes 3–8) or phosphorylation-deficient PhoP-D71N (lanes 10–15) preincubated in phosphorylation mixture with or without AcP, respectively. Lane 1 shows the free probe. Note that binding mixtures in lanes 3–5, 6–8, 10–12, and 13–15 contained the indicated proteins at 0.1, 0.2 and 0.3 μM . To probe the core-binding site of PhoP within the regulatory region of *pks2* (D) and *msl3* (E), PhoP binding to indicated DNA fragments was carried out with 200 and 50 nM of PhoP, respectively, preincubated in phosphorylation mixture with or without AcP. The position of the radioactive material was determined by exposure to a phosphor storage screen, and bands were quantified in the phosphorimager (Fuji). Open and filled arrowheads indicate origins of the polyacrylamide gel and slower moving complexes with band shifts produced in presence of PhoP, respectively.

we conclude that specific interaction(s) between PhoP and the *msl3* and/or *pks2* regulatory region is dependent on PhoP phosphorylation.

Using a number of PCR-amplified overlapping fragments, we next addressed a more precise characterization of the *pks2* and *msl3* regulatory regions important for PhoP binding. Fig. 4D shows that in contrast to pks2up1 (–166 to +80 with respect to the transcription origin) as positive control, pks2up2 (–166 to –10) was not shifted upon incubation with phospho-PhoP (compare lanes 1–3 and lanes 5–7). However, pks2up3 (–85 to +60) generated a stable slower moving complex with phospho-PhoP (Fig. 4D, lanes 9–11), indicating that there was no high

affinity PhoP binding sequence upstream of –85 nucleotide of the *pks2* regulatory region.

However, whereas msl3up1 (–350 to +60 with respect to the transcription origin) and msl3up2 (–300 to +60) fragments were shifted by phospho-PhoP (lanes 1–3 and lanes 5–7, respectively; Fig. 4E), msl3up3 (–200 to +10) was not shifted upon incubation with phospho-PhoP (lanes 9–11). In contrast, incubation of phospho-PhoP with an ≈ 150 -bp msl3up4 DNA fragment (–300 to –150) induced complete band shift of the DNA fragment (lanes 12–14). Thus, phosphorylated PhoP is apparently binding to the *msl3* regulatory region spanning –300 to –150 relative to the transcription

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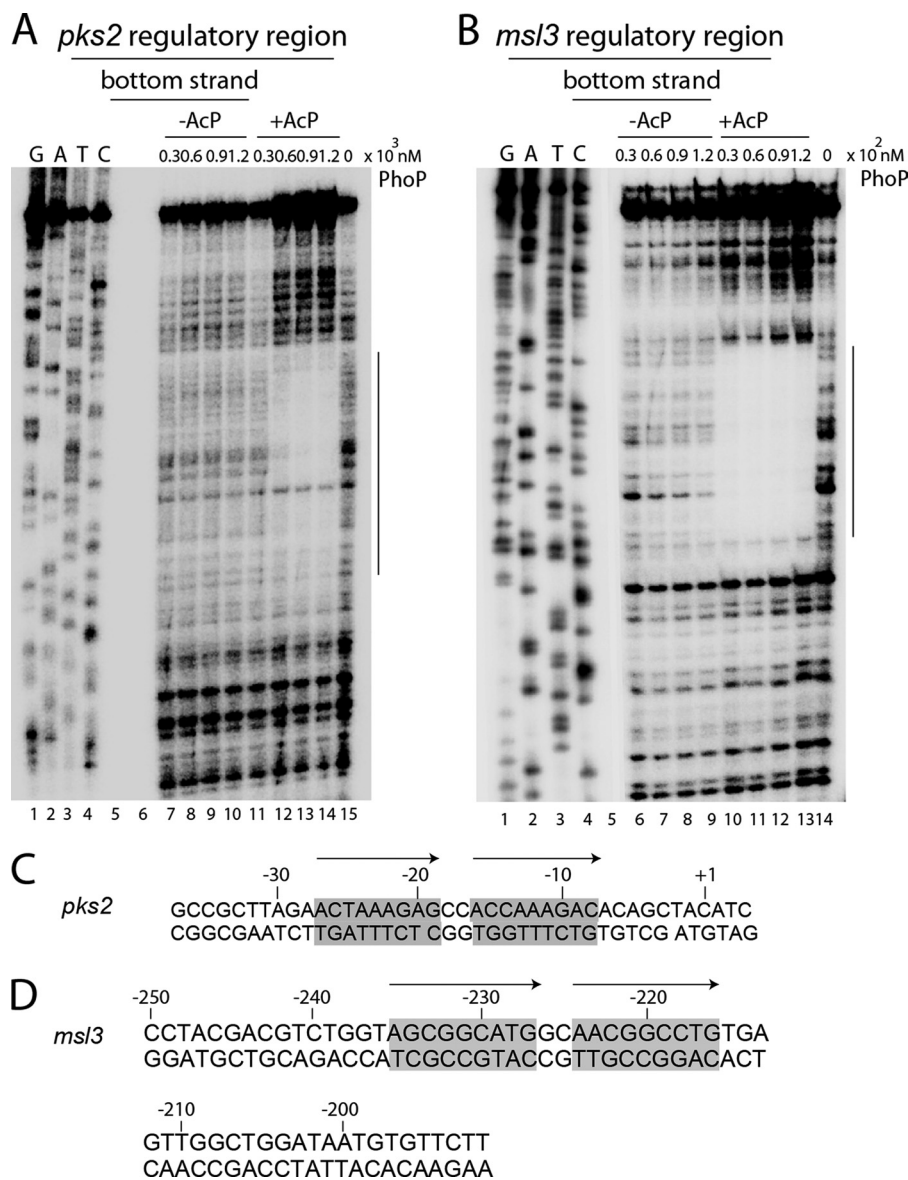


