Convergence of Ser/Thr and Two-component Signaling to Coordinate Expression of the Dormancy Regulon in Mycobacterium tuberculosis

Joseph D. Chao, Kadamba G. Papavinasasundaram, Xingji Zheng, Ana Chávez-Steenbock, Xuetao Wang, Guinevere Q. Lee, and Yossef Av-Gay

From the Department of Microbiology and Immunology and the Division of Infectious Diseases, Department of Medicine, University of British Columbia, Vancouver, British Columbia V5Z 3J5, Canada

Signal transduction in Mycobacterium tuberculosis is mediated primarily by the Ser/Thr protein kinases and the two-component systems. The Ser/Thr kinase PknH has been shown to regulate growth of M. tuberculosis in a mouse model and in response to NO stress in vitro. Comparison of a pknH deletion mutant (ΔpknH) with its parental M. tuberculosis H37Rv strain using iTRAQ enabled us to quantify >700 mycobacterial proteins. Among these, members of the hypoxia- and NO-inducible dormancy (DosR) regulon were disregulated in the ΔpknH mutant. Using kinase assays, protein-protein interactions, and mass spectrometry analysis, we demonstrated that the two-component response regulator DosR is a substrate of PknH. PknH phosphorylation of DosR mapped to Thr196 and Thr206 on the key regulatory helix α10 involved in activation and dimerization of DosR. PknH Thr phosphorylation and DosS Asp phosphorylation of DosR cooperatively enhanced DosR binding to cognate DNA sequences. Transcriptional analysis comparing ΔpknH and parental M. tuberculosis revealed that induction of the DosR regulon was subdued in the ΔpknH mutant in response to NO. Together, these results indicate that PknH phosphorylation of DosR is required for full induction of the DosR regulon and demonstrate convergence of the major signal transduction systems for the first time in M. tuberculosis.

Mycobacterium tuberculosis, the causative agent of tuberculosis, is a human intracellular pathogen that is phagocytosed by alveolar macrophages and subsequently “walled off” by the host immune response within granulomas (1). M. tuberculosis is able to persist within the hostile microenvironment of the granuloma, which is thought to include hypoxic, acidic, and nutrient-poor conditions and immune effectors such as nitric oxide (NO) (2). The survival and persistence of M. tuberculosis in this environment requires the ability to sense external signals and mount an effective adaptive response. M. tuberculosis possesses multiple families of signal transduction systems, including the Ser/Thr protein kinases (STPKs) and the two-component regulatory systems (TCSs) (3).

In a previous study, we found that the STPK PknH functions as an in vivo growth regulator (4). Hypervirulence was consistently detected in BALB/c mice infected with a pknH deletion mutant in M. tuberculosis after 3–4 weeks of infection (4), corresponding to the onset of adaptive immunity. Therefore, we hypothesized that M. tuberculosis uses the PknH kinase-mediated pathways to respond to host-induced signals to regulate its in vivo growth. Nitric oxide produced by the inducible nitric-oxide synthase of the host macrophages plays a key role in controlling bacillary growth during the chronic phase of infection following activation of the host immune response (5). In vitro experiments revealed that the ΔpknH mutant is more resistant to NO compared with WT (4), indicating that PknH may act as a sensor of NO to regulate M. tuberculosis growth in vivo.

Predictions from bioinformatics analysis and studies using in vitro kinase assays have identified three endogenous substrates of PknH kinase: EmbR (6), a transcriptional regulator of the embCAB genes involved in lipoarabinomannan and arabinogalactan synthesis; DacB1, a cell division-related protein; and Rxv0681, a putative transcriptional regulator (7). However, the substrates and downstream effectors of PknH signaling in response to NO stimulus have yet to be discovered.

The DosR system, also known as DevR, is one of 11 pairs of TCSs present in M. tuberculosis (3). It is well established that DosR responds to hypoxia, NO, and CO via signaling through two cognate sensor kinases, DosS (DevS) and DosT (8, 9) to activate transcription of a defined set of ~50 genes termed the “dormancy” or DosR regulon (10). Genes belonging to the DosR regulon, including dosR, are up-regulated in the Wayne model of dormancy (10, 11), under low-oxygen tension (12–14), and in response to NO (10) and CO (15, 16) and are believed to be involved in the adaptation of M. tuberculosis to a non-replicating persistent state in latent tuberculosis infection.
PknH Phosphorylation of DosR

