

Inhibition of the DevSR Two-Component System by Overexpression of *Mycobacterium tuberculosis* PknB in *Mycobacterium smegmatis*

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The DevSR (DosSR) two-component system, which is a major regulatory system involved in oxygen sensing in mycobacteria, plays an important role in hypoxic induction of many genes in mycobacteria. We demonstrated that overexpression of the kinase domain of *Mycobacterium tuberculosis* (*Mtb*) PknB inhibited transcriptional activity of the DevR response regulator in *Mycobacterium smegmatis* and that this inhibitory effect was exerted through phosphorylation of DevR on Thr180 within its DNA-binding domain. Moreover, the purified kinase domain of *Mtb* PknB significantly phosphorylated RegX3, NarL, KdpE, TrcR, DosR, and MtrA response regulators of *Mtb* that contain the Thr residues corresponding to Thr180 of DevR in their DNA-binding domains, implying that transcriptional activities of these response regulators might also be inhibited when the kinase domain of PknB is overexpressed.

Keywords: DevSR, mycobacterium, Ser/Thr protein kinase, two-component system

INTRODUCTION

Mycobacterium tuberculosis (*Mtb*) is an obligatory aerobic bacterium that is a causative agent of tuberculosis. *Mtb* can persist in a non-replicating form in the immune-competent host and establish latent infection without exhibiting any

symptoms. When *Mtb* is phagocytosed by host macrophages, it encounters hostile environments such as hypoxic, low pH, and nutrient-limiting conditions, as well as reactive oxygen species- and reactive nitrogen species-challenging conditions (Russell, 2007; Rustad et al., 2009; Schnappinger et al., 2003), which are thought to lead to transition of *Mtb* to a latent state. Since gradual depletion of oxygen from mycobacterial cultures has been shown to lead to the transition of their growth state to a latency-like state, low oxygen tension was suggested as one of the most plausible determinants for latency transition of mycobacteria (Wayne and Sohaskey, 2001).

Phosphorelay through proteins in signal transduction pathways is a major mechanism by which environmental cues are transduced into appropriate cellular responses and adaptations. The two-component systems (TCSs) consisting of sensory histidine kinases (HKs) and response regulators (RRs) constitute the major signal transduction systems for regulation of gene expression and chemotaxis in prokaryotes (Stock et al., 2000; West and Stock, 2001). A HK normally phosphorylates only its cognate RR through specific protein-protein interactions, ensuring a linear signal transduction between a signal and its relevant effector (Laub and Goulian, 2007; Stock et al., 2000; West and Stock, 2001). In addition to TCSs, the importance of eukaryotic-like Ser/Thr protein kinases (STPKs) in prokaryotic signal transduction becomes increasingly magnified. In contrast to TCSs, a STPK can normally

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phosphorylate multiple substrates, thereby eliciting branched signaling and pleiotropic responses from a single signal in the signal transduction pathway (Leonard et al., 1998; Pereira et al., 2011; Shi et al., 1998; Wehenkel et al., 2008).

The genome of *Mtb* contains 11 paired TCSs as well as an equal number of STPKs (Cole et al., 1998; Fontan, 2004). Of 11 paired TCSs, the DosSR (DevSR) TCS is involved in the adaptation of mycobacteria to hypoxic and NO conditions (hereafter we use the terms “DosSR” for *Mtb* and “DevSR” for *Mycobacterium smegmatis*) (Kendall et al., 2004; Lee et al., 2008; Park et al., 2003; Sherman et al., 2001; Voskuil et al., 2003). In *Mtb*, the DosR RR is phosphorylated by the paralogous DosS and DosT HKs that contain *b*-type hemes in their N-terminal GAF-A domains (Cho et al., 2009; Park et al., 2003; Podust et al., 2008; Roberts et al., 2004; Saini et al., 2004). The kinase activity of DosS and DosT was shown to be controlled by the ligand-binding and redox state of the heme iron (Kumar et al., 2007; Sousa et al., 2007). The unliganded ferrous (deoxyferrous) forms of the HKs as well as NO- and CO-bound forms are active to phosphorylate DosR, while the O₂-bound (oxyferrous) and ferric forms are inactive. The DosR regulon in *Mtb* comprises approximately 48 genes whose expression is induced under hypoxic conditions as well as on exposure to NO (Kendall et al., 2004; Kim et al., 2010; Park et al., 2003; Sherman et al., 2001). The genes encoding the DevSR TCS are duplicated in *M. smegmatis* (*MSMEG_5241* and *MSMEG_3941* for DevS; *MSMEG_5244* and *MSMEG_3944* for DevR). However, only *MSMEG_5241* and *MSMEG_5244* appear to encode the functional DevSR TCS since the *hspX* gene, which is under the control of DevR, is not induced in *MSMEG_5241* or *MSMEG_5244* mutants grown under hypoxic conditions (Kim et al., 2010; Lee et al., 2008; 2012). The transcriptional activity of DosR was shown to be controlled not only by DosS/DosT, but also by PknH STPK in *Mtb* in a study conducted by Chao et al., (2010) who were the first to report the convergence of two major signaling systems (TCS and STPK) that utilize phosphorylation/dephosphorylation of proteins in mycobacteria.

The membrane-bound PknB is one of 11 STPKs in *Mtb* and conserved in all mycobacteria. PknB was shown to be essential for *Mtb* and *M. smegmatis* and to be implicated in cell elongation, division, cell wall synthesis, and regulation of oxygen-dependent cell replication (Chawla et al., 2014; Fernandez et al., 2006; Kang et al., 2005; Ortega et al., 2014).

The adaptive ability of *Mtb* to hypoxic conditions was recently reported to be impaired when *pknB* was overexpressed (Ortega et al., 2014). Based on this report and the fact that the DosSR (DevSR) TCS is involved in the hypoxic adaptation of mycobacteria, we hypothesized that there might be an interplay between the DosSR (DevSR) TCS and PknB for the mycobacterial adaptation to hypoxia. Here, we provide several lines of evidence that overexpression of *Mtb pknB* strongly inhibits the DevSR TCS in *M. smegmatis*, and that this inhibitory effect is exerted through phosphorylation of DevR on Thr180 in its DNA-binding domain.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in [Supplementary Table S1](#). *M. smegmatis* strains were grown in Middlebrook 7H9 medium (Difco, USA) supplemented with 0.2% (w/v) glucose as a carbon source and 0.02% (v/v) Tween 80 as an anticlumping agent at 37°C. *M. smegmatis* strains were grown either aerobically in a 500 ml flask filled with 100 ml of 7H9-glucose medium on a gyratory shaker (200 rpm) to an optical density at 600 nm (OD₆₀₀) of 0.4-0.5 or under hypoxic conditions in a 100 ml flask filled with 60 ml of 7H9-glucose medium and tightly sealed with a rubber stopper (the ratio of headspace volume to culture volume = 1) on a gyratory shaker (200 rpm) for 11 or 20 h following inoculation of the medium with aerobically grown preculture to an OD₆₀₀ of 0.05, which allowed a gradual depletion of O₂ from the growth medium. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37°C. Ampicillin (100 µg/ml for *E. coli*), kanamycin (50 µg/ml for *E. coli* and 15 µg/ml for *M. smegmatis*), and hygromycin (200 µg/ml for *E. coli* and 50 µg/ml for *M. smegmatis*) were added to the growth medium when required. Overexpression of STPKs from pMH201-derived plasmids was induced by the addition of acetamide to the growth medium to a final concentration of 0.2% (w/v). The construction of the plasmids used in this study is described in the Supplementary materials.