FIGURE 5. DNase I protection mapping of PhoP binding to *pks2* and *msl3* regulatory region(s). ≈ 50 fmol of *pks2*up3 (A) and *msl3*up4 (B) fragments each carrying the label at the bottom strands were incubated with increasing concentrations of PhoP preincubated in phosphorylation mixture in the absence (lanes 7–10 for *pks2*up3; lanes 6–9 for *msl3*up4) or presence of AcP (lanes 11–14 for *pks2*up3; lanes 10–13 for *msl3*up4) and in the absence of PhoP protein (lane 15 for *pks2*up3; lane 14 for *msl3*up4) prior to digestion with DNase I as described under “Experimental Procedures.” G, A, T, and C designate the DNA sequencing ladder generated for each strand. The protected regions on each of the bottom strands are indicated by vertical lines. Nucleotide sequences within the DNase I-protected region(s) of *pks2*up3 (C) and *msl3*up4 (D) (numbered with respect to the transcription origins) include a conserved 9-bp direct repeat motif shaded in gray and indicated with arrows.

start site. Together, these EMSA experiments suggest that PhoP binding is likely to be sequence-specific because purified PhoP was able to recognize the specific regulatory region of *pks2* and *msl3* and not any sequence stretch within the regulatory regions.

PhoP Protects Nucleotide Sequences Containing a Direct Repeat Motif Upstream of *pks2* and *msl3* Genes—To determine whether a specific nucleotide sequence motif within ≈ 145 -bp *pks2*up3 and ≈ 150 -bp *msl3*up4 is recognized by PhoP, the precise area of DNA bound by the protein was determined using DNase I footprint mapping. The purified DNA substrates carrying radioactive label either at the top or the bottom strands were incubated with PhoP, and DNase I was added to partially digest the DNA. The resulting fragments were analyzed on a

denaturing sequencing gel. Fig. 5A shows that phospho-PhoP, in a concentration-dependent manner, protected a stretch of ≈ 41 -bp of the bottom strand of *pks2*up3, beginning 78 nucleotides upstream of the ATG start site. An almost identical pattern of protection was observed with phospho-PhoP when the top strand-labeled substrate was used during DNase I footprinting (supplemental Fig. S1A). Similarly, a stretch of ≈ 60 bp from both the bottom and top strands of *msl3*up4 was protected by PhoP, beginning 250 nucleotides upstream of the GTG start site (Fig. 5B and supplemental Fig. S1B, respectively). In agreement with the EMSA data (Fig. 4), clearly protection of the nucleotide sequence from both DNA substrates was significantly influenced by phosphorylation of PhoP. It is noteworthy that we consistently observed

