Interplay of PhoP and DevR response regulators defines expression of the dormancy regulon in virulent *Mycobacterium tuberculosis*

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Atul Vashist[‡1,2], **Vandana Malhotra**[§¶1,3], **Gunjan Sharma**[‡], **Jaya Sivaswami Tyagi**[‡4], **and Josephine E. Clark-Curtiss**[§‖5]

From the [¶]*Department of Biochemistry, Sri Venkateswara College, University of Delhi, New Delhi 110021, India, the* [§]*Center for Infectious Diseases and Vaccinology, Biodesign Institute, and the* [‖]*School of Life Sciences, Arizona State University, Tempe, Arizona 85287, and the* [‡]*Department of Biotechnology, All India Institute of Medical Sciences, New Delhi 110029, India*

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The DevR response regulator of *Mycobacterium tuberculosis* is an established regulator of the dormancy response in mycobacteria and can also be activated during aerobic growth conditions in avirulent strains, suggesting a complex regulatory system. Previously, we reported culture medium–specific aerobic induction of the DevR regulon genes in avirulent *M. tuberculosis* H37Ra that was absent in the virulent H37Rv strain. To understand the underlying basis of this differential response, we have investigated aerobic expression of the *Rv3134c-devR-devS* operon using *M. tuberculosis* H37Ra and H37Rv *devR* overexpression strains, designated as LIX48 and LIX50, respectively. Overexpression of DevR led to the up-regulation of a large number of DevR regulon genes in aerobic cultures of LIX48, but not in LIX50. To ascertain the involvement of PhoP response regulator, also known to co-regulate a subset of DevR regulon genes, we complemented the naturally occurring mutant $phoP_{Ra}$ gene of LIX48 with the WT $phoP_{Rv}$ gene. $PhoP_{Rv}$ dampened the induced expression of the DevR regulon by >70–80%, implicating PhoP in the negative regulation of *devR* expression. Electrophoretic mobility shift assays confirmed phosphorylation-independent binding of $PhoP_{Rv}$ to the *Rv3134c* promoter and further revealed that DevR and $PhoP_{Rv}$ proteins exhibit differential DNA binding properties to the target DNA. Through co-incubations with DNA, ELISA, and protein complementation assays, we demonstrate that DevR forms a heterodimer with $PhoP_{Rv}$ but not with the mutant $PhoP_{Ra}$ protein. The study puts forward a new possible mechanism for coordinated expression of the dormancy regulon, having implications in growth adaptations critical for development of latency.

Despite global initiatives to control tuberculosis, it continues to be an enigma. *Mycobacterium tuberculosis*, the causative agent of tuberculosis has evolved into a highly efficient human pathogen due to its innate ability to adapt to the diverse host environments during infection. Although great strides have been made to elucidate the transcriptomic and proteomic profiles of *M. tuberculosis* in various cellular environments *in vitro* and *in vivo*, our understanding of the mechanisms that are responsible for growth adaptation are far from clear.

Long-term survival of mycobacteria in the host requires the bacteria to transition to a slow-growth phenotype resulting in dormant bacilli that may undergo reactivation when exposed to appropriate stimuli. The DevR (also called DosR) response regulator (RR)[6] of the DevR-DevS (DevRS) TCS has been extensively studied for its role in mycobacterial dormancy and virulence (1–10). A wide variety of cellular stimuli are known to activate the DevRS TCS (11–16), highlighting its central role in metabolic and growth-adaptive strategies of mycobacteria. The DevR RR is activated via phosphorylation by two histidine sensor kinases, DevS and DosT (17), and also by Ser/Thr protein kinases, PknH (18) and PknB (19), that facilitate fine tuning of the dormancy response. This, in addition to the growing list of *M. tuberculosis* RRs that interact with DevR and/or co-regulate the DevR regulon, such as MprA, NarL, and PhoP, indicate overlapping regulatory pathways as a means for expansion of signaling networks (20–23). Indeed, such a strategy will enable coordinated gene expression across the perpetually changing metabolic status of the cell.

The constitutive aerobic induction of the DevR regulon in the hypervirulent *M. tuberculosis* W/Beijing (24) and avirulent H37Ra strains (25) has sparked significant interest in the under-

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The microarray data presented in this study have been deposited in the NCBI Gene Expression Omnibus (GEO) under the GEO series accession number GSE103551.

This article contains Figs. S1 and S2 and Tables S1–S3.

[1] Both authors contributed equally to this work.

[2] Supported by Department of Science and Technology, Government of India, Start-Up Research Grant YSS/2015/000562/LS.

[3] Supported by Department of Biotechnology, Government of India, Grant BT/Bio-CARe/07/9843. To whom correspondence should be addressed: Dept. of Biochemistry, Sri Venkateswara College, University of Delhi, New Delhi 110021, India. E-mail: vmal71@gmail.com.

[4] Supported by the Department of Science and Technology, Government of India, J. C. Bose National Fellowship.

[5] Supported by National Institutes of Health Public Health Service Grant AI46428 and Arizona State University start-up funding. Present address: Division of Infectious Diseases and Global Medicine, Dept. of Medicine, College of Medicine, University of Florida, Gainesville, FL 32610.

[6] The abbreviations used are: RR, response regulator; EMSA, electrophoretic mobility shift assay; M-PFC, mycobacterial protein fragment complementation; mDHFR, murine dihydrofolate reductase; TCS, two-component system; TRIM, trimethoprim; qRT-PCR, quantitative RT-PCR; LB, Luria broth; Kan, kanamycin; Hyg, hygromycin; EV, empty vector.

lying regulatory mechanisms governing DevR regulon expression during aerobic growth conditions. *M. tuberculosis* strains belonging to the W/Beijing lineage were shown to harbor point mutations in the *devR* promoter region and a naturally occurring frameshift mutation in the gene encoding the DosT sensor kinase (26) that were responsible for the constitutive expression of the regulon (27). These observations have enormous implications physiologically, particularly if the pre-induced dormancy regulon in W-lineage Beijing *M. tuberculosis* strains adds a survival advantage against intracellular stress signals, possibly contributing toward their hypervirulent and drug-tolerant phenotypes (24). Our previous studies have shown aerobic induction of the *Rv3134c-devR-devS* operon and DevR-regulated genes in *M. tuberculosis* H37Ra but not in H37Rv cultivated in Dubos Tween-albumin medium (25), highlighting differential regulation of this locus in these isogenic strains. Considering our limited understanding of the early transitioning events that lead to switching of growth rates and the onset of dormancy, it becomes relevant to understand the overall mechanisms directing expression of the DevR-dependent dormancy regulon.

The *devRS* genes are known to be differentially expressed in virulent *M. tuberculosis* H37Rv and its attenuated counterpart H37Ra strain (28, 29), with culture medium constituents like asparagine contributing to the difference in expression (25). Although the exact mechanism is presently unknown, annotation of the *M. tuberculosis* H37Ra genome sequence has provided valuable insights. Specifically, a point mutation at codon 219 (serine to leucine) in the DNA-binding domain of PhoP RR of H37Ra ($PhoP_{Ra}$) has been shown to be crucial for mycobacterial virulence (30–33). The complementation of avirulent *M. tuberculosis* H37Ra with a WT copy of the *phoP* gene from H37Rv ($phoP_{Rv}$) led to restoration of its virulence properties (32, 34). Among other notable differences is the up-regulation of the *sigC* gene encoding σ factor C in H37Ra, likely due to a mutation in its promoter region (33). Interestingly, both PhoP and SigC have been implicated in the regulation of DevR regulon genes (23, 35, 36). Whereas it has been suggested that PhoP participates in the transcriptional regulation of *devRS* TCS (23), direct evidence and the physiological role of such regulation has yet to be demonstrated.

The present study aims to investigate the aerobic expression of the *devRS* TCS in *M. tuberculosis* H37Ra and H37Rv to obtain insights into the mechanisms that regulate expression of the DevR regulon under aerobic (non-inducing) growth conditions. Transcription profiling of aerobically grown *M. tuberculosis* H37Ra and H37Rv *devR* overexpression strains, LIX48 and LIX50, respectively, revealed a distinct dysregulation of *devRS* genes in H37Ra that was conspicuously absent in H37Rv. Significantly, DevR overexpression resulted in up-regulation of 38 genes belonging to the DevR regulon in H37Ra in a phosphorylation-independent manner. Complementation of the LIX48 strain with an exogenous copy of $phoP_{Rv}$ decreased aerobic expression of *devR* and its target gene, *hspX*, by >70–80%. Through DNA binding, and protein–protein interaction assays, we demonstrate $PhoP_{Rv}$ binding to the *Rv3134c* promoter and, more importantly, formation of $PhoP_{Rv}$–DevR heterodimers as a plausible mechanism for co-regulation of the DevR regulon in H37Rv. A model highlighting the roles of PhoP and DevR RRs in regulating the dormancy regulon of *M. tuberculosis* H37Ra and H37Rv under aerobic and hypoxic growth conditions is discussed.