In this work, we demonstrate convergence of the two major signal transduction systems, the STPK and the TCS, for the first time in *M. tuberculosis*. Using a global proteomics approach, we identified members of the DosR regulon to be disregulated in a pknH deletion mutant in *M. tuberculosis*. We show that DosR is a substrate of PknH Thr phosphorylation and that cooperative DosS Asp phosphorylation and PknH Thr phosphorylation enhance DosR-DNA binding. Enhanced binding *in vitro* correlates with up-regulation of the DosR regulon in WT *M. tuberculosis* compared with ΔpknH in response to NO. These results suggest that PknH and the Dos TCS coordinately regulate expression of a key physiological response of *M. tuberculosis*.

**EXPERIMENTAL PROCEDURES**

*M. tuberculosis* Growth/Stress Conditions—*M. tuberculosis* H37Rv and a mutant strain lacking pknH, described previously (4), were grown in Middlebrook 7H9 broth supplemented with 10% albumin/dextrose/sodium chloride and 0.05% Tween 80. For iTRAQ analysis, strains were grown in rolling cultures to *A*~600~ μl~1~ 1.0, harvested, washed, and resuspended in acidified (pH 5.4) Middlebrook 7H9 broth/Tween 80% albumin/dextrose/sodium chloride with 3.0 mM NaN~3~ and harvested after 48 h in standing cultures as described previously (4). For qRT-PCR analysis, cultures were grown in rolling cultures to *A*~600~ μl~1~ 0.3 and treated for 4 h with NaN~3~ or diethylenetriamine/NO as indicated. Cells were washed and resuspended when using acidified media.

iTRAQ and LC-MS/MS—The iTRAQ assay and phosphopeptide identification were performed by the University of Victoria Proteomics Centre (British Columbia, Canada; see supplemental “Methods”).

RNA Extraction and qRT-PCR—Previously described procedures were followed for RNA extraction and qRT-PCR analysis (4). Primers for qRT-PCR are listed in supplemental Table S1. Results were analyzed using GraphPad Prism software. All values were normalized to cDNA expression levels of sigA.

Cloning, Expression, and Purification—Plasmids and primers used for cloning and site-directed mutagenesis are listed in supplemental Table S1. The genes pknH-(1–402), dosR, and dosS-(378–578) were amplified from *M. tuberculosis* H37Rv genomic DNA using standard methods. The dosR gene was cloned into the pET22b vector; dosS-(378–578) was cloned downstream of *G*.protein coding sequence into a modified pGEV2 vector (17), pJC8 (see supplemental “Methods”). Site-directed mutagenesis was performed as described previously (7). For cell-based phosphorylation experiments, dosR was transferred into the pET30b kanamycin-resistant vector (producing an identical DosR recombinant protein), pknH was cloned into the pGEX-4T3 ampicillin-resistant vector, and both were cotransformed into *Escherichia coli* BL21. Expression of all proteins was carried out in *E. coli* BL21(DE3) as described (7), followed by purification on nickel-nitrilotriacetic acid columns (Qiagen) according to the supplied protocol.

In Vitro Kinase Assays—*In vitro* kinase assays were carried out as described previously (7). For EMSA, PknH and DosS were autophosphorylated in 25 mM Tris-HCl (pH 7.5), 5 mM MgCl~2~, 1 mM MnCl~2~, 20 mM KCl, 1 mM DTT, and 1.0 mM unlabeled ATP.

Phosphoamino Acid Stability and Analysis—PknH-phosphorylated DosR was separated by SDS-PAGE and transferred onto 0.45-μm PVDF membranes. Stability of the incorporated phosphate was tested by treating membranes with 1 N HCl, 3 N NaOH, or ddH~2~O overnight at room temperature and visualized by phosphorimaging. Phosphoamino acid analysis was performed as described (18) using cellulose plates and resolved in one dimension with isobutyric acid and 0.5 M NH~4~OH (5:3, v/v).

Protein-Protein Interaction Assays—See supplemental Table S1 for primers and plasmids. The mycobacterial protein fragment complementation assay was performed as described (19). *M. tuberculosis* dosR and pknH-(1–401) genes were amplified by PCR and cloned into pUA100 (expressing murine dihydrofolate reductase (mDHFR) fragment F1,2) and pUA200 (expressing mDHFR fragment F3), producing pKP366 and pKP369, respectively. *Mycobacterium smegmatis* was cotransformed with both plasmids, and the cotransformants were selected on 7H11/kanamycin/hygromycin plates and tested for growth over 3–4 days on kanamycin/hygromycin plates supplemented with 0, 10, and 20 μg/ml trimethoprim.