DNA manipulation and electroporation

Recombinant DNA manipulation was performed according to standard protocols or the manufacturers' instructions (Sambrook, 2012). The introduction of plasmids into *M. smegmatis* strains was accomplished by electroporation as previously described (Snapper et al., 1990).

Yeast two-hybrid assay

Saccharomyces cerevisiae AH109 strains cotransformed with both pGBKT7 and pGADT7linker derivatives were grown at 30°C in synthetic defined dropout (SD) medium (Q-Biogene, Canada) lacking leucine and tryptophan (SD/-Leu/-Trp). For spotting assay, all yeast cultures were harvested, resuspended in distilled water to an OD₆₀₀ of 0.5-0.6, and spotted onto both SD/-Leu/-Trp plates and histidine-deficient SD/-Leu/-Trp/-His plates containing appropriate concentrations of 3-amino-1,2,4-triazole (3AT; Sigma, USA). The SD/-Leu/-Trp and SD/-Leu/-Trp/-His plates were incubated at 30°C for 3 and 5 days, respectively.

β-galactosidase assay and determination of protein concentrations

β-galactosidase activity was measured spectrophotometrically as previously described (Oh and Kaplan, 1999). The protein concentration was determined using a BioRad protein assay kit (Bio-Rad, USA) with bovine serum albumin as a standard protein.

Reverse-transcription PCR

RNA isolation from *M. smegmatis* strains, preparation of cDNA, and reverse-transcription PCR (RT-PCR) were performed as previously described (Kim et al., 2010). The primers used in RT-PCR are as follows: the 16S rRNA gene

(5'-CTGGGACTGAGATACGGC-3' and 5'-ACAACGCTCGGACCCTAC-3'), the *hspX* gene (5'-GGGTCTGCCGTCGTGGGCC TC-3' and 5'-CGCCCGTTGGTCTCCTTCTTC-3'), and the *devS* (*MSMEG_5241*) gene (5'-GAGTTCATCTACGAGGGCAT-3' and 5'-CCGGAACGCCGAGGAACGTG-3').

Protein purification

The C-terminally His₆-tagged kinase domain (KD, amino acids 1-330) of *Mtb* PknB was overexpressed in the *E. coli* BL21 (DE3) strain harboring pETpknBhis, while the C-terminally His₆-tagged RR proteins were overexpressed in *E. coli* BL21 (DE3) strains harboring the corresponding pT7-7 derivatives (Supplementary Table S1). The strains were grown aerobically at 37°C in LB medium containing 50 µg/ml kanamycin (for pETpknBhis) or 100 µg/ml ampicillin (for the pT7-7 derivatives) to an OD₆₀₀ of 0.4 to 0.6. Expression of the *pknB* and RR genes was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, after which cells were further grown at 30°C for 4 h. Cells harvested from 200 ml culture were resuspended in 10 ml buffer A [20 mM Tris-HCl (pH 8.0) and 100 mM NaCl] and disrupted by two passages through a French pressure cell. Following DNase I treatment (10 units/ml) in the presence of 10 mM MgCl₂ for 30 min on ice, cell-free crude extracts were obtained by centrifugation twice at 10,000 × *g* for 20 min. Next, a 0.4 ml of the 80% (v/v) slurry of Ni-Sepharose high-performance resin (GE Healthcare, USA) was added to the crude extracts and mixed gently by shaking for 2 h on ice. The protein-resin mixture was then packed into a column. The resin was washed with 40 bed volumes of buffer A containing 5 mM imidazole, 40 bed volumes of buffer A containing 10 mM imidazole, 40 bed volumes of buffer A containing 50 mM imidazole, and then His₆-tagged proteins were finally eluted with 6 ml of buffer A containing 200 mM imidazole. Fractions from the elution step were collected and desalted by dialysis against 20 mM Tris-HCl (pH 8.0), after which the dialyzed proteins were concentrated using a centrifugal filter device (Millipore, USA).

In vitro kinase assay

Purified PknB KD was mixed with purified RR in 20 µl of reaction buffer [20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂]. Reactions were started by adding 100 µM cold ATP and 1,000 Ci/mol [γ -³²P]ATP and incubated at 30°C. Reactions were terminated by adding 5 µl of gel-loading buffer [50 mM Tris-HCl (pH 8.0), 4% (w/v) sodium dodecyl sulfate (SDS), 20% (w/v) glycerol, 20 mM dithiothreitol (DTT), 1% (v/v) β-mercaptoethanol, and 0.1% (w/v) bromophenol blue] containing 100 mM EDTA. Proteins were resolved by SDS-PAGE. The gel was dried for 2 h and exposed to a film at room temperature overnight.

Western blotting analysis

To determine the cellular abundance of expressed DevR, PknB, PknH, and PknG, Western blotting analysis was performed as described elsewhere (Mouncey and Kaplan, 1998). Mouse monoclonal IgG against His₆-tagged proteins (Santa Cruz Biotech., USA) was used at a 1:2,000 dilution.

Alkaline phosphatase-conjugated anti-mouse IgG (Sigma, USA) was used at a 1:10,000 dilution for detection of the primary antibody.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was conducted using an EMSA kit (Invitrogen, USA) according to the manufacturer's instructions. Briefly, 204 bp DNA fragments containing a DevR-binding site upstream of the *hspX* gene were generated by PCR using pNChspX as a template and the primers F_hspX_EMSA (5'-ACTCCGACGACGAGTTTCGC-3') and R_hspX_ClaI (5'-ATATATCGATCGTTCAGGAAGTTGG-3'). DNA-binding reaction mixtures were composed of appropriate amounts of DNA (2 µl), either purified wild-type (WT) DevR or T180E DevR (5 µl), 1 µl of 20 mM Tris (pH 8.0), and 2 µl of 5× binding buffer [750 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, and 50 mM Tris-HCl (pH 7.5)]. The binding reaction mixtures were incubated for 20 min at room temperature. After the addition of 2 µl of 6× loading buffer (included in the kit), the samples were subjected to nondenaturing PAGE [6% (w/v) acrylamide] in running buffer (pH 8.3) containing 83 mM Tris-borate and 1 mM EDTA at 60 V for 5 h at 4°C. The gels were stained with SYBR green staining solution (included in the kit) and the DNA bands were visualized by a UV-transmitter.