Results

Overexpression of devR in aerobic cultures of M. tuberculosis H37Ra and H37Rv results in significantly different transcription profiles

The transcription of *devRS* TCS in virulent *M. tuberculosis* H37Rv is marked by basal level expression under aerobic growth conditions and up-regulation upon exposure to hypoxia, NO, CO, vitamin C, and nitrite (1, 2, 11, 12, 14, 16, 37). In addition, we have shown that the *Rv3134c-devR-devS* operon in avirulent H37Ra is induced during aerobic growth in asparagine-containing medium (25). However, the question remained whether aerobic DevR regulon expression observed in H37Ra was directly due to DevR or other transcriptional regulators.

To avoid misinterpretation due to medium components, we simulated constitutively expressed *devR* by constructing recombinant *M. tuberculosis* H37Ra strain overexpressing the *devR* gene. Mycobacterial integrating plasmid pMG85 (38) overexpressing *devR* from the acetamide-inducible amidase promoter ($P_{amidase}$) was electroporated into *M. tuberculosis* H37Ra to construct strain LIX48 (Fig. 1). Because $P_{amidase}$ in *M. tuberculosis* is constitutive (38), no acetamide was used in these studies. An analogous *devR* overexpression strain in *M. tuberculosis* H37Rv (LIX50; Fig. 1) was constructed in parallel. All experiments were done in Middlebrook 7H9 medium, a standard laboratory culture medium for mycobacteria cultivation that does not contain asparagine, to rule out any medium-specific induction of DevR regulon genes.

RNA isolated from logarithmic phase, aerobic cultures of LIX48 and LIX50, and their respective control strains LIX47 and LIX49 transformed with empty vector (EV) were subjected to microarray analysis as described under "Experimental procedures." Forty-six genes exhibited differential expression (up-regulated, $n = 44$; down-regulated, $n = 2$) in *M. tuberculosis* H37Ra *devR* overexpression strain (LIX48) (Table 1, Tables S2 and S3), compared with the EV control strain (LIX47). By contrast, only eight genes were up-regulated in *M. tuberculosis* H37Rv *devR* overexpression strain (LIX50), relative to LIX49 control strain (Table 1, Tables S2 and S3). The DevR RR regulates expression of ~50 genes that are collectively termed the DevR regulon (1). Of the 44 genes up-regulated in LIX48, 38 belonged to the DevR regulon (Table 1, *shaded regions*). Notably, no overlap among up-regulated genes was observed with the LIX50 strain. The drastic up-regulation of a majority of DevR regulon genes in LIX48 points toward a dysregulation of the *devRS* locus in H37Ra as a result of *devR* overexpression, which is absent in H37Rv.

DevR overexpression–mediated aerobic induction of the DevR regulon in M. tuberculosis H37Ra is phosphorylation-independent

We validated the microarray results by analyzing expression of the *Rv3134c-devR-devS* operon and three known DevR-reg-

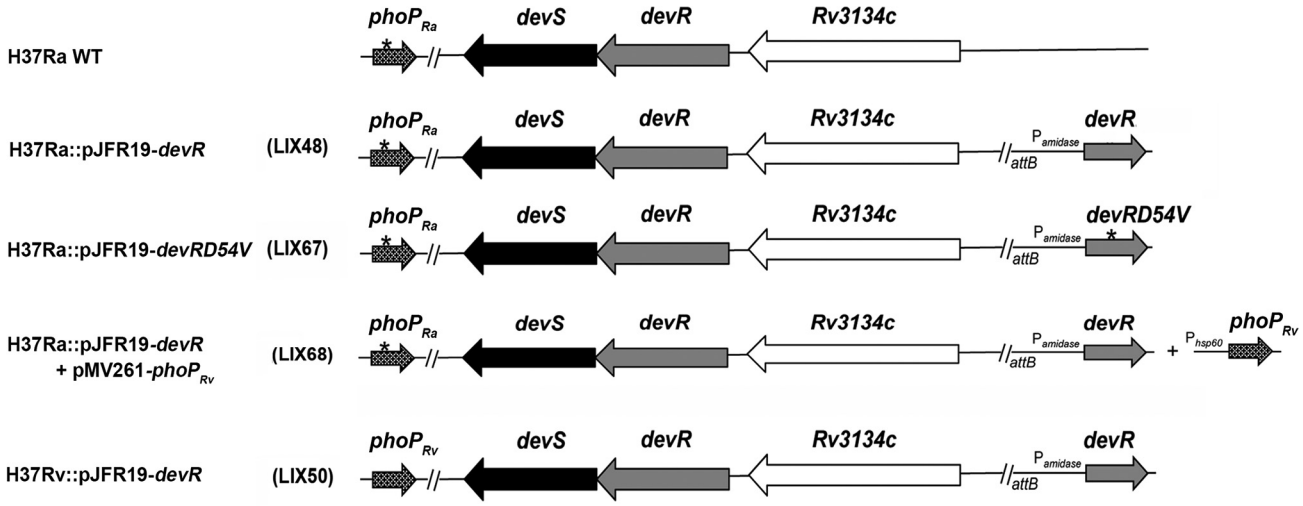

Figure 1. Schematic representation of genetic backgrounds of the *M. tuberculosis* H37Ra and H37Rv strains used in this study. The genetic backgrounds of *M. tuberculosis* H37Ra and various recombinant derivatives thereof along with the *M. tuberculosis* H37Rv *devR* overexpression strain used in this study are presented as a *schematic*. The site-specific integration of mycobacterial integrative vector pJFR19 is shown at the *attB* site. The mutation in the *phoP* coding region in H37Ra is depicted with an *asterisk*.

ulated genes, namely *hspX*, *narK2*, and *fdxA*, in LIX48 and LIX50 strains relative to the EV control strains. The up-regulation in the overexpression strains is reported as -fold induction with respect to baseline expression in the EV control strains (set at 1.0). As shown in Fig. 2*A*, qRT-PCR analysis revealed significant -fold induction of *Rv3134c* (~2638-fold), *devR* (~422-fold), *devS* (~111-fold), *hspX* (~5275-fold), *fdxA* (~1055-fold), and *narK2* (~1000-fold) genes in the LIX48 strain. In contrast, only a marginal increase in transcript levels of *Rv3134c* (~1.3-fold), *devR* (~1.75-fold), *devS* (~1.75-fold), *hspX* (~5-fold), *fdxA* (~2-fold), and *narK2* (~2-fold) was observed in the LIX50 strain (Fig. 2*A*). Western blot analysis of whole-cell lysates prepared from LIX48 and LIX50 strains corroborated the qRT-PCR results (Fig. 2*B*). Densitometric analysis revealed that the DevR protein increased ~1.5-fold and at least 6-fold in H37Rv and H37Ra overexpression strains, respectively. These data suggest two things: first, that *devR* overexpression from the amidase promoter during aerobic growth of *M. tuberculosis* H37Ra leads to induction of the *Rv3134c-devR-devS* operon and DevR-regulated genes, and, second and more importantly, that there are significant differences in overexpression-mediated induction of the DevR regulon in *M. tuberculosis* H37Ra *versus* H37Rv strains.

To rule out possible involvement of DevR phosphorylation in the aerobic dysregulation of the *devRS* TCS in LIX48, we constructed an *M. tuberculosis* H37Ra strain overexpressing the *devR* gene carrying a mutation in aspartic acid residue at position 54 (LIX67; Fig. 1). The D54V substitution mutation in *devR* was shown previously to abolish phosphorylation of the DevR protein (3), thereby resulting in negligible expression of DevR regulon genes (1). The *devR*D54V gene was PCR-amplified from plasmid $pKKP_{operon}$ *devR*D54V-Cmyc (57) and cloned into pJFR19 to yield plasmid pYA1626. The sequence-verified plasmid pYA1626 was electroporated into *M. tuberculosis* H37Ra to generate LIX67 strain (Fig. 1). Overexpression of *devR*D54V in LIX67 did not perturb gene induction. In fact, *devR* (~329-fold) and *hspX* (~4020-fold) (Fig. 2*C*) transcript levels in LIX67 were found to be comparable with those observed in LIX48 (Fig. 2*A*). Collectively, these observations dismiss any role for phosphoactivation of DevR through signal transduction as the underlying basis for the aerobic induction of DevR regulon genes in H37Ra *devR* overexpression strain.