The Trp auxotrophic strain of *M. smegmatis* and plasmids pL240 and pL242, containing the *N*- and *C*-terminal fragments (N~Trp~ and C~Trp~) of *N*(′)-phosphoribosyl)anthranilate isomerase, respectively, were generously provided by Helen O’Hare. The Split-Trap experiment was performed as described (20), with the following modifications. N~Trp~ and C~Trp~ were transferred from pL240 and pL242 into pALACE (21) and pPE207 (22) and designated pJC10 (hygromycin-resistant) and pJC11 (apramycin-resistant), respectively, to place the resulting fusion proteins under control of the inducible acetalidase promoter (see supplemental “Methods”). The indicated genes were PCR-amplified and cloned into pJC10 and pJC11. All inserts were sequenced. Cotransformed *M. smegmatis* Trp~−~ was spotted (5 μl) onto Middlebrook 7H10 broth, 1% glucose, 60 μg/ml histidine, 50 μg/ml hygromycin, and 30 μg/ml apramycin plates; supplemented or not with either 0.02% acetalide or 120 μg/ml Trp; and grown for 2–3 weeks at 30°C.

EMSA—Oligonucleotides corresponding to the combined central and proximal (CP) DosR boxes and the distal (D) box upstream of hspX were designed with 5′-guanine overhangs when annealed (supplemental Table S1). Radioactive [α-³²P]dCTP was incorporated by Klenow (Fermentas) according to the supplied protocol. DosR (64 pmol) was phosphorylated by incubation with and without 0.2 μg each of pre-autophosphorylated PknH, DosS, and both PknH and DosS, followed by incubation with 4 pmol of radiolabeled CP or D DosR boxes. Binding conditions were as described previously (23). Samples were resolved by 5% nondenaturing Tris borate/EDTA PAGE. Gels were dried, and radiolabeled DNA bands were detected by phosphorimaging.

**RESULTS**

PknH-dependent Protein Expression—To draw a global picture of the regulation mediated by PknH kinase, we compared the protein expression profiles of WT and ΔpknH *M. tubercu-
PknH Phosphorylation of DosR

DosR is dependent on phosphorylation of Asp54 by its cognate histidine kinases, DosS and DosT, in response to hypoxia, NO, and CO (9, 12). However, on the basis of our data, we hypothesized that PknH kinase regulates DosR activity by Ser/Thr phosphorylation. We therefore conducted in vitro kinase assays to test whether PknH phosphorylates DosR. As shown in Fig. 2A, DosR was phosphorylated when incubated with recombinant PknH, whereas DosR alone did not undergo autophosphorylation.

## TABLE 1

| Gene   | Function                          | Untreated ratio | NO-treated ratio | DosR-inducible Ref. |
|--------|-----------------------------------|-----------------|------------------|---------------------|
| Rv0079 | Hypothetical                      | 2.31            | 1.04             | (10, 12, 32)*       |
| Rv1738 | Conserved                         | 1.90            | 1.35             | (10, 12, 32)        |
| Rv2030c| Conserved                         | 1.16            | 1.07             | (10, 12, 32)        |
| Rv2031c| α-Crystallin                      | 2.32            | 1.12             | (10, 12, 32)        |
| Rv2032 | USPA motif                        | 1.93            | 1.08             | (10, 12, 32)        |
| Rv2623 | Conserved                         | 1.46            | 1.16             | (10, 12, 32)        |
| Rv2626c| Conserved                         | 1.72            | 1.28             | (10, 12, 32)        |
| Rv2627c| Conserved                         | 2.05            | 0.99             | (10, 12, 32)        |
| Rv3127 | Conserved                         | 1.31            | 0.92             | (10, 12, 32)        |
| Rv3130c| Conserved                         | 1.41            | 0.96             | (10, 12, 32)        |
| Rv3131 | Conserved                         | 1.97            | 1.18             | (10, 12, 32)        |
| Rv3133c| dosR                              | 1.69            | 1.05             | (10, 12, 32)        |
| Rv3134c| USPA motif                        | 1.96            | 1.13             | (10, 12, 32)        |
| Rv1177 | TCS response regulator            | 1.49            | 0.76             | (32)                |
| Rv0231 | fadE4                             | 0.86            | 0.98             | (32)                |
| Rv3841 | Bacterioferritin                  | 0.81            | 0.98             | (12)                |

* Kendall et al. (32) identified Rv0080, which belongs to the same operon as Rv0079.