RESULTS

Identification of *Mtb* RRs that are phosphorylated by PknB in vitro

The stringent regulation of *pknB* expression is important for mycobacterial fitness and survival: e.g., overexpression of the *pknB* gene in *Mtb* and *M. smegmatis* was demonstrated to result in a slowdown of their growth and even cell death (Chawla et al., 2014; Kang et al., 2005), indicating that protein phosphorylation by overexpressed PknB disturbs metabolism or gene regulation critical for mycobacterial growth. Since TCSs play important roles in both bacterial signal transduction and gene regulation and some TCSs are known to be essential for mycobacterial survival, it is reasonable to assume that there might be TCSs whose functionality is controlled by PknB. We initially attempted to identify the RRs of *Mtb* that are phosphorylated by the purified KD (amino acids 1-330) of *Mtb* PknB in vitro. For this experiment, 11 RRs and PknB KD of *Mtb* were overexpressed as His₆-tagged proteins in *E. coli*, then purified by means of affinity chromatography. In vitro phosphorylation assay using the purified proteins revealed that PknB KD was autophosphorylated in the presence of ATP and that RegX3, NarL, KdpE, TrcR, DosR, and MtrA were more strongly phosphorylated by PknB KD than were PhoP, PrrA, MprA, TcrX, and PtdaR as judged by the intensity of the phosphorylated bands (Fig. 1). It is worth noting that PtdaR containing no DNA-binding domain was only very marginally phosphorylated by PknB KD. In the absence of PknB KD in the reaction mixtures, no phosphorylation of the RRs occurred, ruling out autophosphorylation of the RRs.

Multiple alignment of the amino acid sequences of the *Mtb* RRs revealed that the effector domains of the RRs

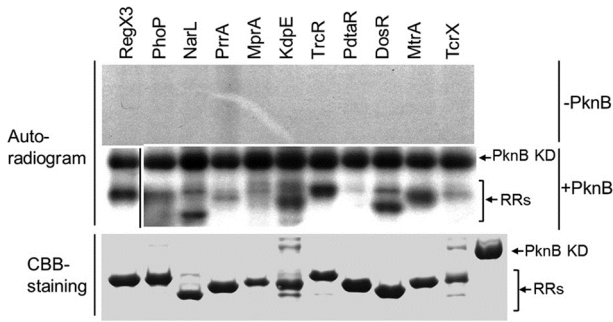


Fig. 1. *In vitro* phosphorylation of *Mtb* RRs by the KD of *Mtb* PknB. Phosphorylation reactions of the RRs (0.2 nmol each) were conducted in the presence (+PknB) and absence (-PknB) of 0.2 nmol of purified PknB KD. Reactions were started by adding 100 μ M cold ATP and 1,000 Ci/mole [γ - 32 P] ATP and incubated at 30°C for 30 min. After reactions were terminated, proteins were resolved by SDS-PAGE. Phosphorylated proteins were detected by autoradiography. 0.2 nmol each of purified RRs and 0.2 nmol of PknB KD were subjected to SDS-PAGE and the gel was stained by Coomassie brilliant blue (CBB). The stained gel is presented below the autoradiogram to compare the size and amounts of the purified proteins used in the experiment.

belonging to the OmpR family (RegX3, KdpE, TrcR, MtrA, PrrA, PhoP, MprA, and TcrX) are well aligned with one another, while the effector domains of DosR and NarL of the NarL family are not well aligned with those of the OmpR family RRs. The Thr residues, which are located within the helix-turn-helix motifs and likely correspond to Thr180 of DosR, are conserved in all strongly phosphorylated RRs with the exception of PrrA (Fig. 2A), indicating the possibility that the Thr residues are the candidates that can be phosphorylated by PknB. Of six RRs that were strongly phosphorylated by PknB, we chose DosR to study further regarding the regulation of RR transcriptional activity by PknB.

Protein-protein interactions between DosR and PknB

We used the yeast two-hybrid (Y2H) assay to assess protein-protein interactions between DosR and PknB of *Mtb*. At the same time, the other 10 STPKs of *Mtb* were also examined for protein interactions with DosR (Fig. 3). The *dosR* gene was cloned into the prey vector pGADT7linker and the gene portions of the STPK KDs were cloned into the bait vector pGBKT7. The bait plasmids containing the gene portions of the DosS and DosT KDs were employed in Y2H assay as positive

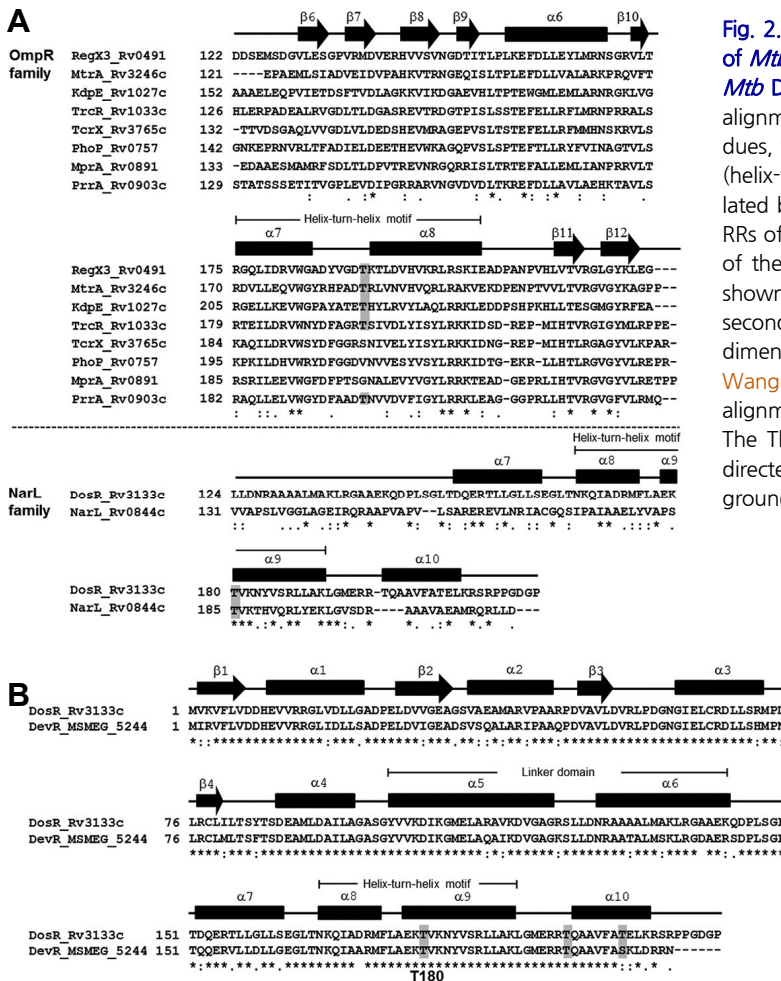


Fig. 2. Multiple alignment analysis of the effector domains of *Mtb* RRs and comparison of the amino acid sequence of *Mtb* DosR with that of *M. smegmatis* DevR. (A) Multiple alignment was generated using ClustalW2. The Thr residues, which are located within the DNA-binding domains (helix-turn-helix motifs) and predicted to be phosphorylated by PknB, are indicated by the gray background. The RRs of the OmpR family are aligned separately from those of the NarL family. The relevant secondary structures are shown above the aligned sequences. The position of the secondary structures is based on the determined three-dimensional structures of PhoP and DosR (Menon and Wang, 2011; Wisedchaisri et al., 2008). (B) Sequence alignment indicates 85% identity between DevR and DosR. The Thr and Ser residues, which were subjected to site-directed mutagenesis, are indicated by the gray background (Thr180, Thr198, and Ser205).