Complementation of M. tuberculosis H37Ra devR overexpression strain with WT $PhoP_{Rv}$ mitigates dysregulation of devR-devS TCS

In subsequent experiments, we investigated the possible role of $PhoP_{Ra}$ in regulation of the DevR regulon in *M. tuberculosis* H37Ra. Toward this end, the *$phoP_{Rv}$* gene was amplified from *M. tuberculosis* H37Rv DNA and cloned downstream from an *hsp60* promoter in pMV261 to yield the recombinant plasmid pYA1630. The *M. tuberculosis* H37Ra strain overexpressing *devR* (LIX48) was transformed with plasmid pYA1630 to generate LIX68 strain, wherein defective endogenous $PhoP_{Ra}$ is complemented with WT $PhoP_{Rv}$. LIX47 strain transformed with the empty vector pMV261 served as control in this experiment. Expression of *$phoP_{Rv}$* in LIX68 led to a >70–80% reduction in the transcription of *devR* (~120-fold *versus* 422-fold) and *hspX* (~800-fold *versus* 5275-fold), compared with the levels obtained in LIX48 (Fig. 3). These observations establish that presence of WT $PhoP_{Rv}$ down-regulates *devR* transcription. Based on these results, we propose that, in the *M. tuberculosis* H37Rv *devR* overexpression strain, WT PhoP negatively regulates aerobic expression of the *devRS* TCS and that this phenomenon is absent/inefficient in H37Ra owing to the presence of a mutant PhoP protein.

M. tuberculosis $PhoP_{Rv}$ and DevR response regulators exhibit differential binding properties to Rv3134c promoter

To determine whether PhoP mediates regulation of *Rv3134c-devR-devS* genes through direct binding or through indirect effects, we investigated the ability of $PhoP_{Rv}$ and $PhoP_{Ra}$ proteins to bind the *Rv3134c* promoter region. The consensus DNA-binding sequence for *M. tuberculosis* $PhoP_{Rv}$ from

Table 1
Genes up-/down-regulated in *M. tuberculosis* H37Ra and H37Rv *devR* overexpression strains during aerobic growth

S. No	Gene Name / Product	[a] Fold Induction in LIX48/LIX47	SD
1	*Rv3130c*	6.45	0.782
2	*Rv2627c*	6.30	0.522
3	*acg*	5.97	0.739
4	*Rv3131*	5.97	1.154
5	*Rv2030c*	5.92	0.984
6	*Rv3128c*	5.79	1.429
7	*Rv0080*	5.69	1.322
8	*Rv2624c*	5.63	1.232
9	*narK2*	5.40	1.321
10	*Rv2625c*	5.39	1.137
11	*fdxA*	5.21	1.368
12	*Rv0079*	5.15	1.044
13	*Rv2028c*	5.12	1.285
14	*Rv1733c*	5.06	1.095
15	*pfkB*	5.03	1.439
16	*Rv1996*	4.98	1.745
17	*Rv2628*	4.90	1.939
18	*Rv2630*	4.70	1.655
19	*ctpF*	4.48	1.703
20	*Rv2629*	4.47	1.612
21	*Rv0572c*	4.42	1.962
22	*Rv2004c*	4.41	1.686
23	*narX*	4.39	1.293
24	*Rv2003c*	4.24	1.853
25	*devS*	4.07	2.127
26	*Rv1734c*	4.07	1.649
27	*Rv0081*	3.92	1.012
28	*Rv1735c*	3.8	1.325
29	*Rv0082*	3.41	0.864
30	*Rv0574c*	3.37	1.908
31	*Rv3129*	3.06	1.868
32	*Rv0571c*	2.96	1.864
33	*nrdZ*	2.88	1.728
34	*Rv0083*	2.71	1.045
35	*Rv0573c*	2.18	1.787
36	*hycP*	2.04	0.758
37	*Rv3126c*	2.0	0.607
38	*Rv1812c*	1.94	1.605
39	*otsB1*	1.91	0.91
40	*Rv1732c*	1.86	1.192
41	*hycE*	1.41	0.611
42	*hycQ*	1.41	0.614
43	*PE15*	1.0	0.212
44	*PPE50*	0.99	0.252
45	*trcR*	-1.64	0.581
46	PPE1	-1.02	0.135

S. No	Gene Name / Product	[b] Fold Induction in LIX50/LIX49	SD
1	*Rv1057*	2.02	0.097
2	*Rv3269*	1.61	0.548
3	*Rv1955*	1.35	0.25
4	*ahpC*	1.15	0.054
5	*Rv1956*	1.1	0.375
6	*Rv0967*	1.06	0.102
7	*Rv3183*	0.98	0.176
8	*whiB7*	0.96	0.175
9	*ppsC*	-2.81	0.005
10	*ppsB*	-2.78	0.132
11	*ppsA*	-2.26	0.308
12	*Rv0188*	-0.96	0.078

[a] Geometric mean of log $_2$ ratio of expression in *M. tuberculosis* H37Ra *devR* overexpression strain (LIX48) relative to EV control (LIX47) during aerobic growth.
[b] Geometric mean of log $_2$ ratio of expression in *M. tuberculosis* H37Rv *devR* overexpression strain (LIX50) relative to EV control (LIX49) during aerobic growth.
SD represents standard deviation between replicates.
Shaded boxes represent overlap with the hypoxia-induced and NO-induced DevR regulon (1,2)
Red and Green boxes indicate fold change > - 0.8 and < - 0.8 value in *devR* overexpression strain relative to EV control.

