Title: Phosphatase-defective DevS sensor kinase mutants permit constitutive expression of DevR-regulated dormancy genes in Mycobacterium tuberculosis

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Abstract

The DevR-DevS/DosR-DosS two-component system of *Mycobacterium tuberculosis*, that comprises of DevS sensor kinase and DevR response regulator, is essential for bacterial adaptation to hypoxia by inducing dormancy regulon expression. The dominant phosphatase activity of DevS under aerobic conditions enables tight negative control, whereas its kinase function activates DevR under hypoxia to induce the dormancy regulon. A net balance in these opposing kinase and phosphatase activities of DevS calibrates the response output of DevR. To gain mechanistic insights into the kinase-phosphatase balance of DevS, we generated alanine substitution mutants of five residues located in DHp α1 helix of DevS, namely Phe-403, Gly-406, Leu-407, Gly-411 and His-415. For the first time, we have identified kinase positive phosphatase negative (K⁺P⁻) mutants in DevS by single-site mutation in either Gly-406 or Leu-407. *M. tuberculosis* Gly-406A and Leu-407A mutant strains constitutively expressed the DevR regulon under aerobic conditions despite of the presence of negative signal, oxygen. These mutant proteins exhibited ~2-fold interaction defect with DevR. We conclude that Gly-406 and Leu-407 residues are individually essential for phosphatase function of DevS. Our study provides new insights into negative control mechanism of DevS by demonstrating the importance of an optimal interaction between DevR and DevS, and local changes associated with individual residues, Gly-406 and Leu-407, which mimic ligand-free DevS. These K⁺P⁻ mutant strains are expected to facilitate the rapid aerobic screening of DevR antagonists in *M. tuberculosis*, thereby eliminating the requirement for hypoxic culture conditions.
List of Abbreviations

Mtb, Mycobacterium tuberculosis; TCS, two-component system; SK, sensor kinase; RR, response regulator; His/H, histidine; Asp/D, aspartic acid residue; Gln/Q, glutamine; Phe/F, phenylalanine; Gly/G, glycine; Leu/L, leucine; DevS WT, DevSTM Wild Type; DHp, Dimerization and Histidine phosphotransfer; CA, catalytic and ATP-binding domain; DevR~P, phosphorylated DevR; DevS~P, phosphorylated DevS; SM, sensory module; TM, transmitter module; O₂, oxygen; w.r.t, with respect to
Introduction

*Mycobacterium tuberculosis* (Mtb) is capable of establishing a persistent infection in the host despite the generation of a productive cell-mediated immune response, due to its ability to attain a state of dormancy. Two-component systems (TCSs) serve as a major signaling device which help the bacteria to sense different stimuli or environmental stresses and respond adequately to survive under fluctuating conditions. TCSs are involved in diverse processes such as chemotaxis, cell division, metabolic pathways, nitrogen fixation, stress responses and virulence [1]. A typical TCS comprises of a histidine sensor kinase (SK) which is phosphorylated at a conserved histidine (His) in response to an environmental signal and a cytoplasmic response regulator (RR) which receives the phosphosignal at a conserved aspartic acid residue (Asp) from SK, and functions as a transcription factor to mediate a downstream adaptive response [2, 3].

DevR-DevS/DosT is a widely studied TCSs of Mtb wherein, DevR is the RR and DevS/DosT are the two SKs which control its activity [4, 5]. DevR and its ~48-gene regulon play an essential role in bacterial adaptation to dormancy in response to various gaseous signals such as hypoxia, CO, NO or vitamin C [6]. Therefore it is considered as a novel target for developing strategies against non-replicating or dormant bacteria [6-9]. DevS, like NarX and DesK, belongs to HisKA_3 subfamily of sensor kinases and we showed that in the presence of negative signal such as oxygen (O2), under aerobic conditions the phosphatase activity of DevS is dominant which prevents regulon induction, while under inducing conditions (hypoxia, CO, NO or vitamin C), the kinase activity is prominent which favors phosphorylation of DevR [10]. We further showed the involvement of the highly conserved His-395 residue and the DxxQ motif (residues 396 to 400 in DevS) located in the α1 helix of Dimerization and Histidine phosphotransfer (DHp) domain of
DevS in both kinase (positive) and phosphatase (negative) functions of DevS; and found that a fine tuning of these opposing activities determines the output response of this TCS [10]. A complete understanding of the negative control mechanism of DevS is limited by the absence of kinase-positive, phosphatase-defective (K+P-) missense substitutions in DevS. Furthermore, the identification of residues exclusively involved in negative control will provide a DevS mutant strain (having constitutive DevR regulon expression) that would facilitate the rapid screening of anti-DevR/DevS compounds under aerobic conditions.