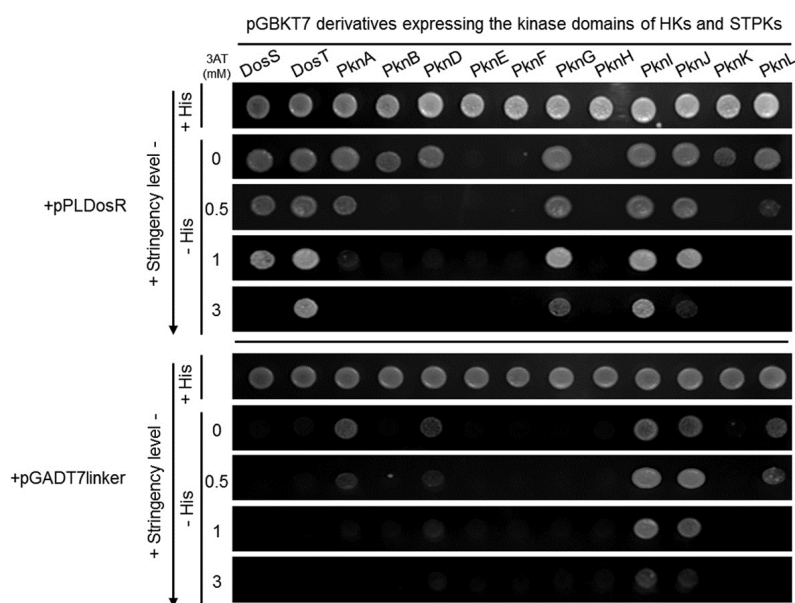


Fig. 3. Determination of protein-protein interactions between DosR and STPKs of *Mtb* in Y2H assay. The gene portions encoding the KDs of *Mtb* STPKs, DosS, and DosT were cloned into pGBKT7 (encoding the GAL4 DNA-binding domain). The *dosR* gene was cloned into pGADT7linker (encoding the GAL4 activation domain), resulting in pPLDosR. The yeast strains cotransformed with both pGBKT7 derivatives and pPLDosR were used for Y2H assay. To discriminate false-positive interactions, the empty pGADT7linker was introduced into the yeast strains with the pGBKT7 derivatives in place of pPLDosR and the resulting yeast strains were used as negative controls. All yeast strains were spotted onto SD/-Leu/-Trp plates (+His) and histidine-deficient SD/-Leu/-Trp/-His plates containing 3AT (-His).

controls. Consistent with the previously reported results (Lee et al., 2012), the yeast strains coexpressing DosR and either DosS or DosT grew well in the absence of histidine (-His) with a stringency level of 1 or 3 mM 3AT, indicating protein-protein interactions between DosR and its cognate HKs (DosS and DosT). Coexpression of PknA, PknB, PknD, PknG, PknI, PknJ, PknK, or PknL with DosR led to growth of yeast strains in the absence of histidine. However, in the case of PknA, PknD, PknI, PknJ, and PknL, the yeast strains expressing the STPKs alone grew in the absence of histidine as well. The yeast strain coexpressing PknB and DosR did not grow on -His medium in the presence of 3AT, implying weak and transient interactions between PknB and DosR. Although we do not know whether PknA, PknD, PknI, PknJ, and PknL interact with DosR due to the false-positive results observed in Y2H assay, this result indicates the possible interaction of DosR with PknB, PknG, and PknK.

Overexpression of *pknB* inhibits DevSR TCS

We used *M. smegmatis* expressing the KD of *Mtb* PknB in place of *Mtb* to examine the *in vivo* effects of *pknB* overexpression on the transcriptional activity of DosR based on the following facts: *M. smegmatis* contains DevR (MSMEG_5244) whose primary structure is 85% identical to that of *Mtb* DosR. Thr180 is conserved in both DevR and DosR. Thr198 and Thr205 in DosR, which were reportedly phosphorylated by PknH (Chao et al., 2010), are also present as Thr198 and Ser205 in DevR, respectively (Fig. 2B).

We examined whether overexpression of *Mtb* PknB KD influences the transcriptional activity of DevR in *M. smegmatis* (Fig. 4). The overexpression effect of PknG was assessed due to the strong interaction between PknG and DosR observed in the Y2H assay. Since it was reported that phosphorylation of DosR on Thr198 and Thr205 by PknH led to an increase in DosR transcriptional activity (DNA-binding affinity) in *Mtb*

(Chao et al., 2010), PknH was also included in this experiment. Overexpression of PknK was reported to lead to an overall reduction in protein synthesis by inhibiting transcription, translation, and tRNA synthesis (Malhorta et al., 2012). Therefore, we did not examine the effects of PknK overexpression on DevR transcriptional activity. The gene portions encoding the cytoplasmic KDs of PknB and PknH were overexpressed from an acetamide-inducible promoter on the pMH201 integration vector, while the whole gene of *pknG* was expressed from the same vector because PknG KD alone without the C-terminal domain is known to be inactive (Baer et al., 2014).

The transcriptional activity of DevR in *M. smegmatis* was determined by measuring the expression level of *hspX* that is under the control of the DevSR TCS. *M. smegmatis* strains harboring an *hspX::lacZ* transcriptional fusion plasmid (pNChspX) and an integrated STPK-expressing plasmid (pMHPknB, pMHPknG, or pMHPknH) were grown under 20 h hypoxic conditions and β -galactosidase activities were determined. The *M. smegmatis* strain containing both pNChspX and the empty vector pMH201 grown under aerobic and 20 h hypoxic conditions was included in the experiment as a negative control. As shown in Fig. 4A, *hspX* was only marginally expressed in the control strain grown aerobically, and its expression was strongly induced in the same strain grown under hypoxic conditions. The expression level of *hspX* was drastically reduced in the *M. smegmatis* strain with pMHPknB grown under hypoxic conditions relative to the control strain with pMH201 grown under the same conditions. In contrast, overexpression of *pknG* and *pknH* did not affect expression of *hspX* under hypoxic conditions. Crude extracts of *M. smegmatis* strains with the integrated STPK-expressing plasmids showed the corresponding STPK bands in Western blotting analysis using a His-tag antibody, whereas those of the control strain with pMH201 did not, confirming expression