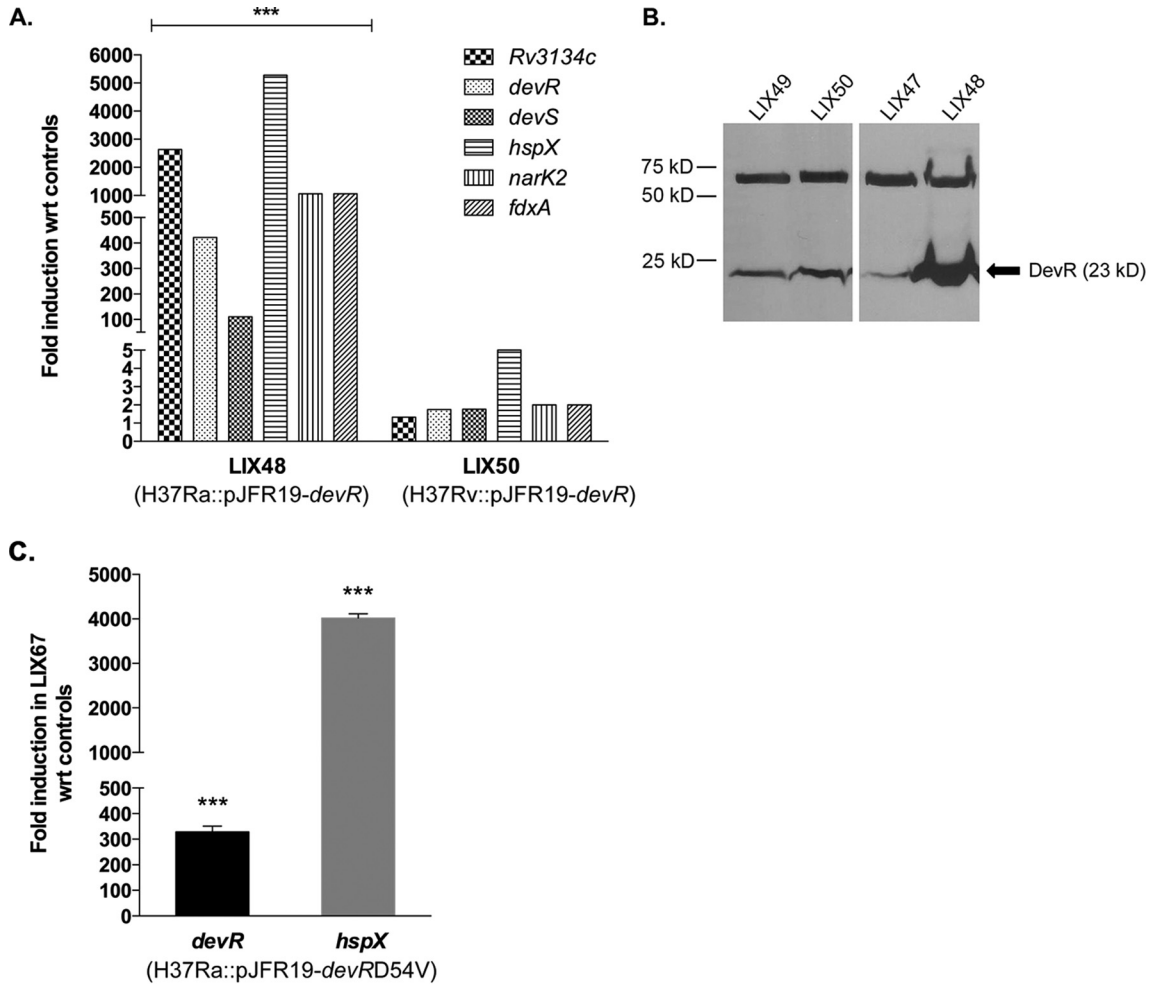

Figure 2. Expression analysis of DevR regulon genes in aerobically grown *M. tuberculosis* H37Rv and H37Ra *devR* overexpression strains. *A*, -fold induction of the *Rv3134c-devR-devS* operon and three DevR-regulated genes (*narK2*, *hspX*, and *fdxA*) in aerobic cultures of H37Ra *devR* (LIX48) and H37Rv *devR* (LIX50) overexpression strains relative to LIX47 and LIX49, EV control strains, respectively (baseline expression set to 1.0). ***, $p < 0.001$ for the differences in expression between LIX48 and LIX50 strains. *B*, Western blot analysis of whole-cell lysates of LIX49 (*lane 1*), LIX50 (*lane 2*), LIX47 (*lane 3*), and LIX48 (*lane 4*) with polyclonal rabbit anti-DevR antibodies (1:3000). *Arrow*, DevR protein. The top band in the blot was used as an internal control to normalize protein amounts in all lanes. *C*, -fold induction of *devR* and *hspX* in RNA isolated from aerobic cultures of *M. tuberculosis* H37Ra overexpressing phosphorylation-defective *devR*D54V gene (LIX67) relative to the EV control (baseline expression set to 1.0). ***, $p < 0.001$ for the differences in expression between LIX67 and control strains. Results are presented as mean ± S.D. (*error bars*) of three independent experiments.

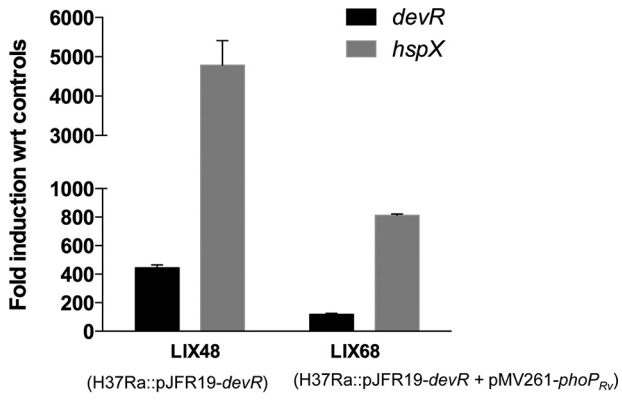

Figure 3. qRT-PCR analysis of *M. tuberculosis* H37Ra *devR* overexpression strain complemented with *phoP*$_{Rv}$ gene (LIX68). -Fold induction of *devR* and *hspX* genes in LIX68 and its parent strain (LIX48) with respect to EV control strains (baseline expression set to 1.0) is shown. Results are presented as mean ± S.D. (*error bars*) of three independent experiments.

PhoP ChIP-Seq data (39) was used to predict putative PhoP binding sites in a ~350-bp upstream region of the *Rv3134c-devR-devS* operon. This region harbors multiple operon promoters (35, 40, 41) and DevR binding sites (35), and in addition, 5 putative PhoP-binding sites were detected (Fig. 4*A*). A 218-bp DNA fragment in the *Rv3134c* promoter region containing all five putative PhoP binding sites along with previously established DevR-binding sites was PCR-amplified from H37Rv DNA and examined for PhoP$_{Rv}$, PhoP$_{Ra}$, and DevR binding. We used small molecule phosphodonor acetyl phosphate to phosphorylate DevR and PhoP proteins because they can be phosphorylated by acetyl phosphate (42–44). All three proteins were confirmed to be phosphorylation-proficient (data not shown). Different concentrations (4 and 8 μM) of DevR, PhoP$_{Rv}$, and PhoP$_{Ra}$ proteins were incubated separately with DNA. PhoP$_{Rv}$ was found to bind the target DNA in a concentration-dependent but phosphorylation-independent manner (Fig. 4, *B* and *C*). In contrast, PhoP$_{Ra}$ did not bind DNA (Fig. 4*C*, *right*). This

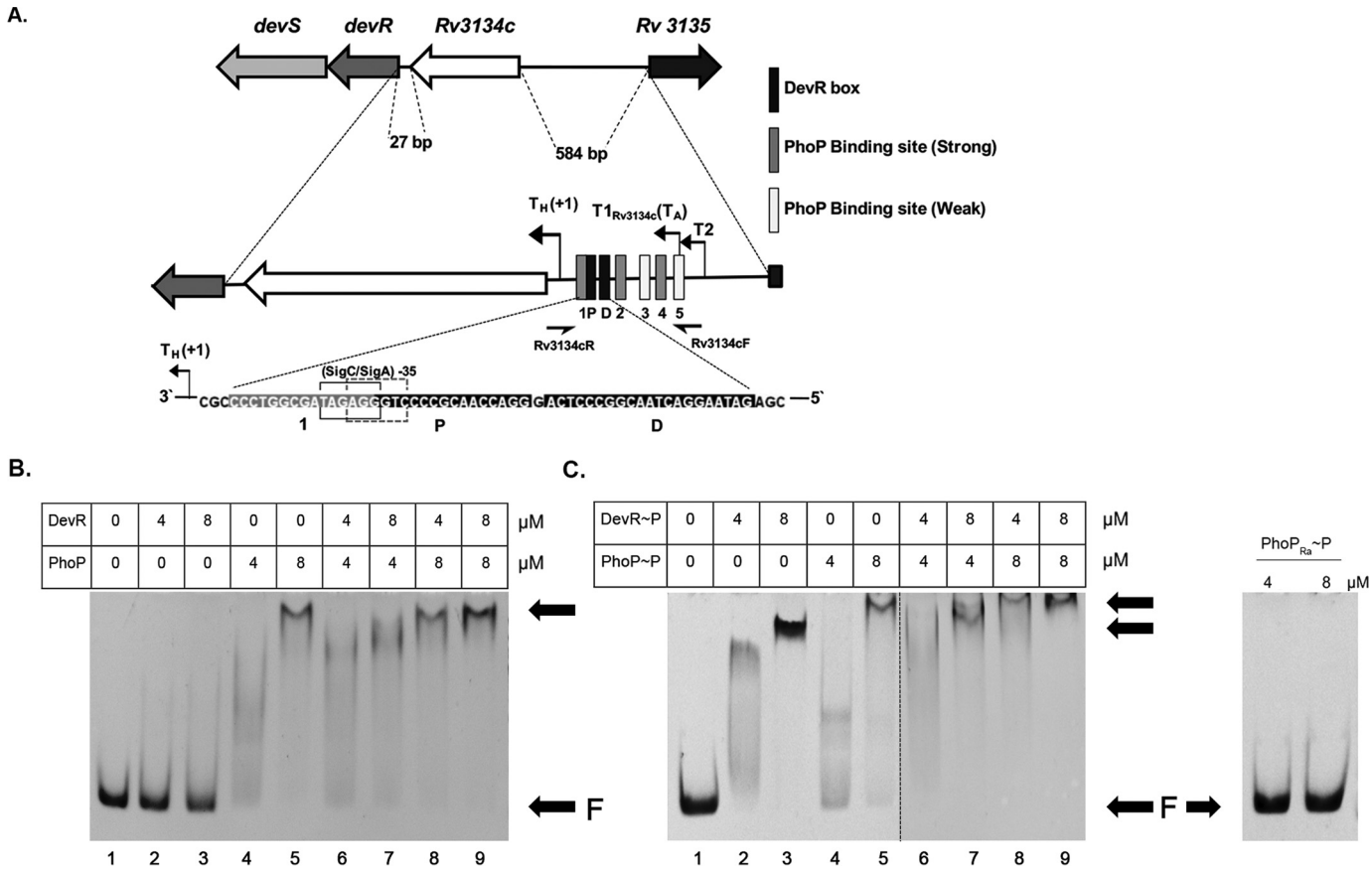

Figure 4. DNA-binding studies of PhoP RR with *Rv3134c-devR-devS* promoter region. *A, in silico* analysis of the upstream region of *Rv3134c-devR-devS* operon revealed five putative sites for PhoP binding depicted as *1–5*. Regulatory features including DevR binding boxes (*P* and *D*) and transcription start sites (aerobic and hypoxic) are indicated. Primers used for amplifying the 218-bp DNA probe for use in EMSAs are shown as *half-arrows*. *B*, binding of unphosphorylated DevR and PhoP proteins. *C*, binding of phosphorylated DevR, PhoP (*left*), and $PhoP_{Ra}$ (*right*) proteins to target DNA. *Arrows*, free DNA (*F*) and bound DNA.

result is consistent with the reported DNA-binding defect of $PhoP_{Ra}$ protein (45). In agreement with previous data (43), unphosphorylated DevR bound to DNA poorly, whereas DevR~P formed a stable complex with DNA (compare *lanes 2* and *3* in Fig. 4, *B* and *C*). At equimolar concentrations, unphosphorylated $PhoP_{Rv}$ exhibited a higher DNA binding property compared with unphosphorylated DevR (Fig. 4*B*, compare *lanes 2* and *4* and *lanes 3* and *5*), suggesting differential binding affinities of the two proteins.