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Phosphoamino acid analysis identified that DosR was phosphorylated on Thr (Fig. 2B). Phosphorylation was acid-stable and alkali-labile, characteristic of Thr phosphorylation, but not of Asp phosphorylation (supplemental Fig. S2) (25). MS/MS analysis identified PknH-monophosphorylated DosR at Thr\textsuperscript{198} (supplemental Fig. S1A) and Thr\textsuperscript{205} (supplemental Fig. S1B) of the trypsin-digested \textsuperscript{198}TQAAVFATELKR\textsuperscript{209} peptide located in the C-terminal domain of DosR. Site-directed mutagenesis of DosR confirmed these findings: DosR(T198A) had reduced ability to be phosphorylated by PknH, and DosR(T205A) and DosR(T198A/T205A) were nearly abolished for PknH phosphorylation (Fig. 2C).

Next, we used \textit{E. coli}, which lacks any known STPKs, as a surrogate host to test PknH phosphorylation of DosR in a cell-based system. The active kinase domain of PknH and full-length recombinant DosR were coexpressed in \textit{E. coli}. MS/MS analysis of DosR purified from the PknH-expressing strain identified monophosphorylated (supplemental Fig. S1, C and D) and diphosphorylated (Fig. 3 and supplemental Fig. S1E) DosR at the previously identified Thr\textsuperscript{198} and Thr\textsuperscript{205} residues, with diphosphorylation being the predominant species.

\textbf{PknH Interaction with DosR in Mycobacteria}—To determine whether PknH interacts with DosR \textit{in vivo}, we performed two separate protein-protein interaction assays in \textit{M. smegmatis}: the Split-Trp assay (20) and mycobacterial protein fragment complementation assay (19). In the former, protein-protein interaction leads to the reassembly of the N- and C-terminal fragments (NTrp and CTrp) of \textit{N}-(5'-phosphoribosyl)anthranilate isomerase, an enzyme required for Trp biosynthesis. In the

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure2.png}
\caption{\textbf{PknH phosphorylation of DosR.} A, \textit{in vitro} kinase assay demonstrated phosphorylation of DosR by PknH using [\textgamma\textsuperscript{32}P]-ATP. Upper, phosphorimage; lower, silver stain of DosR protein bands. B, one-dimensional phosphoamino acid analysis of PknH-phosphorylated DosR identified phosphorylation on Thr. Control phospho-Tyr (Y), phospho-Thr (T), and phospho-Ser (S) were visualized by spraying with ninhydrin, and radiolabeled DosR residues were visualized by phosphorimaging. Retention factors were calculated as follows: Tyr, 0.37; Thr, 0.31; Ser, 0.25; and DosR, 0.30. C, \textit{in vitro} kinase assay confirmed that DosR(T198A) (A\textsubscript{1}), DosR(T205A) (A\textsubscript{2}), and the double mutant DosR(T198A/T205A) (AA) are defective for phosphorylation by PknH. Upper, phosphorimage; lower, silver stain. Arrowheads point to DosR.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3.png}
\caption{\textbf{Identification of DosR phosphorylation sites.} The MS/MS spectra represent peptide positions 198–209 with a monoisotopic mass of 1493.69 Da from DosR phosphorylated in a cell-based system showing diphosphorylation of Thr\textsuperscript{198} and Thr\textsuperscript{205}. Phosphorylation at Thr\textsuperscript{198} is shown by the b N-terminal daughter ion series, where all b ions identified lose phosphoric acid (~98 Da). Phosphorylation at Thr\textsuperscript{205} is shown by the y C-terminal daughter ion series, where all y ions after Thr\textsuperscript{205} lose phosphoric acid. pT, phosphothreonine; amu, atomic mass units.}
\end{figure}

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latter, reassembly of complementary fragments F1,2 and F3 of mDHFR confers resistance to trimethoprim.