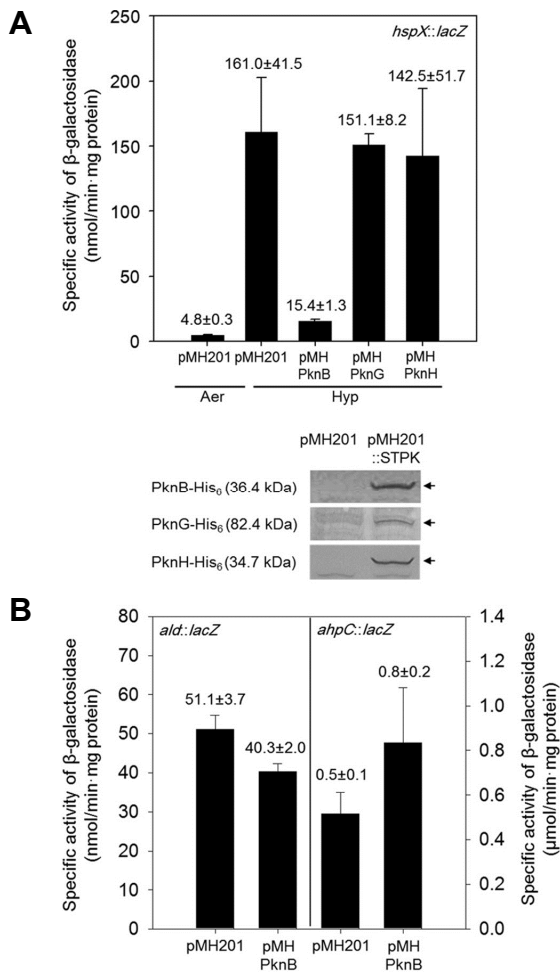


Fig. 4. Effect of PknB overexpression on expression of *hspX*, *ald*, and *ahpC* in *M. smegmatis*. For overexpression of PknB KD, PknH KD, and PknG in *M. smegmatis*, the pMH201 derived plasmids, pMHPknB, pMHPknH, and pMHPknG, were employed, respectively. (A) The promoter activity of *hspX* was measured by determining β -galactosidase activity in *M. smegmatis* strains harboring both an *hspX::lacZ* transcriptional fusion plasmid (pNChspX) and one of pMHPknB, pMHPknH, or pMHPknG. As a negative control, the *M. smegmatis* strain with both pNChspX and pMH201 was included in the experiment. The *M. smegmatis* strains were grown either aerobically to an OD₆₀₀ of 0.4 to 0.5 (Aer) or under hypoxic conditions for 20 h (Hyp) in the presence of 0.2% (w/v) acetamide. (B) The promoter activity of *ald* was determined using the *M. smegmatis* strains harboring an *ald::lacZ* transcriptional fusion plasmid (pALDLACZ) and either pMH201 or pMHPknB, which were grown aerobically to an OD₆₀₀ of 0.4 to 0.5 in the presence of both 0.2% (w/v) acetamide and 25 mM alanine. The promoter activity of *ahpC* was determined using an *ahpC::lacZ* transcriptional fusion plasmid (pNCM3) in *M. smegmatis* strains grown aerobically to an OD₆₀₀ of 0.4–0.5 in the presence of 0.2% (w/v) acetamide. All values provided are the averages of the results from three independent determinations. Error bars indicate standard deviations. Western blot analysis was conducted for detection of the expressed PknB KD, PknH KD, and PknG. Cell-free crude extracts (20 μ g) were separated on SDS-PAGE, followed by Western blot analysis with a His-tag antibody.

of the STPK genes in *M. smegmatis*. There is the possibility that overexpressed PknB could inhibit transcription and translation machinery or β -galactosidase itself by protein phosphorylation, similar to PknK, thereby decreasing the synthesis and activity of β -galactosidase from the *lacZ* transcriptional fusion. To evaluate this possibility, we determined the expression level of the *ald* gene, which encodes alanine dehydrogenase and is not regulated by the DevSR TCS, by using an *ald::lacZ* transcriptional fusion (pALDLACZ) (Jeong et al., 2013). When aerobically grown in the presence of alanine and acetamide, the *M. smegmatis* strain with pMHPknB exhibited only a 21% decrease in *ald* expression relative to the control strain with pMH201 (Fig. 4B). We also examined the effects of *pknB* overexpression on expression of the *ahpC* gene encoding alkyl hydroperoxide reductase. For this experiment, we used an *ahpC::lacZ* transcriptional fusion (pNCM3) that contains mutations within the FurA-binding site and is therefore regulated only by CRP (cAMP receptor protein) (Lee et al., 2014). Overexpression of *pknB* resulted in a rather modest increase in *ahpC* expression compared to the control strain with pMH201 (Fig. 4B). Taken together, our results indicate that the strong inhibition of *hspX* expression by *pknB* overexpression is not caused by nonspecific general inhibition of gene expression.

Before further studying the effects of *pknB* overexpression on the transcriptional activity of DevR in detail, we examined the overexpression effect of *Mtb* PknB KD on hypoxic growth of *M. smegmatis*. When *M. smegmatis* strains were grown under hypoxic conditions, the *M. smegmatis* strain with pMHPknB grew to an OD₆₀₀ of 0.3–0.4 (11 h after inoculation) equally well to the control strain with pMH201. After that point, growth of *M. smegmatis* was negatively affected by overexpression of PknB KD and the *M. smegmatis* strain with pMHPknB reached a plateau at the lower OD₆₀₀ value than the control strain with pMH201 (Fig. 5A). The OD₆₀₀ value of the *M. smegmatis* strain with pMHPknB decreased rapidly after 20 h of hypoxic growth, which was attributable to cell aggregations in liquid medium. This phenomenon (cell aggregations) was also observed in aerobic cultures of *M. smegmatis* with pMHPknB when the OD₆₀₀ reached 0.45–0.5 (Supplementary Fig. S1), indicating that overexpression of PknB KD results in changes in cell surface properties. Overexpression of PknB KD of *M. smegmatis* also inhibited the growth of *M. smegmatis* (Supplementary Fig. S1).

To confirm that overexpression of *Mtb* PknB KD led to a decrease in *hspX* expression at the transcriptional level, we determined the level of the *hspX* transcript by means of RT-PCR. We also examined the effects of *pknB* overexpression on expression of *devS* that also belongs to the DevR regulon (Mayuri et al., 2002). As shown in Fig. 5B, expression of *hspX* was induced in the *M. smegmatis* strain with pMH201 grown under 11 h hypoxic conditions and even more induced under 20 h hypoxic conditions relative to that in the same strain grown aerobically, while the hypoxic (11 and 20 h) induction of *hspX* was almost completely abolished in the *M. smegmatis* strain harboring pMHPknB. The hypoxic (20 h) induction of *devS* was also inhibited in the *M. smegmatis* strain with pMHPknB, confirming the inhibition of DevSR TCS by *pknB* overexpression (Fig. 5B).