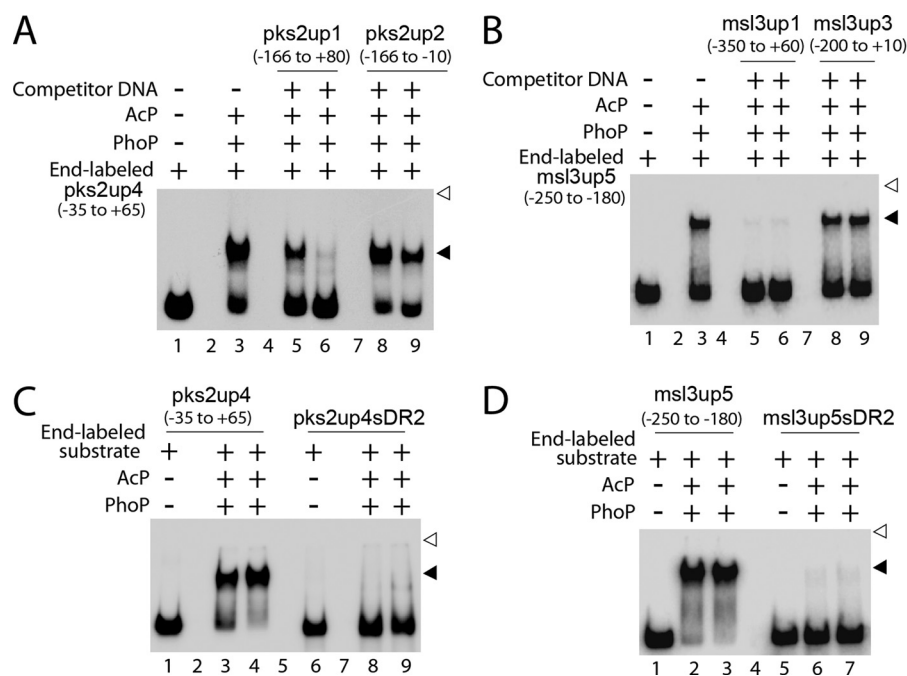


FIGURE 6. EMSA experiments examined sequence-specific binding of phospho-PhoP to *pks2up4* (A) and *msl3up5* (B) in the absence (*lane 3*) or presence of 12.5- and 25-fold excess of specific (*lanes 5 and 6*) and nonspecific (*lanes 8 and 9*) competitors at 100 and 25 nM of PhoP, for A and B, respectively. *Lane 1* shows the free probe. Additional EMSA experiments examined indicated end-labeled probes *pks2up4* and *pks2up4sDR2* (C) and *msl3up5* and *msl3up5sDR2* (D) for binding of phospho-PhoP at 0.1 and 0.2 μ M PhoP (*lanes 3 and 4*, and *lanes 8 and 9* (C)) and 50 and 100 nM of PhoP (*lanes 2 and 3*, and *lanes 6 and 7* (D)), respectively. *Lanes 1 and 6* of C and *lanes 1 and 5* of D show indicated free probes used. Open and filled arrowheads indicate origins of the polyacrylamide gel and slower moving complexes with band shifts produced in the presence of PhoP, respectively. Note that nucleotides outside the direct repeats, in both cases, included identical extensions of natural sequence at both the 5'- and 3'-ends. The gels are representative of at least three independent experiments.

an ≈ 10 -fold difference in protein concentration to achieve comparable protection to DNase I cleavage at *msl3* and *pks2* regulatory regions. This is in agreement with the EMSA experiments suggesting that PhoP shares ~ 5 –10-fold higher binding affinity with *msl3* regulatory region.

When we examined each protected region more closely, strikingly a conserved 9-bp direct repeat motif was apparent within each PhoP-protected sequence. For example, the PhoP-protected *pks2* promoter region contained a direct repeat motif with two 9-bp repeat units (comprising nucleotides -28 to -20 and -17 to -9 relative to the transcription start site) separated by an intervening spacer sequence of two nucleotides (Fig. 5C). Similarly a 9-bp direct repeat motif (comprising nucleotides -235 to -227 and -224 to -216 relative to the transcription start site) separated by a spacer of two nucleotides (Fig. 5D) was identified within the PhoP-protected sequence of the *msl3* regulatory region. From these results, we surmise that PhoP directly binds to regulatory regions of *pks2* and *msl3* containing a 9-bp direct repeat motif. It should be noted that although the two repeat units consisting of a direct repeat motif, both within the *pks2* and *msl3* regulatory regions, share a significant similarity in nucleotide sequence (7 of 9 nucleotides are identical in both cases), there exists striking differences in the nucleotide sequence of the repeat units present within the regulatory regions of two genes (8 of 9 nucleotides are different).

Transcriptional Activation of *pks2* and *msl3* Involves Recognition of the Direct Repeat Motif by *M. tuberculosis* PhoP—To investigate whether the 9-bp direct repeat motif upstream of both *pks2* and *msl3* alone was critical for DNA binding, PhoP binding to the 95-bp *pks2up4* (-35 to $+65$ relative to the *pks2*

transcription start site) and the 70-bp *msl3up5* (-250 to -180 relative to the *msl3* transcription start site) DNA fragments containing the putative direct repeat motif(s) was investigated by EMSA in the absence (*lane 3*, Fig. 6A) or presence of either a 12.5- and 25-fold excess of specific (*lanes 5 and 6*) or nonspecific competitor (*lanes 8 and 9*). Although unlabeled *pks2up1* at a 25-fold molar excess efficiently competed PhoP binding (only $5 \pm 0.5\%$ of binding; Fig. 6A, *lane 6*), identical fold excess of *pks2up2* as nonspecific competitor DNA resulted in a minor variation of DNA binding efficiency ($57 \pm 0.8\%$ binding; *lane 9*) compared with no competitor control ($68 \pm 1\%$ binding; *lane 3*), suggesting that PhoP recruitment at *pks2up4* is sequence-specific. Similarly, whereas unlabeled *msl3up1* at a 12.5-fold molar excess efficiently competed PhoP binding to *msl3up5* ($<2\%$ of binding; *lane 5*, Fig. 6B), even 25-fold excess of *msl3up3* as nonspecific competitor DNA resulted in an insignificant variation of $12(\pm 2)\%$ DNA binding efficiency compared with no competitor control (compare *lane 9* to *lane 3*, Fig. 6B), suggesting that PhoP recruitment at *msl3up5* is sequence-specific.