Next we examined whether DevR and PhoP affect each other's binding to target DNA. Significantly, co-incubation of phosphorylated DevR and PhoP resulted in a "supershift" of the protein–DNA complex (Fig. 4*C*, compare *lanes 2* and *4* with *lane 6*, and compare *lanes 3* and *5* with *lanes 7–9*), which was not distinctly evident when unphosphorylated proteins were co-incubated (Fig. 4*B*, *lanes 6–9*). Taken together, the electrophoretic mobility shift assay (EMSA) results validate the DNA-binding defect of $PhoP_{Ra}$ and indicate that $PhoP_{Rv}$ binds to the *Rv3134c* upstream region independent of phosphorylation. Moreover, the observed "supershift" in DNA binding suggests a possible interaction between DevR and $PhoP_{Rv}$ RRs.

DevR and $PhoP_{Rv}$ form heterodimers in vitro and in vivo

The interaction between *M. tuberculosis* DevR and PhoP RRs was assessed *in vitro* in an ELISA format using purified $PhoP_{Ra}$/$PhoP_{Rv}$-His_6 protein (10 pmol) that was immobilized in triplicate in a 96-well plate overnight in coating buffer. DevR-His_6 protein (480, 240, and 120 ng) was incubated with $PhoP_{Ra}$/$PhoP_{Rv}$, and the interaction was probed using anti-DevR antibody as primary antibody (1:5000) and horseradish peroxidase–conjugated anti-rabbit antibody as secondary antibody (1:10,000). The background values from control wells were subtracted from test well readings, and ΔA_{450} values were plotted as shown in Fig. 5*A*. A strong interaction was observed between DevR–$PhoP_{Rv}$ as compared with DevR–$PhoP_{Ra}$ proteins ($p < 0.001$; Fig. 5*A*).

Because *in vivo* interaction in a related host, *Mycobacterium smegmatis*, is anticipated to provide functionally and physiologically relevant environment to study protein–protein interaction, we investigated DevR–PhoP interactions using the mycobacterial protein fragment complementation (M-PFC) assay that is well-established to study interactions between mycobacterial proteins *in vivo* (46). Briefly, when two mycobacterial interacting proteins are independently fused with domains of murine dihydrofolate reductase (mDHFR), functional reconstitution of the two mDHFR domains can occur in mycobacteria, allowing for the selection of mycobacterial resistance against trimethoprim (TRIM). The plasmid pairs (pUAB300::*devR* and pUAB400 carrying $phoP_{Rv}$ and $phoP_{Ra}$, respectively) were generated as C-terminal fusions with the complementary fragments of mDHFR. The plasmid pairs were

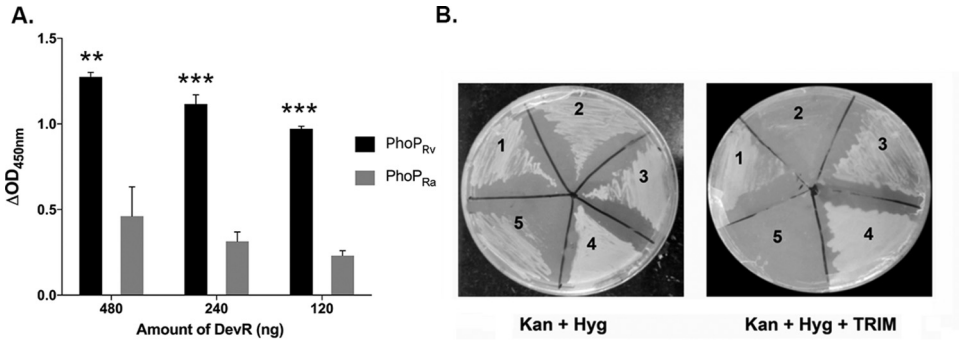

Figure 5. *In vitro* and *in vivo* protein–protein interaction of DevR and PhoP RRs. *A*, the interaction between *M. tuberculosis* DevR–$PhoP_{Rv}$ (*black bars*) and DevR–$PhoP_{Ra}$ (*gray bars*) proteins was assessed *in vitro* in an ELISA format using 10 pmol of purified $PhoP_{Rv}$/$PhoP_{Ra}$ proteins as described under "Experimental procedures." The background values from the control wells were subtracted from the test well readings, and ΔA_{450} values are plotted. ** and ***, $p < 0.01$ and 0.001, respectively, for the difference in interaction between DevR–$PhoP_{Rv}$ and DevR–$PhoP_{Ra}$ proteins. *B*, *in vivo* mycobacterial protein fragment complementation assay. Shown is *M. smegmatis* transformed with the following pairs of plasmid constructs encoding recombinant fusion proteins: recombinant $PhoP_{Rv}$-[F3]/DevR-[F1,2] (*1*) and $PhoP_{Ra}$-[F3]/DevR-[F1,2] (*2*). Positive controls (*3* and *4*) and negative control (*5*) were streaked on Kan + Hyg (*left*) and Kan + Hyg + TRIM plates (*right*). One representative picture from three independent experiments is shown. *Error bars*, S.D.

electroporated into *M. smegmatis* mc^2155 to generate the following protein expression pairs, DevR/$PhoP_{Rv}$ and DevR/$PhoP_{Ra}$. The cotransformants were screened on MB7H11 plates + kanamycin (Kan) (25 μg/ml) + hygromycin (Hyg) (50 μg/ml) with or without TRIM (30–50 μg/ml) (Fig. 5*B*). The protein pairs GCN4/GCN4 encoded by pUAB100/pUAB200 and GCN4/DevR encoded by pUAB100/pAVDevR200 were used as positive and negative control, respectively. Robust growth was observed in the positive controls (Fig. 5*B*, *sections 3* and *4*) as well as in transformants co-expressing DevR/$PhoP_{Rv}$ (Fig. 5*B*, *section 1*), suggesting that these pairs of proteins interact *in vivo*. We observed interaction between DevR and $PhoP_{Rv}$ even in the presence of 50 μg/ml TRIM, confirming strong interactions *in vivo*. Importantly, no growth was observed in the transformant co-expressing DevR/$PhoP_{Ra}$ proteins (Fig. 5*B*, *section 2*). Furthermore, the negative control, co-expressing DevR and GCN4, did not show growth, ruling out a spontaneous association of mDHFRF[1,2] and F[3] (Fig. 5*B*, *section 5*). Based on these observations, we infer that $PhoP_{Ra}$ is defective in interaction with DevR owing to the point mutation. Collectively, these findings indicate that formation of $PhoP_{Rv}$–DevR heterodimer and its interaction with DNA facilitate co-regulation of DevR regulon genes.

Discussion

The attenuated *M. tuberculosis* H37Ra strain is an isogenic variant of virulent H37Rv and thus provides a unique opportunity to understand and dissect regulatory mechanisms that might otherwise be difficult to unravel. Of significant interest is the culture medium-specific aerobic induction of the *Rv3134c-devR-devS* genes in *M. tuberculosis* H37Ra but not in H37Rv, suggesting differential regulation of these genes in H37Ra and H37Rv (25). Here, we have investigated aerobic expression of the *Rv3134c-devR-devS* genes by comparing transcription profiles of *M. tuberculosis* H37Ra and H37Rv *devR* overexpression strains. We report striking up-regulation of the *Rv3134c-devR-devS* operon and several members of the DevR regulon in *M. tuberculosis* H37Ra as a result of *devR* overexpression during aerobic growth that is absent in the analogous H37Rv strain. Our results implicate the inability of the mutant $PhoP_{Ra}$ protein to bind DNA (Fig. 4*C*) and interact with DevR RR (Fig. 5) as the foremost factors responsible for aerobic dysregulation of the *devRS* TCS observed in H37Ra. Further, we identified a role for the WT PhoP RR in blocking unwarranted induction of the DevR regulon in *M. tuberculosis* H37Rv during aerobic growth.

Typically, TCS systems are responsive to specific stimuli, and activation usually leads to an increase in concentration of the phosphorylated RR species above a threshold. Thus, a balance in ratio between the unphosphorylated and phosphorylated RR molecules dictates the direction of response. Due to a dynamic equilibrium between the inactive and active forms, an increase in unphosphorylated RR above the threshold value can bypass activation requirements, resulting in transcriptional activation in the absence of any natural stimulus (47). Recent findings suggest that aerobic overexpression of *devR* in *M. tuberculosis* can override the need for signal activation (47, 48). Therefore, we utilized *devR* overexpression strains of *M. tuberculosis* H37Ra and H37Rv to investigate regulation of aerobic transcription of *devRS* genes. With pleiotropic effects associated with gene deletions and the fact that culture medium components modulate *devRS* expression, use of these strains provided an unequivocal advantage.