As shown in Fig. 4A, using the mycobacterial protein fragment complementation system, coexpression of DosR-F1,2 and PknH-(1–401)-F3 reconstituted mDHFR expression as determined by trimethoprim resistance, indicating that PknH interacts with DosR in vivo. The interaction between PknH and DosR in M. smegmatis was slightly weaker than the positive control obtained with the interaction of the yeast Gcn4 dimerization domains but is consistent with the transient nature of kinase-substrate interactions. Using the less sensitive Split-Trp system, interaction between PknH and WT DosR was not observed; however, interaction between PknH and the phosphorylation-defective DosR(T198A/T205A) mutant restored growth of the Trp auxotrophic strain of M. smegmatis in the absence of exogenous Trp (Fig. 4B). This result suggests that the PknH-DosR interaction is dependent on the phosphorylation status of DosR. DosR(T198A/T205A) likely acted as a kinase-trapping mutant, where PknH was able to bind but not release DosR(T198A/T205A) due to its inability to be phosphorylated. This result is in agreement with Split-Trp studies related to another M. tuberculosis protein kinase, PknG, which interacts significantly better with its phosphorylation-defective substrate, GarA(T21A), compared with WT GarA (26). Taken together, these results provide further evidence that PknH interacts with and phosphorylates DosR in mycobacteria.

Enhanced DNA Binding of PknH-phosphorylated DosR—DosR is able to bind its cognate DNA sequence, the DosR box (12), and Asp phosphorylation enhances DosR-DNA binding (27). We therefore assessed the effect of PknH on DosR-DNA binding using EMSA. We compared the DNA-binding ability of PknH Thr-phosphorylated DosR with unphosphorylated and DosS Asp-phosphorylated DosR. We tested the binding of DosR to the D and CP DosR boxes of the hspX promoter. In vitro phosphorylation of DosR by PknH and/or DosS increased binding to the D box (A) and CP box (B). Cell-based phosphorylation of DosR by PknH increased binding and caused an addition shift to the D box (C) and CP box (D). DosR was incubated with radiolabeled DNA and run on a nondenaturing gel. Radiolabeled DNA was titrated with excess unlabeled probe by the dilution factor (DF) indicated to show specific binding. Arrowheads show unbound DNA. Data are representative of three separate experiments.

PknH Phosphorylation of DosR

FIGURE 4. PknH interaction with and dimerization of DosR in vivo. A, PknH and DosR protein-protein interaction facilitated the reassembly of the F1,2 and F3 domains of mDHFR, enabling growth of M. smegmatis strains coexpressing DosR-F1,2 and PknH-(1–401)-F3 fusion proteins in the presence of 20 μg/ml trimethoprim (TMP). Identical spots on control plates without trimethoprim revealed growth of all strains. Positive Control, Saccharomyces cerevisiae Gcn4 dimerization domains fused to F1,2 and F3, respectively; Negative Control, mDHFR fragments alone. The experiment is shown in duplicate, B, the specific interaction between PknH and the phosphorylation-defective DosR(T198A/T205A) (DosR$^\text{Thr}$) mutant facilitated the reassembly of the F1,2 and C terminal fragments required for Trp biosynthesis, thus enabling growth of M. smegmatis strains coexpressing N$_\text{Trp}$-PknH-(1–401) with DosR(T198A/T205A)-C$_\text{Trp}$, but not with WT DosR-C$_\text{Trp}$ (upper row). The positive control consisted of N$_\text{Trp}$-Cfp10 and Esat6-C$_\text{Trp}$. The negative control consisted of N$_\text{Trp}$ and C$_\text{Trp}$ alone. C, the growth of M. smegmatis Trp$^\text{Thr}$ mutant was dependent on the reassembly of N$_\text{Trp}$ and C$_\text{Trp}$ mediated by the dimerization of the C-terminal domains (amino acids 145–217) of the phosphomimetic DosR(EE) mutant (DosR$^\text{Thr}$), but not of WT DosR (DosR$^\text{WT}$) or the WT DosR/DosR(EE) (DosR$^\text{WT}$/DosR(EE)) combination (upper row). B and C, middle rows, Trp supplied exogenously; lower rows, no acetamide (Acet) induction of the fusion proteins. Data are representative of three separate experiments.
PknH Phosphorylation of DosR

We also tested the DNA-binding characteristics of DosR that had been phosphorylated by PknH in our cell-based system. Equal amounts of DosR (as determined by Bradford assay and had been phosphorylated by PknH in our cell-based system. As shown in Fig. 4C, interaction between the two C-terminal domains of the phosphomimetic DosR(EE) mutant restored growth of M. smegmatis Trp +, whereas interaction between WT DosR proteins did not enable growth. Growth was not observed with either full-length WT or DosR(EE) proteins (data not shown) and was expected, as full-length DosR exists in an inactive conformation (31).