We next examined PhoP binding to variants of *pks2up4* and *msl3up5*, which were altered in the downstream repeat sequence of the direct repeat motif (*pks2up4sDR2* and *msl3up5sDR2*, respectively) as described under “Experimental Procedures.” Although *pks2up4* was shifted completely by phospho-PhoP, *pks2up4sDR2* probe was completely ineffective for PhoP binding (<2 and $<5\%$ of DNA binding at 0.1 and 0.2 μ M PhoP, respectively; compare *lanes 3 and 4* to *lanes 8 and 9*, Fig. 6C). Similarly, compared with *msl3up5*, *msl3up5sDR2* probe was significantly ineffective for PhoP binding (undetected

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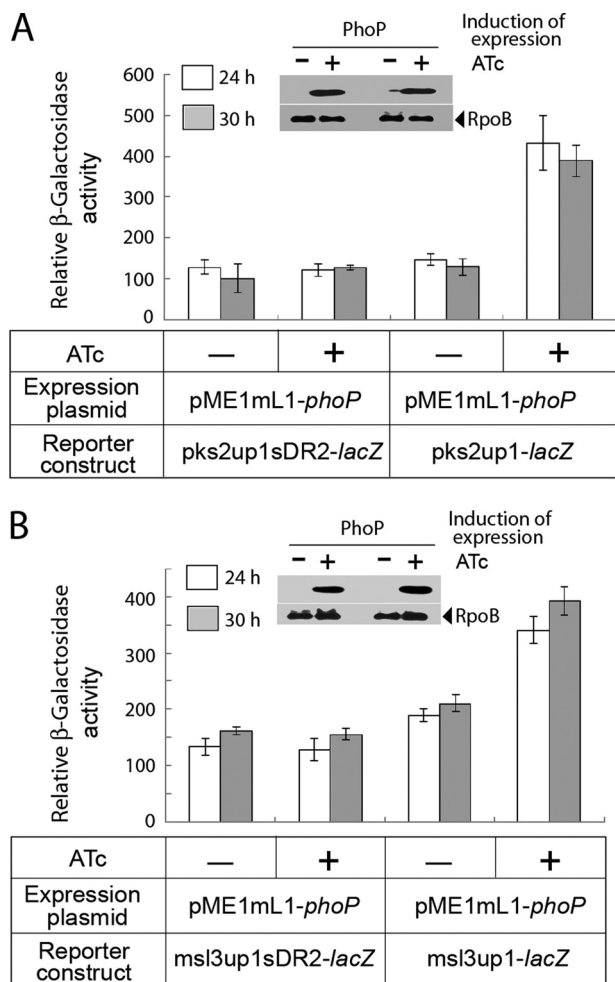


FIGURE 7. PhoP regulates expression of *pks2* and *msl3* by specific recognition of direct repeat motif. To examine the importance of predicted PhoP-binding sites in PhoP-dependent transcription regulation of *pks2* and *msl3*, *M. smegmatis* strains harboring *pks2up1sDR2* (A) and *msl3up1sDR2* (B) (see “Results” for details), as well as the corresponding wild-type regulatory regions, were grown in the absence or presence of inducing PhoP expression as indicated on the figure and described in the legends to Fig. 3. β -Galactosidase activity from the transcription fusion was measured at indicated times. Insets compare expression of PhoP in crude extracts (24-h time point) containing equal amounts of total protein by Western blot using anti-PhoP antibody (Abxome Biosciences). As loading control, crude extracts were probed with antibody against β -subunit of RNA polymerase (Abcam).

able binding at both 0.05 and 0.1 μ M PhoP (based on the limits of detection in this assay); compare lanes 2 and 3 to lanes 6 and 7, Fig. 6D). Thus, specific interaction(s) of PhoP at the regulatory region(s) of *pks2* and *msl3* is exclusively dependent on the presence of the direct repeat motif(s).