The net effect of *devR* overexpression driven from a strong promoter is an increased level of unphosphorylated DevR leading to signal-independent activation of the DevR regulon. In contrast to previous studies that have reported significant induction of the DevR regulon in aerobic cultures of *M. tuberculosis* H37Rv strains overexpressing *devR* (39, 47–49), we observed a fairly moderate response. We reason that promoters of varying strengths in overexpression constructs, differences in genetic backgrounds, and media used are sources of discrepancy between different studies (47–49). A critical concentration of DevR essential for activation of *devRS* TCS has been previously established (10). We rationalize that the marginal overexpression of DevR observed in H37Rv (Fig. 2) is probably insufficient to override the threshold for transcriptional activation. Thus, it follows that expression of the DevR regulon under non-inducing conditions, such as aerobic growth, is a function of cellular levels of the DevR protein. The substantially high levels of DevR in *M. tuberculosis* H37Ra *devR* overexpression strain used in the present study are indicative of a signal-inde-

pendent, hyperactivated DevR regulon. It is noteworthy that such levels of DevR regulon have not been observed in any of the *devR* overexpression strains in H37Rv background (39, 47–49) or in W-Beijing lineage of *M. tuberculosis* (24) reported so far. Thus, it is not surprising that hyperactivation of the DevRS TCS in *M. tuberculosis* H37Ra *devR* overexpression strain resulted in significant clumping and poor aerobic growth (Fig. S1). Our results point toward differential autoregulatory effects of DevR in H37Ra and H37Rv overexpression strains. Because these strains were constructed identically, the differences in the level of DevR protein and downstream regulon expression are intriguing and may be due to disparities in their genetic backgrounds.

The *M. tuberculosis Rv3134c-devR-devS* operon characteristically contains multiple transcription start sites, aerobic (basal) and hypoxia-inducible promoters, binding sites of more than one transcriptional regulator, and/or alternative σ factors (such as SigA and SigC) that are suggestive of complex regulation of this operon (35, 36, 40, 41). However, little is known about how *M. tuberculosis* H37Rv precludes up-regulation of the DevR regulon during aerobic growth. Unlike the *M. tuberculosis* Beijing strain, wherein aerobic induction of the DevR regulon was linked to specific mutations (24, 26, 27), DNA sequences of the *Rv3134c-devR-devS* operon and its promoter region in H37Rv and H37Ra are identical. Thus, we hypothesized that aerobic dysregulation of this locus in *M. tuberculosis* H37Ra may be attributed to a defect in some regulatory protein(s). The marginal aerobic expression of DevR regulon genes in *M. tuberculosis* H37Ra Δ*devR* (Fig. S2) and H37Rv Δ*devR* mutant strains (35, 40) supports the role of another transcriptional regulator driving aerobic expression of these genes.

We focused on the possible involvement of *M. tuberculosis* PhoP in regulation of aerobic expression of *devRS* genes based on three key observations. First, a subset of the DevR regulon is down-regulated in aerobically grown *M. tuberculosis* H37Rv Δ*phoP* mutant bacteria (32); second, *M. tuberculosis* PhoP is implicated in co-regulation of the hypoxic response in mycobacteria, which is further supported by identification of multiple PhoP binding sites in the *Rv3134c* promoter region (this study), and third, PhoP in *M. tuberculosis* H37Ra has impaired DNA binding affinity as a result of a point mutation in its DNA recognition helix (45, 50). Partial restoration by complementation of the *M. tuberculosis* H37Ra *devR* overexpression strain with WT $PhoP_{Rv}$ highlighted the role of mutant $PhoP_{Ra}$ protein in aerobic dysregulation of the *devRS* TCS in H37Ra. A key finding of this study is that in addition to its positive regulatory effect on *devR* expression (23), PhoP is seen to exert a negative regulatory effect (Fig. 3). We propose that $PhoP_{Rv}$ maintains the aerobic expression of *devR* gene to a level well below the threshold for DevR regulon activation. At present, it is unclear whether *sigC* promoter mutation in *M. tuberculosis* H37Ra also contributes toward regulation of *devRS* expression; thus, SigC involvement cannot be completely discounted. To the best of our knowledge, this is the first report demonstrating direct *in vitro* binding of $PhoP_{Rv}$ to the *devRS* upstream region. Although the significance of a strong PhoP binding site within the −35 element of a SigA/SigC promoter is not clear at present, interaction of PhoP with the transcriptional machinery is a distinct possibility. In line with this hypothesis, PhoP has been shown to interact with SigE in a recent report (51).

Based on our observations and previously reported data, we propose a model for possible regulatory mechanisms exerted by PhoP and DevR on expression of the *Rv3134c-devR-devS* operon in *M. tuberculosis* H37Ra and H37Rv (Fig. 6). During aerobic growth (non-inducing conditions) (Fig. 6*A*), DevR is unphosphorylated and does not bind to DNA (Fig. 4*B*). The *devRS* genes are expressed at lower basal level in H37Ra compared with H37Rv (29). Moreover, unlike $PhoP_{Rv}$ that binds to *Rv3134c* upstream DNA (Fig. 4, *B* and *C*), $PhoP_{Ra}$ mutant protein is defective in DNA binding (Fig. 4*C*, *right*). Under DevR overexpression conditions (Fig. 6*B*), unphosphorylated DevR mediates positive autoregulation (47), thereby increasing the level of total DevR protein, resulting in hyperactivation of the DevR regulon in H37Ra. By contrast, in the *M. tuberculosis* H37Rv overexpression strain, $PhoP_{Rv}$ is bound to the *Rv3134c* promoter and interacts with DevR to block autoregulation. In the presence of inducing signals, such as hypoxia (Fig. 6*C*), DevR is phosphorylated in both *M. tuberculosis* H37Rv (1, 40) and H37Ra strains (32) and induces downstream expression of the DevR regulon, albeit to higher levels in H37Ra (32), highlighting the negative effect of PhoP. These findings suggest that PhoP plays a secondary but repressive role in hypoxia-induced DevR regulon expression.

Heterodimers between RR proteins have now been discovered in multiple instances (52–55), suggesting that heterodimer formation may be a fundamental mechanism contributing to the concerted regulation of overlapping regulons in *M. tuberculosis.* We recently elucidated heterodimeric interactions between *M. tuberculosis* DevR and NarL RRs for co-regulation of gene expression during aerobic nitrate metabolism (22). Overlap of DevR- and PhoP-binding sites on the DNA raises the possibility of protein–protein interactions between them. Such a situation may arise during hypoxia, as both DevR and PhoP are known to regulate overlapping sets of genes during hypoxia (23). Our results establish that DevR and $PhoP_{Rv}$ RRs interact *in vitro* and *in vivo*. Notably, DevR did not exhibit robust interaction with the mutant $PhoP_{Ra}$ protein, suggesting that the S219L mutation disrupts heterodimer formation. Previous reports have suggested that the dimeric interface of PhoP monomers from *B. subtilis* retains a second surface that is available for further interactions (56), raising the likelihood that homodimer and heterodimer interfaces in PhoP may be different.

In conclusion, comparative analysis of the interplay between DevR and PhoP RRs in virulent H37Rv and avirulent H37Ra strains of *M. tuberculosis* provides novel insights into $PhoP_{Rv}$-mediated negative regulation of the *devRS* TCS, its interaction with DevR, and their differential DNA binding affinities as key regulatory mechanisms that fine-tune expression of the dormancy regulon in mycobacteria.