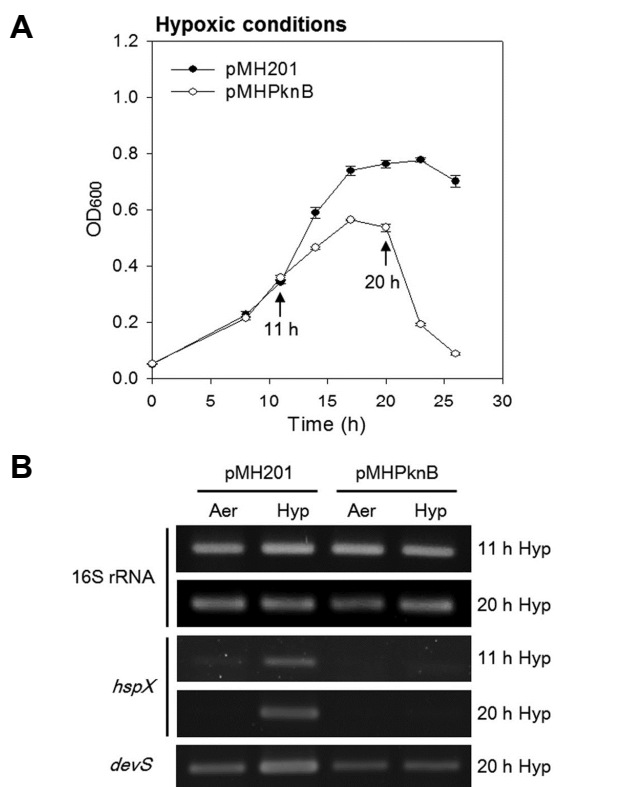


Fig. 5. Effect of PknB overexpression on hypoxic growth of *M. smegmatis* and expression of *hspX* and *devS* at the transcriptional level. (A) The *M. smegmatis* strains harboring either pMHPknB or pMH201 were grown in 7H9 medium containing 0.2% (w/v) acetamide under hypoxic conditions and their growth was monitored spectrophotometrically at 600 nm. All values provided are the averages of the results from two independent determinations. Error bars indicate deviations from the means. (B) The *M. smegmatis* strains harboring pMHPknB or pMH201 were grown either aerobically to an OD₆₀₀ of 0.4 to 0.5 (Aer) or under hypoxic conditions for 11 and 20 h (Hyp) in the presence of 0.2% (w/v) acetamide. The expression levels of *hspX* and *devS* were determined by RT-PCR. RT-PCR of the 16S rRNA gene was conducted to ensure that the same amounts of total RNA were employed for RT-PCR.

Phosphorylation of Thr180 in DevR is responsible for inhibition of DevR transcriptional activity by PknB

In vitro kinase assay with purified RRs and multiple alignment analysis (Figs. 1 and 2) strongly suggested that Thr180 of DevR is the amino acid residue phosphorylated by PknB KD. To investigate whether Thr180 in DevR undergoes phosphorylation by PknB, we conducted an *in vitro* phosphorylation assay using the WT and T180A mutant forms of DevR as well as the purified KD of *Mtb* PknB. The T198A and S205A mutant forms of DevR were also included in this phosphorylation experiment, since the corresponding amino acid residues of DosR had been reported to be phosphorylated by PknH of *Mtb* (Chao et al., 2010). As shown in Fig. 6A, phosphorylation assay revealed that phosphorylation of

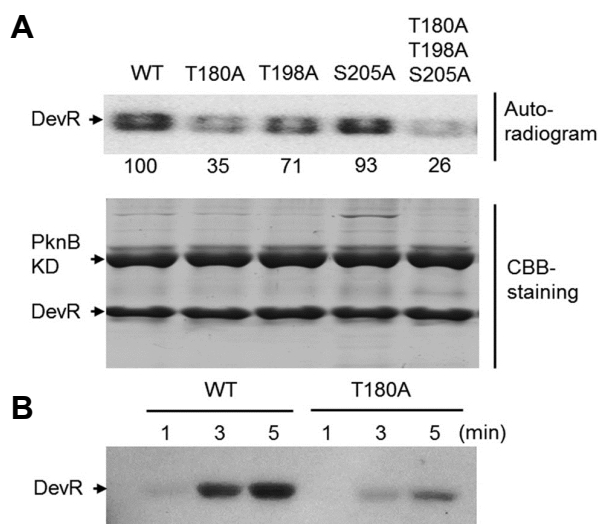


Fig. 6. Effect of point mutations on phosphorylation of DevR by PknB KD. (A) 0.1 nmol of PknB KD was mixed with 0.1 nmol of WT or mutant forms of DevR in 20 μ l of reaction buffer. Reactions were started by adding 100 μ M cold ATP and 1,000 Ci/mole [γ -³²P] ATP, incubated at 30°C for 5 min, and terminated. Phosphorylated proteins were detected by autoradiography. The phosphorylation extent of the WT and mutant forms of DevR was estimated by using a densitometer program, ImageJ (v1.37). The phosphorylation extent of WT DevR is set at 100 and the relative values are expressed for the mutant forms of DevR. Before phosphorylation assay, the mixtures of 0.1 nmol of purified PknB KD and 0.1 nmol each of DevR protein were subjected to SDS-PAGE and the gel was stained by Coomassie brilliant blue (CBB). As a loading control, the stained gel is presented below the autoradiogram to compare the amounts of purified WT and mutant forms of DevR used in the experiment. (B) Phosphorylation reactions were performed using 0.05 nmol of PknB KD and 0.1 nmol of WT and T180A DevR in the time course. The reactions were terminated at the time points indicated. Phosphorylated proteins were detected by autoradiography.

T180A DevR by PknB was strongly reduced when compared with WT DevR. Inhibition of DevR phosphorylation also occurred for T198A DevR, but the extent of inhibition was much less prominent than that observed for T180A DevR. In contrast, the S205A mutation did not affect DevR phosphorylation by PknB. A cumulative effect of T180A and T198A mutations on DevR phosphorylation was observed in DevR with triple mutations (T180A/T198A/S205A). The T180A/T198A/S205A mutant form of DevR was slightly phosphorylated, which could be explained by the fact that the substrate specificity of mycobacterial STPKs is not stringent (Prisic et al., 2010). The phosphorylation assay in the time course confirmed that the phosphorylation rate of T180A DevR by PknB was severely affected relative to that of WT DevR (Fig. 6B), indicating that Thr180 in DevR is the major phosphorylation site.