To investigate the role of the newly identified PhoP-binding site in PhoP-coupled transcription activation of *pks2* and *msl3*, changes were introduced in the nucleotide sequence of the downstream repeat unit of *pks2up1* and *msl3up1* template DNA as described under “Experimental Procedures.” The DNA fragments carrying changes in the PhoP-binding site (*pks2up1sDR2* and *msl3up1sDR2*, respectively) were subsequently cloned in pSM128 and used as transcription fusions to examine the effect of PhoP on the regulation of expression (Fig. 7). Interestingly, with induction of PhoP expression by ATc, there was no significant change in the level of β -galactosidase

activity from the promoters carrying changes at PhoP-binding sites. Multiple replicates of experiments confirmed that the difference of β -galactosidase activity in the presence and absence of induction of PhoP expression was 0.94(\pm 0.05)-fold (compare *empty columns*) and 1.07(\pm 0.03)-fold (compare *filled columns*) from the *pks2up1sDR2-lacZ* at 24 and 30 h, respectively (Fig. 7A). Likewise, we obtained 0.95(\pm 0.01)-fold (compare *empty columns*) and 0.96(\pm 0.03)-fold (compare *filled columns*) difference of β -galactosidase activity at 24 and 30 h, respectively, from the *msl3up1sDR2-lacZ* construct in presence and absence of induction of PhoP expression (Fig. 7B). These results are in striking contrast with PhoP-dependent 2.9(\pm 0.2)- and 1.9(\pm 0.3)-fold activation of wild-type *pks2up1* and *msl3up1* expression, respectively, under identical conditions examined (Fig. 7, A and B, respectively). From these results, we conclude that phosphorylated PhoP binding at the newly identified repeat motif within the regulatory regions of *pks2* and *msl3* is necessary and sufficient for transcription activation of these genes. The fact that PhoP-binding site(s) within the regulatory regions of *pks2* and *msl3* are located at distinctly different distances from the corresponding transcription start sites possibly indicates mechanistic variation of PhoP-mediated activation in the presence of the RNA polymerase.

Notably, within the *msl3* regulatory region, the PhoP-binding site is located very far from the promoter (Fig. 5D), suggesting that any regulatory role of PhoP on transcription would require constraints on DNA topology.

DISCUSSION

Interesting similarities in growth attenuation and morphological and cytochemical properties between H37Ra and *phoP* mutant of *M. tuberculosis* H37Rv have been proposed to be a direct consequence of the absence of three kinds of complex lipids, SL, DAT, and PAT (5, 6, 18). It is noteworthy that these lipids are relatively restricted to the virulent strains of the *M. tuberculosis* complex, suggesting their role in pathogenicity of the tubercle bacilli (42, 43). These reports coupled with recent studies show that among the reasons for the attenuation of *M. tuberculosis* the H37Ra strain is a single nucleotide polymorphism within the *phoP* gene that (a) is incapable of restoring polyketide-derived acyltrehalose synthesis in a *phoP-phoR* knock-out mutant of H37Rv (19) and (b) inhibits secretion of proteins that are important for virulence (18). Although these results establish a strong link between PhoP with lipid biosynthesis and virulence regulation, studies using a *pks2-3/4* knock-out mutant of *M. tuberculosis* H37Rv clearly show that not polyketide-derived acyltrehaloses but rather phthiocerol dimycocerosates, which are not regulated by PhoP, are the major contributor of virulence of the tubercle bacillus (19, 43, 44). However, it should be noted that proteins encoded by genes of the *pks2* cluster (Rv3825c-3824c-3823c) and *msl3* cluster (Rv1180-Rv1182-Rv1183) (supplemental Fig. S2) are shown to be regulated by PhoP (5), and some of these proteins critically contribute to regulation of *M. tuberculosis* virulence (45, 46). In agreement with these studies, more recently acyltrehaloses have been shown to influence phagosome maturation in macrophages (47).

Given the link that exists between *M. tuberculosis* PhoP and complex lipid biosynthesis on the one hand (5, 6) and between the presence of polyketide-derived lipids and maintenance of cell morphology on the other hand (18, 19), we sought to investigate the mechanism of how PhoP regulates expression of genes involved in lipid biosynthesis. To circumvent the problem of PhoP being phosphorylated *in vivo* by a noncognate sensor kinase or by some other mechanisms, we utilized the phosphorylation-deficient PhoP-D71N mutant in both *in vivo* and *in vitro* experiments. Our results show that PhoP in the phosphorylated form directly regulates expression of *pks2* and *msh3*, products of which are essential components of *M. tuberculosis* cell wall (40, 41). What offers a new mechanistic insight is the finding that phosphorylation at Asp⁷¹ of PhoP is critically important for the control mechanism of complex lipid biosynthesis, which in turn regulates cell morphology. This is clearly shown by our results on cell morphology of *M. tuberculosis* H37Ra, H37Rv, and H37Ra complemented with H37Rv *phoP* along with analyses of lipid profile (Fig. 1) and concomitant restoration of expression of *pks2* and *msh3* (Fig. 2). Thus, PhoP contributes to the regulation of cell morphology of the tubercle bacilli most likely through its regulatory influence on complex lipid biosynthesis and facilitates an integrated view of our results. However, additional experiments are needed to examine and understand the signal sensed by the cognate kinase PhoR (38), which in turn regulates phosphorylation of PhoP. The fact that H37Ra carries an identical copy of *phoR* as H37Rv appears to suggest that PhoR, under the conditions examined, triggers phosphorylation of PhoP. However, PhoP-dependent up-regulation of *pks2* and *msh3* in *M. smegmatis* in the absence of PhoR is most likely suggestive of PhoP phosphorylation by cross-talk with other two-component systems (21). It is tempting to speculate that a relatively lower level of activation of *pks2* and *msh3* (Fig. 3) by PhoP is possibly due to the low level of phosphorylation of PhoP in the absence of cognate kinase.