Experimental procedures

Bacterial strains and plasmids

Escherichia coli JM109 and DH5α strains were used as host strains for genetic manipulations and plasmid constructions. *E. coli* C43 (DE3) strain was used for overexpression and puri-

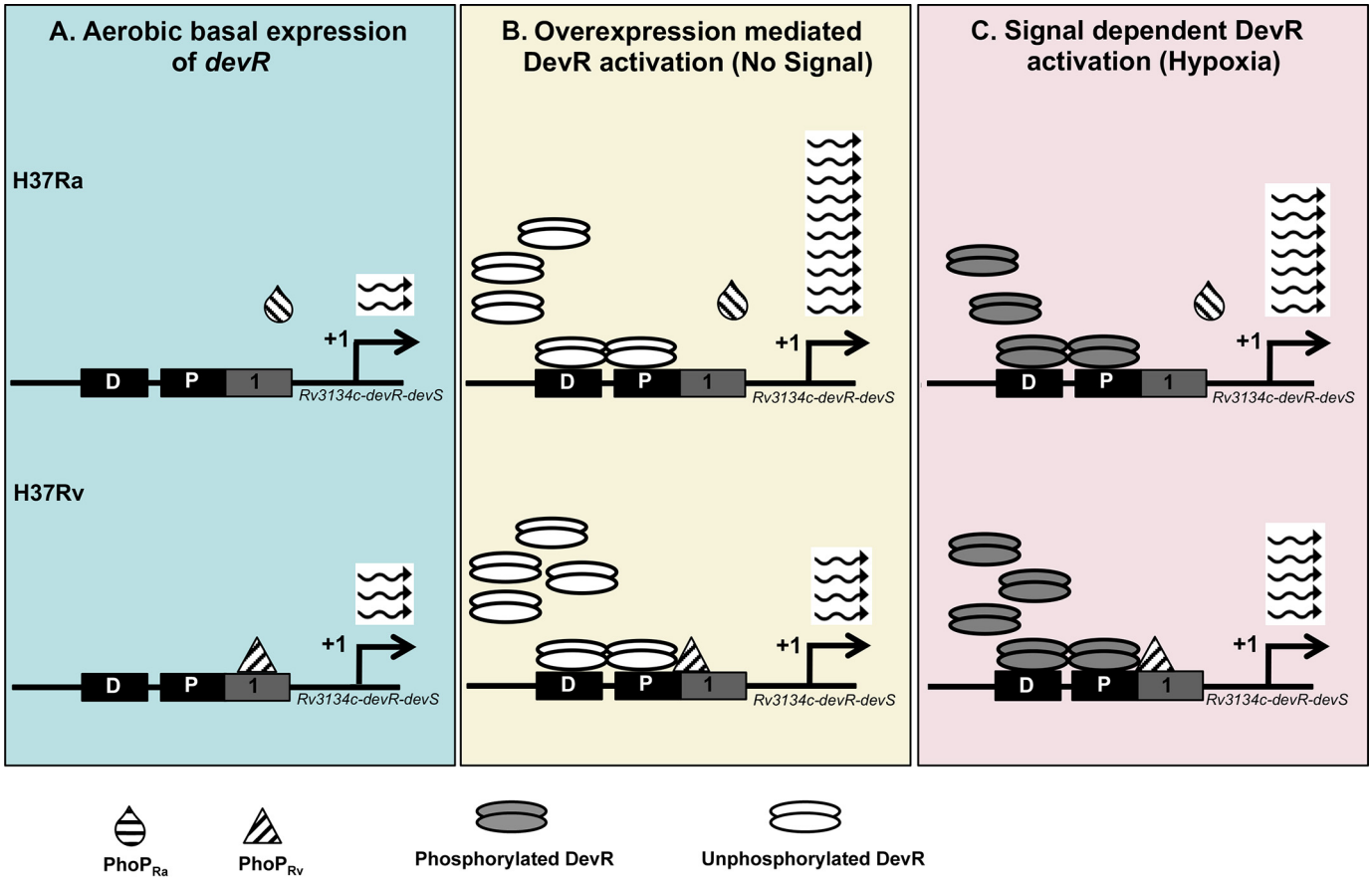

Figure 6. Model depicting the role of PhoP and DevR in regulating the expression of DevR regulon genes in *M. tuberculosis* H37Rv and H37Ra. The DevR binding sites upstream of *Rv3134c-devR-devS* genes responsible for activation of DevR regulon are depicted as described (39, 40). The most probable PhoP-binding site identified in this study (*1*, *solid gray box*) overlaps the proximal DevR-binding box (*P*, *solid black box*). *A*, aerobic basal *devR* expression. There is a basal level expression of the *devR* gene that is marginally lower in *M. tuberculosis* H37Ra as compared with H37Rv. *B*, overexpression-mediated activation of DevR. In *M. tuberculosis* H37Ra *devR* overexpression strain, the absence of PhoP$_{Ra}$ binding enables unphosphorylated DevR to autoregulate and cause downstream expression of the DevR regulon. This sequence of events is prevented in *M. tuberculosis* H37Rv due to binding of PhoP$_{Rv}$ to the *Rv3134c* upstream region and its interaction with DevR. *C*, signal dependent activation of DevR. Hypoxia leads to phosphorylation of DevR and its cooperative binding to the Dev boxes, resulting in induction of the DevR regulon genes in *M. tuberculosis* H37Rv and H37Ra strains. The inhibitory effect of PhoP$_{Rv}$ on *devR* expression and differential interactions between these RRs explain the lesser number of transcripts in H37Rv compared with H37Ra during hypoxic growth conditions.

fication of His-tagged DevR and PhoP$_{Rv}$ proteins. Purified His-tagged PhoP$_{Ra}$ protein was obtained as a gift from Dr. Dibyendu Sarkar (IMTECH, Chandigarh, India). Virulent *M. tuberculosis* H37Rv, avirulent *M. tuberculosis* H37Ra, and *M. smegmatis* mc^{2}155 strains were used in the present study. Table S1 summarizes bacterial strains and plasmids used in this study. Detailed plasmid constructions are available upon request to V. M.

Media, chemicals, and culture conditions

E. coli cultures were grown in 2× YT or Luria–Bertani (LB) broth or on LB-agar plates at 37 °C. *M. smegmatis* cultures were grown at 37 °C with aeration in Middlebrook 7H9 (Difco) or LB medium supplemented with 0.05% Tween 80 or on LB-agar plates. For the M-PFC assay, *M. smegmatis* strains were plated on Middlebrook 7H11 agar (MB7H11, Difco) supplemented with 0.5% glycerol, 0.5% glucose, and 0.2% Tween 80 and grown at 37 °C. *M. tuberculosis* strains were grown in Middlebrook 7H9 medium supplemented with 0.05% Tween 80 and 10% ADS (0.5% albumin, 0.2% dextrose, 0.085% Saline) or on Middlebrook 7H10 agar plates at 37 °C to an A_{600} ~0.2–0.3 Antibiotics and chemicals were added as required at the following concentrations: ampicillin, 100 μg/ml; Kan, 50 μg/ml; Hyg, 50 and 100 μg/ml for *M. smegmatis* and *M. tuberculosis*, respectively, and 150 μg/ml for *E. coli*; isopropyl β-D-thiogalactopyranoside, 1 mM; and trimethoprim (TRIM), 20–50 μg/ml. All chemicals were obtained from Sigma-Aldrich, unless stated otherwise.

RNA isolation

RNA isolation of exponentially grown cultures of *M. tuberculosis* was performed as described previously (25). Total RNA integrity was assessed using RNA 6000 Nano Lab Chip on the 2100 Bioanalyzer (Agilent) according to the manufacturer's protocol. Total RNA purity was assessed by the NanoDrop® ND-1000 UV-visible spectrophotometer (NanoDrop Technologies).

Microarray slide design for M. tuberculosis H37Ra and H37Rv

The Agilent custom *M. tuberculosis* slide was designed by Genotypic Technology Private Ltd. to study the expression of all of the genes of *M. tuberculosis* H37Rv (source: NCBI Accession ID AL123456.2) and *M. tuberculosis* H37Ra strain (NCBI Accession ID NC_009525.1). Probes common to both *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra (n = 15,106) and probes specific to *M. tuberculosis* H37Ra (n = 18) and

M. tuberculosis H37Rv ($n = 84$) were designed. All of the oligonucleotides were designed and synthesized *in situ* as per the standard algorithms and methodologies used by Agilent Technologies. The array format was 8 × 15K (AMADID: 023057) comprising a total number of 15,744 features encompassing 15,208 probes and 536 Agilent control probes.

Microarray hybridization and quantitative RT-PCR

RNA samples from two independent experiments were analyzed by microarrays. Poly(A) tails were added to the 3′-ends of RNA using the A-plus poly(A) polymerase tailing kit (Epicenter Biotechnologies). The samples were labeled using the QuickAmp labeling Kit (Agilent). Five hundred nanograms of each sample was incubated with reverse transcription mix at 42 °C and converted to double-stranded cDNA primed by oligo(dT) with a T7 polymerase promoter. The cDNA was used as template for cRNA generation by *in vitro* transcription and incorporation of the dye Cy3-CTP and Cy5-CTP (Agilent). The cDNA synthesis and *in vitro* transcription steps were carried out at 40 °C. The quality of labeled cRNA was assessed for yields and specific activity followed by hybridization. Cy3 and Cy5-labeled samples (300 ng each) were hybridized using the Gene Expression Hybridization kit (Agilent) in Surehyb Chambers (Agilent) at 65 °C for 16 h. The hybridized slides were washed using Gene Expression wash buffers (Agilent) and scanned using the Microarray Scanner G2505C at 5 μm resolution. Data extraction from images was done using Feature Extraction software version 10.5.1.1 (Agilent), followed by analysis using the GeneSpring GX version 10 software (Agilent). Normalization of the data were done in GeneSpring GX using Lowess (locally weighted scatterplot smoothing) normalization. Samples were grouped based on the replicates, and genes showing up- or down-regulation >0.8-fold among the samples were identified. Results are reported as the geometric means of $\log_2$ expression ratios ± S.D. between the replicates.