Transcription Profiling of the DosR Regulon in M. tuberculosis—As we observed greater DosR-DNA binding upon phosphorylation of DosR by PknH, we hypothesized that this increase in DNA binding would correlate to increased DosR regulon transcription in M. tuberculosis. We therefore used qRT-PCR to measure DosR regulon expression in WT M. tuberculosis compared with ΔpknH. For a broad coverage of the DosR regulon spanning the M. tuberculosis genome, we looked at the expression of eight DosR regulon genes whose products were identified in our iTRAQ analysis and five additional DosR regulon genes not identified by iTRAQ. All values were normalized to the housekeeping sigA gene, whose expression is affected neither by NO stress (32) nor by acidic conditions (33). A heat map of the ΔpknH/WT ratios normalized to sigA expression for all 13 genes tested is shown in Fig. 6A (for graphical analysis, see supplemental Fig. S3). Basal level transcription of the DosR regulon in aerobic early log phase growth was unaffected by pknH deletion (first column). The addition of NaNO2 in resulted in an ~2-fold lower expression of the DosR regulon genes in ΔpknH compared with WT M. tuberculosis (second column). Because NaNO2 can also generate reactive oxygen intermediates, we verified these results using diethylenetriamine/NO, a specific NO donor, and found a similar 2-fold decrease in DosR regulon expression in ΔpknH (third column). Expression of the regulon under standing conditions was also lower in the mutant, but to a lesser extent (fourth column). Finally, to mimic the combined low oxygen and presence of NO likely encountered in the host, standing conditions with the NO donors were tested and resulted in a similar decrease to DosR regulon expression in ΔpknH (fifth and sixth columns). The decreased DosR regulon expression in the pknH mutant was due to an impaired induction of each gene following NO treatment (Fig. 6B). Comparison of gene expression in cultures treated with NO relative to basal level transcription revealed strong induction of the DosR regulon in WT M. tuberculosis (mean of 22.5-fold, maximum of 83.0-fold) but weaker induction in the ΔpknH mutant (mean of 11.6-fold, maximum of 42.4-fold) (Fig. 6B). Although this ~2-fold difference is relatively moderate, it is comparable with the 40–60% impaired induction observed in single knock-out mutants of DosS and DosT under hypoxic conditions (27, 34). The modest expression may also be due to compensating function(s) of other STPKs present in ΔpknH M. tuberculosis, as many of the STPKs appear to have substantial cross-talk activity (35). Nevertheless, these results indicate that PknH is required for full induction of the DosR regulon and agree with our EMSA analysis, suggesting that enhanced DNA binding of jointly Asp- and Thr-phosphorylated DosR leads to an increase in transcription of the regulon.
DISCUSSION

In this study, we have demonstrated for the first time that the dormancy regulon, an important and major regulatory response in the human pathogen *M. tuberculosis*, is controlled by two distinct signal transduction systems, the STPK and the TCS. We have shown that DosR is a substrate of PknH phosphorylation *in vitro* and in multiple cell-based systems. We also provide evidence that PknH phosphorylation of DosR enhances DosR dimerization and DNA binding, resulting in up-regulation of the DosR regulon in response to NO. A correlation between PknH and DosR has been suggested previously (36), and in this study, we provide the experimental basis to support this hypothesis.

Integration of these two types of signaling systems has been reported in other biological systems. In *Streptococcus agalactiae*, the STPK Stk1 phosphorylates the two-component response regulator CovR to repress CovR-dependent transcription of a secreted cytotoxin and to impede CovR transcriptional repression of a β-hemolysin/cytolysin gene (37) by inhibiting CovR-DNA binding (38). In *Myxococcus xanthus*, STPKs and a TCS coordinately regulate developmental changes in response to nutrient depletion. Expression of *mrpC*, encoding a transcription factor involved in fruiting body and myxospore formation, is transcribed by the TCS MrpAB but inhibited by Ser/Thr phosphorylation by the Pkn8/Pkn14 STPK cascade (39, 40). Convergence of STPKs and TCSs is also seen in eukaryotes where TCSs regulate activation of MAPK (Ser/Thr) signaling (41–43). Intriguingly, the HstK protein from the nitrogen-fixing *Anabaena* sp. PCC 7120 (44) and the NTHK2 ethylene receptor in tobacco plants (45) possess both Ser/Thr and histidine kinase activity. These examples demonstrate that STPKs and TCSs can be coupled to control a common signal transduction pathway.