Phosphorylation of response regulators plays an important role to activate or repress gene expression *in vivo* (48). Results reported here validate and expand upon our previous work on *msh3* promoter recognition (17) to show that phospho-PhoP, through sequence-specific recognition of the regulatory regions, directly regulates transcription of *pks2* and *msh3*, the two genes of related metabolic function involving complex lipid biosynthesis. In agreement with data on *in vivo* regulation (Figs. 1–3), phosphorylation of PhoP showed a striking impact on *in vitro* DNA binding, a result that was further confirmed by DNase I footprinting analyses. Interestingly, these results are consistent with the crystal structure of *M. tuberculosis* PrrA (Protein Data Bank code 1YS6 (16)), which shows that the recognition helix is involved in interacting with the regulatory domain and thereby on phosphorylation, significantly enhancing binding affinity of the protein to the regulatory region of the *prxA-prrB* operon (49). Although DNase I footprint mapping clearly identified genetic determinants recognized by phospho-PhoP (Fig. 5), additional EMSA experiments coupled with *in vivo* reporter assays using transcription fusion of wild type and mutant promoters (carrying mutations at the PhoP binding sites; Fig. 7) to *lacZ* clearly establishes the following: (i) the identified direct repeat motif alone is likely responsible for

PhoP-DNA interaction(s) at the relevant regulatory regions, and (ii) recruitment of phospho-PhoP at these sites is essential for PhoP-dependent regulation of *pks2* and *msh3*. The structural data derived from the co-crystals of protein-DNA complexes of *E. coli* PhoB-DNA (50) and *Bacillus subtilis* Spo0A-DNA (51) also exhibit tandem binding of the protein on adjacent repeat motifs where a single protomer recognizes each repeat sequence. Thus, recruitment of PhoP protomers on two adjacently arranged repeat sites consisting of a direct repeat motif provides an interesting example of how similar sequence modules with variations of nucleotide sequence enable functional diversification by the same family of response regulator.

Although expression of PhoP is known to be critical for *M. tuberculosis* virulence (4–8), the genetic determinant(s) in the downstream effector regions that are recognized by PhoP remain largely unknown. PhoP belongs to the OmpR/PhoB subfamily, members of which often recognize repeat DNA sequences. *M. tuberculosis* DosR, a regulator of genes involved in response to hypoxia and NO exposure had been shown to recognize a 20-bp palindromic sequence that is present upstream of almost all genes regulated by it (1, 52). In contrast, *M. tuberculosis* MprA recognizes a direct repeat motif comprising two tandemly arranged 8-bp repeat units, a sequence present upstream of *mprA* and *pepD* genes (53). Here, we show recruitment of phosphorylated PhoP to a 20-bp stretch of *pks2* and *msh3* regulatory region(s) comprising a 9-bp direct-repeat motif separated by a two-nucleotide spacer. Together, these results suggest that differences in nucleotide sequences, orientation of repeat units, and/or intervening spacer lengths at the recognition sites of different promoters most likely regulate transcription factors with largely conserved structures to control diverse biological responses. Interestingly, our results on newly identified PhoP-binding sites are in broad agreement with the presence of three 9-bp repeat units within the PhoP-protected region of the *phoP* promoter, including a 23-bp sequence containing a 9-bp direct repeat motif (38). However, the nucleotide sequences of the repeat sites identified here (within the regulatory regions of *pks2* and *msh3*; Fig. 5, C and D), consistent with their variable affinity of interaction with PhoP (Fig. 5, A and B), are significantly different from each other and also from what we had identified previously within the *phoP* promoter itself. Thus, these results suggest that differences in nucleotide sequences, in addition to displaying differential affinity for the same transcription factor (PhoP), contribute to regulatory mechanisms of variable physiological functions.

In conclusion, our results show that direct interactions between the phosphorylated PhoP and the newly identified PhoP-binding site(s) are essential for activation of the genes, a result confirmed by reporter assays using transcriptional fusion of promoters to *lacZ*. More importantly, we demonstrate a critical role of phosphorylation of a single residue of PhoP contributing to regulation of cell morphology most likely by influencing lipid biosynthesis, a novel result of unusual significance with implications on the molecular mechanism of action of the key regulator. It should be noted that earlier studies link PhoP-PhoR of *Streptomyces lividans* and *Streptomyces coelicolor* to phosphate control of lipid biosynthesis (54, 55). Together, these results provide an interesting example of how similar mecha-

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nisms in actinomycetes exist and operate to regulate biosynthesis of polyketides or polyketide-derived products of diverse structure and function.