Quantitative RT-PCR was performed to validate the microarray results as described previously (25). Briefly, 200 ng of RNA was reverse-transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad). The cDNA was diluted 1:10 and served as the template in real-time PCR using gene-specific primers and SYBR Green dye (Bio-Rad). RNA from three independent experiments was used for expression analysis. Normalization of expression was done using 16S rRNA as an internal control, and the -fold change in expression between test and control samples was calculated using the iQ5 software.

Protein isolation and immunoblot analysis

Total protein was isolated from logarithmic phase cultures of *M. tuberculosis* H37Rv and H37Ra *devR* overexpression strains along with their respective EV control strains. The cell pellets were lysed using a bead beater and subjected to immunoblot analysis using anti-DevR antibody as described previously (3).

Overexpression and purification of His-tagged DevR and $PhoP_{Rv}$ proteins

The $phoP_{Rv}$ coding region (744 bp) was PCR-amplified from *M. tuberculosis* H37Rv genomic DNA and cloned into the NcoI and XhoI sites of the pET28a expression vector to create plasmid pGSPhoP$_{Rv}$-His$_6$. Recombinant plasmid pAVDevR-His$_6$ was used for purification of His-tagged DevR protein (43). *E. coli* C43 (DE3) cultures carrying recombinant pGSPhoP$_{Rv}$-His$_6$ and pAVDevR-His$_6$ plasmids were grown at 37 °C, 180 rpm in 600 ml of 2× YT medium containing Kan (50 μg/ml) to an A_{590} of 0.4–0.5. The production of recombinant proteins, DevR and PhoP$_{Rv}$, was induced by the addition of 1 mM isopropyl β-D-thiogalactopyranoside followed by overnight incubation at 25 °C followed by standing incubation at 4 °C for 4–5 h. Cells were harvested by centrifugation at 6000 rpm for 10 min (Sorvall GSA rotor, Sorvall RC6 Plus centrifuge (Thermo Scientific)). Briefly, the culture pellet was resuspended in 4 ml of lysis buffer containing 20 mM imidazole, 20 mM Na_2HPO_4, and 0.5 M NaCl, Complete EDTA-free protease inhibitor mixture (Roche Diagnostics). The resuspended pellet was sonicated, and the cell lysate was aliquoted into 1.5-ml sterile tubes and centrifuged at 12,000 rpm into Sorvall (F-20 microrotor, Thermo Scientific) for 40 min followed by filter sterilization of the supernatant through 0.45-μm and 0.22-μm filters. The supernatant was loaded onto the Ni^{2+}-nitrilotriacetic acid–agarose column of 5 ml bed volume (Qiagen, GmBH, Germany) connected to an AKTA protein purifier system (GE Healthcare) via an injection valve with the help of a 10-ml Luerlok syringe. The pre-equilibration was done with 10 bed volumes of equilibration buffer (50 mM Na_3PO_4, pH 7.4, 300 mM NaCl). The flow rate was maintained at 0.5 ml/min. The column was washed with 40 bed volumes of equilibration buffer (as above) and then washed with 30 bed volumes of wash buffer (50 mM Na_3PO_4, pH 7.4, 300 mM NaCl, 50 mM imidazole, 10% glycerol). The Ni^{2+}-nitrilotriacetic acid resin with bound protein was treated with the elution buffer (50 mM Na_3PO_4, pH 7.4, 300 mM NaCl, 300 mM imidazole), and the eluant was collected in fractions of 0.5 ml. The fractions were pooled, dialyzed against dialysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50% glycerol, 0.1 mM DTT), and stored at −20 °C. The purified proteins were subjected to SDS-PAGE followed by analysis through Coomassie Brilliant Blue R250 staining and immunoblotting with anti-His antibody (Sigma-Aldrich) at 1:2000 dilution and detected using diaminobenzidine-hydrogen peroxidase as substrate.

Electrophoretic mobility shift assays

In a standard EMSA reaction, phosphorylated protein and the target DNA probe were incubated with binding buffer containing 25 mM Tris-HCl (pH 8.0), 0.5 mM EDTA, 20 mM KCl, 6 mM $MgCl_2$, 5% glycerol for 30 min on ice in a final reaction volume of 20 μl. The reaction was electrophoresed on a 5% nondenaturing acrylamide gel containing 1.5% glycerol at 120 V (constant) in 0.5× TBE buffer at 4 °C after pre-running the gel for 30 min under similar conditions. The gel was then stained with ethidium bromide, and DNA–protein complexes were visualized using the gel documentation system. For phosphorylation, DevR, PhoP$_{Rv}$, and PhoP$_{Ra}$ were incubated with 50 mM acetyl phosphate (Sigma) for 30 min at 25–30 °C in 40 mM Tris-HCl (pH 8.0) and 5 mM $MgCl_2$. After phosphorylation, a DNA-binding assay was performed in a 20-μl reac-

tion. Negative control without any protein was included in all EMSA gels.

Enzyme-linked immunosorbent assay

$PhoP_{Ra}$/$PhoP_{Rv}$-His_6 protein (10 pmol or 283 ng) was coated in triplicate in 100 μl of coating buffer in a 96-well polystyrene microtiter plate incubated overnight at 4 °C in a humidified chamber. After washing once with 1× PBS, the wells were blocked with blocking buffer (5% BSA, 0.25% Tween 20 in 1× PBS) for 2 h at 37 °C. Different amounts of DevR-His_6 protein (in 100 μl of 1× PBS) were then added in triplicate in test ($PhoP^+$) and control wells ($PhoP^-$) and incubated for 1 h at room temperature. The plate was washed once with 1× PBS, followed by incubation with polyclonal rabbit anti-DevR antibody (1:5000) for 1 h under shaking conditions. The plate was washed three times with washing buffer (1× PBS containing 0.1% Tween 20). Detection was done using anti-goat anti-rabbit IgG-horseradish peroxidase–conjugated antibody (1:10,000) in 2% BSA in 1× PBS and 0.05% Tween 20 after incubation for 1 h at room temperature followed by washing and developed using TMB as substrate. Absorbance was recorded at 450 nm using a spectrofluorimeter (Spectra Max, Molecular Devices LLC). Suitable controls (namely antigen control ($PhoP^-$, $DevR^-$, coating buffer only), primary antibody control, secondary antibody control, and interacting partner control ($PhoP^-$, $DevR^+$)) were applied in the assay.

In vivo M-PFC assay

The M-PFC assay was performed to investigate DevR–PhoP interaction *in vivo* as described previously (46). The assay enables visualization of protein–protein interactions within mycobacteria through functional reconstitution of murine DHFR protein and selective targeting of mycobacterial DHFR by TRIM. Positive protein–protein interactions are scored by growth in the presence of TRIM concentrations that do not inhibit the recombinant mDHFR. Plasmids pSSDevR300, pGSPhoP$_{Ra}$400, and pGSPhoP$_{Rv}$400 encoding recombinant DevR-[F1,2], $PhoP_{Ra}$, and $PhoP_{Rv}$ proteins fused with DHFR[F3] domains, respectively, were electroporated into *M. smegmatis*. The co-transformants obtained after electroporation into *M. smegmatis* were screened on MB7H11 plates containing Kan (50 μg/ml) + Hyg (100 μg/ml). Positive clones along with suitable controls were then subcultured or streaked on MB7H11 Kan (50 μg/ml) + Hyg (100 μg/ml) + TRIM (20–50 μg/ml) plates and incubated at 37 °C for 4–5 days to assess protein–protein interactions. Growth of colonies on TRIM-containing medium was indicative of protein–protein interaction. Homodimerization of the GCN4/GCN4 domains encoded by the plasmid pair pUAB100/pUAB200 served as the positive control. The plasmid pair pUAB100/pAVDevR200 was used as a negative control for the assay.

Statistical analyses

Statistical analyses were performed using Student's *t* test or one-way analysis of variance using GraphPad Prism version 6.0 software. A p value <0.05 was considered statistically significant.

Author contributions—A. V. and V. M. data curation; A. V., V. M., J. S. T., and J. E. C.-C. formal analysis; A. V., V. M., J. S. T., and J. E. C.-C. funding acquisition; A. V. and V. M. validation; A. V., V. M., and G. S. investigation; A. V. and V. M. methodology; A. V., V. M., J. S. T., and J. E. C.-C. writing-review and editing; V. M. conceptualization; V. M., J. S. T., and J. E. C.-C. supervision; V. M. visualization; V. M. writing-original draft; J. S. T. and J. E. C.-C. resources; J. S. T. and J. E. C.-C. project administration.

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