To examine the importance of Thr180, Thr198, and Ser205 in the transcriptional activity of DevR *in vivo*, we measured

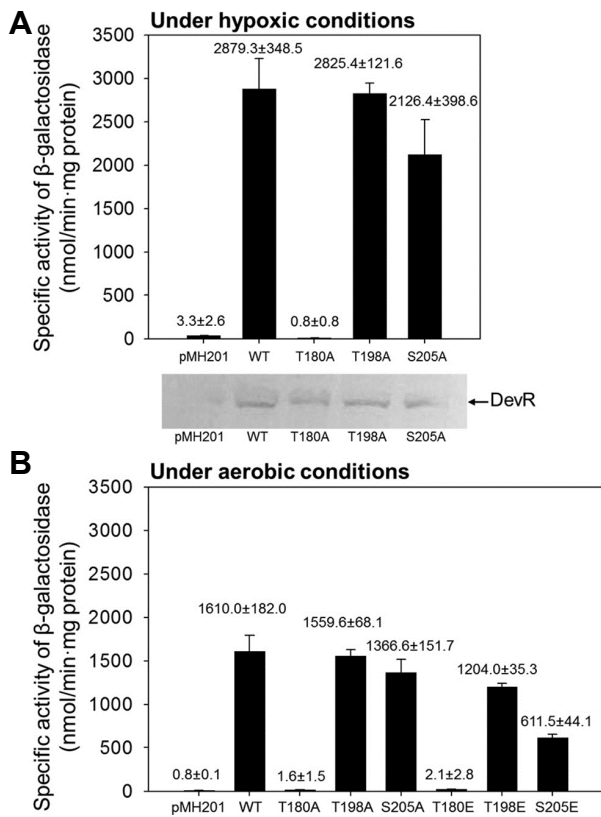


Fig. 7. Effect of point mutations on the transcriptional activity of DevR *in vivo*. The transcriptional activities of the mutant forms of DevR were assessed by determining the expression level of *hspX* in *M. smegmatis* $\Delta devR$ mutant strains complemented with the WT or mutant *devR* genes. To express *devR*, the genes encoding the WT and mutant forms of DevR (T180A, T198A, S205A, T180E, T198E, and S205E) were cloned into pMH201. The empty pMH201 vector and its derivatives were integrated into the chromosomal DNA of the *M. smegmatis* $\Delta devR$ mutant harboring pNChspX. Complementation tests were performed by determining the β -galactosidase activity in strains grown under either 20 h hypoxic (A) or aerobic (B) conditions. All values provided are the averages of the results from three independent determinations. Error bars indicate standard deviations. Western blot analysis was performed for the detection of expressed DevR and DevR derivatives in *M. smegmatis* strains grown under 20 h hypoxic conditions. Crude extracts (50 μ g) were separated on SDS-PAGE, followed by Western blot analysis with a His-tag antibody.

the expression of *hspX* in *M. smegmatis* $\Delta devR$ (*MSMEG_5244*) strains expressing the WT or mutant forms (T180A, T198A, and S205A) of DevR (Fig. 7A). As expected, *hspX* was not expressed in the $\Delta devR$ mutant with the empty vector pMH201 under hypoxic conditions, while the $\Delta devR$ mutant strain complemented with the WT *devR* gene showed strong expression of *hspX* under hypoxic conditions. When the $\Delta devR$ mutant was complemented with T180A *devR*, expression of *hspX* was completely abolished under

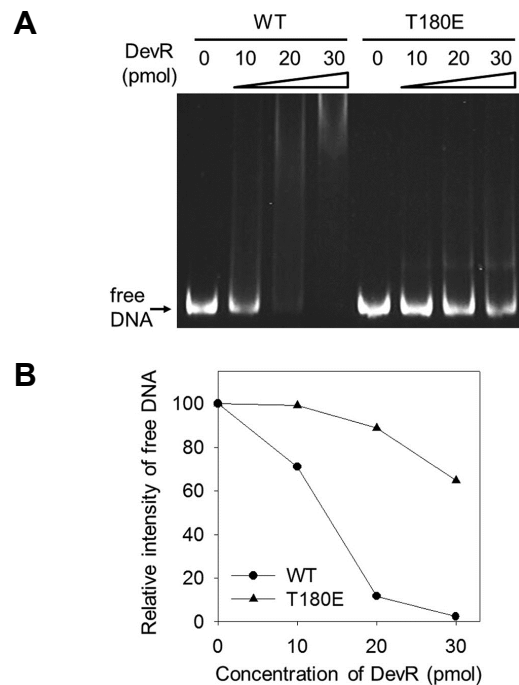


Fig. 8. EMSA showing the binding of purified WT and T180E mutant forms of DevR to the *hspX* control region. (A) The 204 bp DNA fragments (100 fmol corresponding to 12.6 ng) containing the DevR-binding site upstream of *hspX* were incubated with various concentrations of purified WT and T180E DevR. The amounts of WT DevR and T180E DevR are given above the lanes. The arrow indicates the bands of free DNA. (B) Quantitation of the band intensity of free DNA in panel A was performed using the ImageJ densitometry program (v1.37). The band intensity of the free DNA without DevR is set at 100 and the relative values are plotted as a function of the DevR concentration.

hypoxic conditions. In contrast, T198A mutation did not affect *hspX* expression and S205A mutation led to a slight (26%) decrease in *hspX* expression relative to the mutant strain expressing the WT *devR* gene. Western blotting analysis using a His-tag specific antibody showed that the WT and mutant forms of DevR were synthesized in *M. smegmatis* mutant strains. Taken together, these results indicate that Thr180 is critical to the transcriptional activity of DevR.

We performed the experiment using the same *M. smegmatis* strains grown aerobically (Fig. 7B). Overexpression of WT *devR* resulted in strong expression of *hspX* even in the $\Delta devR$ mutant grown aerobically, although the expression level was lower under aerobic conditions than that under hypoxic conditions. Since the DevS HK is known to be inactive for DevR phosphorylation under aerobic conditions, the aerobic expression of *hspX* can be explained by a high dose effect of DevR. The expression pattern of *hspX* in aerobically grown $\Delta devR$ strains expressing the WT and mutant forms (T180A, T198A, and S205A) was very similar to that observed for the same strains grown under hypoxic conditions, confirming the importance of Thr180 in the functionality of DevR. To indirectly assess the phosphorylation effects of

Thr180, Thr198, and Ser205 on the transcriptional activity of DevR, phosphomimetic mutations (T180E, T198E, and S205E) were introduced into DevR and the expression level of *hspX* was measured in the aerobically grown $\Delta devR$ mutants expressing the mutant forms of DevR (Fig. 7B). The T180E mutation abrogated the transcriptional activity of DevR. The T198E and S205E mutations partially inhibited the DevR activity. Since Thr180 in DevR is the major site phosphorylated by PknB *in vitro*, this result implies that inhibition of *hspX* expression by *pknB* overexpression primarily resulted from phosphorylation of DevR on Thr180.

Given that Thr180 is located within the helix-turn-helix motif of DevR and involved in binding of the phosphate backbone on the target DNA (Wisedchaisri et al., 2008), we assumed that phosphorylation of Thr180 by PknB negatively affects the DNA-binding affinity of DevR. To examine this assumption, we compared the DNA-binding affinity of WT DevR with that of T180E phosphomimetic DevR by EMSA using 204 bp DNA fragments containing the upstream control region of *hspX*. As shown in Fig. 8, the DNA fragments were retarded by purified T180E DevR to a much lesser extent than by WT DevR as judged by the level of free DNA. We did not observe the distinct retarded bands in this EMSA, which might be due to a weak DNA-binding affinity of unphosphorylated DevR (the DevR proteins purified from *E. coli* were not phosphorylated on Asp54). These results indicate that phosphorylation of DevR on Thr180 significantly reduces the binding affinity for its target DNA sequence, thereby inhibiting the transcriptional activity of DevR.