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REFERENCES

- Sherman, D. R., Voskuil, M., Schnappinger, D., Liao, R., Harrell, M. I., and Schoolnik, G. K. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98**, 7534–7539
- Zahrt, T. C., and Deretic, V. (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98**, 12706–12711
- Betts, J. C., Lukey, P. T., Robb, L. C., McAdam, R. A., and Duncan, K. (2002) *Mol. Microbiol.* **43**, 717–731
- Pérez, E., Samper, S., Bordas, Y., Guillhot, C., Gicquel, B., and Martín, C. (2001) *Mol. Microbiol.* **41**, 179–187
- Walters, S. B., Dubnau, E., Kolesnikova, I., Laval, F., Daffe, M., and Smith, I. (2006) *Mol. Microbiol.* **60**, 312–330
- Gonzalo Asensio, J., Maia, C., Ferrer, N. L., Barilone, N., Laval, F., Soto, C. Y., Winter, N., Daffé, M., Gicquel, B., Martín, C., Jackson M. (2006) *J. Biol. Chem.* **281**, 1313–1316
- Ludwiczak, P., Gilleron, M., Bordat, Y., Martin, C., Gicquel, B., Puzo, G. (2002) *Microbiology* **148**, 3029–3037
- Martin, C., Williams, A., Hernandez-Pando, R., Cardona, P. J., Gormley, E., Bordat, Y., Soto, C. Y., Clark, S. O., Hatch, G. J., Aguilar, D., Ausina, V., and Gicquel, B. (2006) *Vaccine* **24**, 3408–3419
- Ryndak, M., Wang, S., and Smith, I. (2008) *Trends Microbiol.* **16**, 528–534
- Gonzalo-Asensio, J., Mostowy, S., Harders-Westerveen, J., Huygen, K., Hernández-Pando, R., Thole, J., Behr, M., Gicquel, B., and Martín, C. (2008) *PLoS ONE* **3**, e3496
- Frigui, W., Bottai, D., Majlessi, L., Monot, M., Josselin, E., Brodin, P., Garnier, T., Gicquel, B., Martin, C., Leclerc, C., Cole, S. T., and Brosch, R. (2008) *PLoS Pathog.* **4**, e33
- Li, A. H., Waddell, S. J., Hinds, J., Malloff, C. A., Bains, M., Hancock, R. E., Lam, W. L., Butcher, P. D., and Stokes, R. W. (2010) *PLoS ONE* **5**, e11066
- Abramovitch, R. B., Rohde, K. H., Hsu, F. F., and Russell, D. G. (2011) *Mol. Microbiol.* **80**, 678–694
- Menon, S., and Wang, S. (2011) *Biochemistry* **50**, 5948–5957
- Wang, S., Engohang-Ndong, J., and Smith I. (2007) *Biochemistry* **46**, 14751–14761
- Nowak, E., Panjikar, S., Konarev, P., Svergun, D. I., and Tucker, P. A. (2006) *J. Biol. Chem.* **281**, 9659–9666
- Pathak, A., Goyal, R., Sinha, A., and Sarkar, D. (2010) *J. Biol. Chem.* **285**, 34309–34318
- Lee, J. S., Krause, R., Schreiber, J., Mollenkopf, H. J., Kowall, J., Stein, R., Jeon, B. Y., Kwak, J. Y., Song, M. K., Patron, J. P., Jorg, S., Roh, K., Cho, S. N., and Kaufmann, S. H. (2008) *Cell Host Microbe* **3**, 97–103
- Chesne-Seck, M. L., Barilone, N., Boudou, F., Gonzalo Asensio, J., Kolattukudy, P. E., Martín, C., Cole, S. T., Gicquel, B., Gopaul, D. N., and Jackson, M. (2008) *J. Bacteriol.* **190**, 1329–1334
- Lejona, S., Castelli, M. E., Cabeza, M. L., Kenney, L. J., García Vescovi, E., and Soncini, F. C. (2004) *J. Bacteriol.* **186**, 2476–2480
- Bijlsma, J. J., and Groisman, E. A. (2003) *Trends Microbiol.* **11**, 359–366
- Ferrer, N. L., Gomez, A. B., Neyrolles, O., Gicquel, B., and Martin, C. (2010) *PLoS ONE* **5**, e12978
- Goude, R., and Parish, T. (2008) in *Mycobacteria Protocols* (Parish, T., and Brown, A. C., eds) 2nd Ed., pp. 203–215, Humana Press, London, UK
- Sambrook, J., Fritsch, E., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Timm, J., Lim, E. M., and Gicquel, B. (1994) *J. Bacteriol.* **176**, 6749–6753
- Dussurget, O., Timm, J., Gomez, M., Gold, B., Yu, S., Sabol, S. Z., Holmes, R. K., Jacobs, W. R., Jr., and Smith, I. (1999) *J. Bacteriol.* **181**, 3402–3408