Although further experiments are needed to elucidate the mechanism of action of PknH, the position of PknH phosphorylation suggests a potential means of post-translational regulation. Both phospho-Thr*₁⁹⁹* and phospho-Thr*₂⁰⁵* map to the critical regulatory helix α₁₀ in the crystal structure of DosR (31). As suggested by Wisedchaisri *et al.* (31), DosR activation is dependent on the flexibility of this helix. In their model, helix α₁₀ is in dynamic equilibrium in the closed-inactive conformation, bound to the N-terminal regulatory domain, burying the key Asp*₅⁴* residue, and in an open-inactive conformation, allowing Asp*₅⁴* to be solvent-exposed part of the time and thus available for Asp phosphorylation by DosS/T. Upon activation by Asp phosphorylation, helix α₁₀ provides the DosR dimerization interface in an open-active conformation for DNA binding. Phosphorylation of helix α₁₀ by PknH could potentially shift the equilibrium toward the open-active conformation of DosR, allowing for more efficient phosphorylation by DosS/T and activation of DosR. Alternatively, DosS/T phosphorylation may initiate conformational changes leading to the open-active conformation of DosR, whereas PknH phosphorylation may play a role in DosR dimerization.

Integration of PknH and DosS/T signal transduction systems controlling DosR activity would allow for tighter control of DosR-dependent activity. Activation of DosR by its cognate histidine kinases, DosS and DosT, results in a strong induction of the DosR regulon (27), and this induction is believed to be involved in metabolic changes that result in the pathogen entering a non-replicating persistent state. It is reasonable to expect mechanisms to be in place to prevent the pathogen from entering non-replicating persistence in the absence of an appropriate signal. Furthermore, nonspecific transcription and translation of the ~50 genes encoded in the DosR regulon would be considerably energy-costly. As a required second trigger (in addition to DosS/T) for full induction of the DosR regulon, PknH acts as a “molecular modulator” to repress nonspecific induction of the regulon and as an amplifier of the regulon in the presence of an appropriate signal.

The global proteomics approach proved to be a powerful tool for identifying key components in the PknH signal transduction pathway. However, our transcriptomic results seemingly contradict our proteomic data. The iTRAQ experiment was designed based on the enhanced survival of the *pknH* mutant in stationary phase growth exposed to lethal quantities of NaNO₂ (4) and was not designed to test specific DosR induction conditions. Furthermore, the 48-h time point tested in the iTRAQ experiment was well beyond the short-lived induction of the DosR regulon, whose gene expression largely returns to base line by 24 h (10, 46). Therefore, due to the difference in conditions and time points tested, the iTRAQ and qRT-PCR data cannot be directly compared. The somewhat discrepant results may indicate, however, that PknH also plays a role in inhibiting or turning off DosR regulon expression beyond the 24-h induction period. Further experiments, including a time-dependent analysis of DosR regulon expression under controlled conditions, would be required to test this hypothesis.

Deletion of *pknH* results in hypervirulence after 3–4 weeks of infection (4), corresponding to the induction of the host adaptive immune response and production of NO (47). It is tempting to speculate that the hypervirulence observed in the ΔpknH mutant may be mediated via signaling though DosR. An initial report indicated that deletion of *dosR* results in hypervirulence in mouse models (48). However, subsequent studies indicated that Δ*dosR* displays either attenuation or no difference in pathogenicity in mice, guinea pigs, and rabbits compared with WT *M. tuberculosis* (46, 49–51). Curiously, deletion of at least two members of the DosR regulon, *hspX* and *Rv2623*, each results in hypervirulence in mice (52, 53). Up-regulation of the DosR regulon has also been associated with hypervirulence, as genes belonging to the DosR regulon are constitutively up-regulated in the hypervirulent W-Beijing lineage of *M. tuberculosis* (54). It therefore remains a challenge to identify whether PknH signaling through DosR and/or the other known substrates contributes to the growth regulation and adaptation during the chronic or latent phase of infection.

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