DISCUSSION

PknB has a modular structure composed of an N-terminal cytoplasmic KD and a C-terminal extracytoplasmic domain consisting of four PASTA (penicillin binding protein and Ser/Thr kinase associated) repeats (Yeats et al., 2002; Young et al., 2003). The PASTA domain of PknB was shown to bind muropeptides of peptidoglycan and the binding of muropeptides to the extracellular domain of PknB was suggested to activate its kinase activity (Mir et al., 2011; Shah et al., 2008). PknB proteins are localized to the septa of dividing bacteria and the cell poles, where hydrolysis and assembly of peptidoglycan for cell wall synthesis actively occur (Mir et al., 2011). Furthermore, the cellular abundance of PknB was shown to be drastically reduced in *Mtb* exposed to hypoxic conditions when compared with that in actively dividing cells (Ortega et al., 2014). Both findings imply that PknB activity is likely much higher in actively dividing mycobacteria than in non-replicating mycobacteria.

Constitutive expression of *pknB* under both aerobic and hypoxic conditions in *Mtb* led to impaired survival when growth of *Mtb* was shifted from aerobic to hypoxic conditions (Ortega et al., 2014). Based on these reports and the fact that the DevSR (DosSR) TCS is involved in the hypoxic adaptation of mycobacteria, we assumed that high activity of PknB under aerobic conditions might inhibit the DevSR (DosSR) TCS to some extent, and that the reduced activity of PknB during the transition period from aerobic to hypoxic conditions might mitigate the inhibitory effect of PknB on

DevSR (DosSR), thereby facilitating mycobacterial adaptation to hypoxic conditions. Indeed, overexpression of *Mtb* PknB KD in *M. smegmatis* resulted in growth inhibition under hypoxic conditions, as well as in a robust inhibition of the DevSR TCS, which was manifested by a substantial decrease in *hspX* expression under the conditions of *pknB* overexpression. Since the PknB KDs of *M. smegmatis* and *Mtb* show very high homology at the level of their primary structures (Supplementary Fig. S2), overexpression of *M. smegmatis* PknB KD is likely to inhibit the DevSR TCS as well. Although we did not examine the reverse effect in a *pknB* knockout mutant of *M. smegmatis* due to the indispensability of PknB in mycobacteria, both phosphorylation of DevR on Thr180 by purified PknB KD of *Mtb* and abolishment of the transcriptional and DNA-binding activity of the T180E phosphomimetic DevR indicate that PknB inhibits the transcriptional activity of DevR through phosphorylation of the Thr180 residue located within the DNA-binding helix-turn-helix motif.

Signal convergence between TCSs and STPKs has been reported to occur in several regulatory systems. For example, in group A and group B, streptococci phosphorylation of the CovR RR on Thr65 by a STPK (Stk or Stk1) was shown to inhibit phosphorylation of CovR by acetyl phosphate (therefore, probably by CovS HK), thereby reducing the transcriptional activity of CovR (Horstmann et al., 2014; Lin et al., 2009). The GraR RR of the GraSR TCS of *Staphylococcus aureus* was shown to be phosphorylated on Thr128, Thr130, and Thr149 by a PknB-like STPK, Stk1, which resulted in an increase in DNA-binding affinity of GraR in contrast to DevR and CovR (Fridman et al., 2013). The modulation of TCS activity by a STPK was also reported in mycobacteria. The DosR RR of *Mtb* is phosphorylated on Thr198 and Thr205 by PknH, and phosphorylation of DosR enhances its DNA-binding affinity, thereby increasing DosR transcriptional activity (Chao et al., 2010). However, our study showed that overexpression of *Mtb* PknH KD in *M. smegmatis* did not change the DevR transcriptional activity as judged by the expression level of *hspX* (Fig. 4). This disparity might be a result of the occurrence of Ser205 in DevR in place of Thr205 in DosR. It has been suggested that PknH has a strong preference for Thr over Ser (Prisic et al., 2010). This implies that, if phosphorylation of both Thr198 and Ser205 is necessary for the enhancement of DevR transcriptional activity, overexpression of *pknH* would not increase DevR transcriptional activity in *M. smegmatis*. *M. smegmatis* has 13 STPK genes in its genome. Sequence analysis revealed that there is no gene encoding a close homolog of PknH in the genome of *M. smegmatis* (Narayan et al., 2007). The absence of both the PknH homolog and *pknH* overexpression effect in *M. smegmatis* indicates that the functionality of DevSR TCS from this organism is not regulated by PknH.

We found that the Thr residues corresponding to Thr180 of DevR are conserved in the DNA-binding helix-turn-helix motifs of RegX3, KdpE, TrcR, MtrA, PrrA, and NarL RRs and that the five RRs except PrrA were as strongly phosphorylated as DevR by purified PknB KD. These findings raise the possibility that the transcriptional activities of RegX3, KdpE, TrcR, MtrA, and NarL might be inhibited by *pknB* overexpression. Indeed, when *pknB* was overexpressed in *M. smegma-*

tis, expression of the *phoA* gene, which encodes alkaline phosphatase and is under the control of the SenX3-RegX3 TCS, was significantly (90%) reduced (manuscript in preparation). Furthermore, our *in vitro* phosphorylation assay with the mutant forms of RegX3 revealed that RegX3 RR was phosphorylated on Thr191, corresponding to Thr180 of DevR by purified PknB KD (manuscript in preparation). Previous studies suggested that RegX3 and MtrA were essential for growth of *M. smegmatis* and *Mtb*, respectively (Glover et al., 2007; James et al., 2012; Zahrt and Deretic, 2000). MtrA is also known to be involved in the regulation of mycobacterial proliferation by acting as both an *oriC*-binding protein and a transcriptional activator for the *dnaA* gene whose product is a key initiator protein for DNA replication at *oriC* (Fol et al., 2006). Furthermore, a *mtrB*-knockout mutant of *M. smegmatis* was shown to have a filamentous shape and impaired cell division, which is reminiscent of the phenotype associated with *M. smegmatis* overexpressing *pknB* (Plocinska et al., 2012). Based on these reports, it can be inferred that one of the reasons for the negative effects of *pknB* overexpression on the growth of *Mtb* and *M. smegmatis* might be inhibition of the essential TCSs like MtrBA and SenX3-RegX3. However, further studies are necessary to confirm this assumption.

In conclusion, we found that overexpression of *Mtb* PknB KD inhibits the transcriptional activity of DevR by phosphorylating Thr180 within the DNA-binding domain. The crosstalk between the PknB and DevSR (DosSR) signaling pathways might enable mycobacteria to reflect two different signals, the environmental oxygen level and the replicating state, for the controlled adaptation to hypoxic conditions.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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