- Ehrt, S., Guo, X. V., Hickey, C. M., Ryou, M., Monteleone, M., Riley, L. W., and Schnappinger, D. (2005) *Nucleic Acids Res.* **33**, e21
- Besra, G. S. (1998) *Methods Mol. Biol.* **101**, 91–107
- Bowtell, D., and Sambrook, J. (2003) *DNA Microarrays: A Molecular Cloning Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Mannan, M. A., Sharma, S., and Ganesan, K. (2009) *Anal. Biochem.* **389**, 77–79
- Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F. R., and Smith, I. (1999) *Mol. Microbiol.* **31**, 715–724
- Hu, Y., and Coates, A. R. (1999) *J. Bacteriol.* **181**, 469–476
- Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Gupta, S., Pathak, A., Sinha, A., and Sarkar, D. (2009) *J. Bacteriol.* **191**, 7466–7476
- Glickman, M. S., Cox, J. S., and Jacobs, W. R., Jr. (2000) *Mol. Cell* **5**, 717–727
- Gao, R., Mack, T. R., and Stock, A. M. (2007) *Trends Biochem. Sci.* **32**, 225–234
- Gao, R., and Stock, A. M. (2010) *Curr. Opin. Microbiol.* **13**, 160–167
- Gupta, S., Sinha, A., and Sarkar, D. (2006) *FEBS Lett.* **580**, 5328–5338
- Sinha, A., Gupta, S., Bhutani, S., Pathak, A., and Sarkar, D. (2008) *J. Bacteriol.* **190**, 1317–1328
- Sirakova, T. D., Thirumala, A. K., Dubey, V. S., Sprecher, H., and Kolattukudy, P. E. (2001) *J. Biol. Chem.* **276**, 16833–16839
- Dubey, V. S., Sirakova, T. D., and Kolattukudy, P. E. (2002) *Mol. Microbiol.* **45**, 1451–1459
- Graham, J. E., and Clark-Curtiss, J. E. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 11554–11559
- Rousseau, C., Neyrolles, O., Bordat, Y., Giroux, S., Sirakova, T. D., Prevost, M. C., Kolattukudy, P. E., Gicquel, B., and Jackson, M. (2003) *Cell. Microbiol.* **5**, 405–415
- Rousseau, C., Turner, O. C., Rush, E., Bordat, Y., Sirakova, T. D., Kolattukudy, P. E., Ritter, S., Orme, I. M., Gicquel, B., and Jackson, M. (2003) *Infect. Immun.* **71**, 4684–4690
- Converse, S. E., Mougous, J. D., Leavell, M. D., Leary, J. A., Bertozzi, C. R., and Cox, J. S. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100**, 6121–6126
- Domenech, P., Reed, M. B., Dowd, C. S., Manca, C., Kaplan, G., and Barry, C. E., 3rd (2004) *J. Biol. Chem.* **279**, 21257–21265
- Brodin, P., Poquet, Y., Levillain, F., Peguillet, I., Larrouy-Maumus, G., Gilleron, M., Ewann, F., Christophe, T., Fenistein, D., Jang, J., Jang, M. S., Park, S. J., Rauzier, J., Carralot, J. P., Shrimpton, R., Genovesio, A., Gonzalo-Asensio, J. A., Puzo, G., Martin, C., Brosch, R., Stewart, G. R., Gicquel, B., and Neyrolles, O. (2010) *PLoS Pathog.* **6**, e1001100
- Av-Gay, Y., and Deretic, V. (2005) in *Tuberculosis and the Tubercle Bacillus* (Cole, S. T., ed) pp. 359–367, American Society for Microbiology, Washington, D. C.
- Ewann, F., Loch, C., and Supply, P. (2004) *Microbiology* **150**, 241–246
- Blanco, A. G., Sola, M., Gomis-Rüth, F. X., and Coll, M. (2002) *Structure* **10**, 701–713
- Zhao, H., Msadek, T., Zapf, J., Madhusudan Hoch, J. A., and Varughese, K. I. (2002) *Structure* **10**, 1041–1050
- Voskuil, M. I., Schnappinger, D., Visconti, K. C., Harrell, M. I., Dolganov, G. M., Sherman, D. R., and Schoolnik, G. K., (2003) *J. Exp. Med.* **198**, 705–713
- He, H., and Zahrt, T. C. (2005) *J. Bacteriol.* **187**, 202–212
- Sola-Landa, A., Moura, R. S., and Martín, J. F. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100**, 6133–6138
- Martin, J. F. (2004) *J. Bacteriol.* **186**, 5197–5201
- Studier, F. W., and Moffatt, B. A. (1986) *J. Mol. Biol.* **189**, 113–130
- Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T., and Jacobs, W. R., Jr. (1990) *Mol. Microbiol.* **4**, 1911–1919