Analysis of missense substitution mutants in the transmitter module (TM) of NarX SK, also a member of the HisKA_3 family, has shown that the substitutions exhibiting K+P- phenotype are located in the α1 and α2 helices of the DHp domain [11, 12]. In the present study, we have analyzed five residues, namely Phe-403, Gly-406, Leu-407, Gly-411 and His-415, located in the vicinity of the conserved His residue and DxxxQ motif in the α1 helix of the DHp domain of DevS. The contribution of these residues in negative and positive control of DevS was investigated using biochemical and genetic approaches of alanine substitutions mutants. For the first time we have identified key residues, Gly-406 and Leu-407, that confer a K+P- phenotype which are vital for the negative control function of DevS. These K+P- mutants constitutively express the DevR regulon and provide new insights into negative control mechanisms of DevS.

**Material and methods**

**Homology Modeling of DevS with DesK.** The homology-based three-dimensional structure of DevS was generated using SWISS-model server [13]. DesKC-H188V (PDB ID 3EHX), which shares ~26 % identity with DevS, was used as template. A model was generated using aligned amino acid residues 3-189 from X-ray coordinates of DesKC-H188V in complex with ADP [14].
Amino acid residues Phe-403, Gly-406, Leu-407, Gly-411, His-415 in the DHp α1 helix are marked using PyMOL.

Plasmids, bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are described in Tables S1 and S2. Mtb stocks were sub-cultured twice for establishing the respective culture conditions in (a) Dubos medium containing 0.5% bovine serum albumin (BSA), 0.15% dextrose and 0.085% NaCl (DTA) plus 0.1% Tween-80 with shaking at 220 rpm at 37 °C for aerobic set up and were allowed to stand (10 ml cultures in 50 ml tubes) at 37°C for hypoxic set up, or (b) Middlebrook 7H9 medium (7H9) containing 0.2% dextrose, 0.5% BSA, 0.085% NaCl and 0.05% Tyloxapol (7H9 glucose), or (c) 7H9 containing 0.2% sodium acetate, 0.5% BSA, 0.085% NaCl and 0.05% Tyloxapol (7H9 acetate). The culture conditions for *Escherichia coli* strains were as described earlier [15]. Antibiotics were used at the following concentrations: Hygromycin (Hyg), 50 μg/ml for Mtb, 200 μg/ml for *E. coli*; kanamycin (Kan), 25 μg/ml for Mtb, Ampicillin (Amp), 100 μg/ml for *E. coli*.

Site-directed mutagenesis and construction of Mtb strains that constitutively express DevS WT/mutant protein. For *in vitro* experiments, devS WT gene encoding residues 378 to 578 of DevS protein was used [4] and site-directed mutagenesis was performed as per the manufacturer's protocol (GeneArt® Site-Directed Mutagenesis PLUS Kit). For *in vivo* experiments, plasmid pKKDevS was used (carrying the full-length devS gene, Table S2) and site-directed mutagenesis was performed as per the manufacturer's protocol (QuikChange II XL Site-Directed Mutagenesis Kit, Agilent Technologies). Plasmid pSCS201 was used with mutagenic primers (Table S3) to incorporate alanine substitutions at Phe-403, Gly-406, Leu-407, Gly-411 and His-415 and pKKDevS plasmid, was used for alanine substitutions at Gly-406 and Leu-407 positions by PCR.
All the resultant plasmids (Table S2) were sequence verified and for *in vitro* experiments, were transformed into *E. coli* BL21 to express the recombinant DevS WT/mutant proteins as described earlier [4]. For *in vivo* analysis, the selected plasmids were electroporated into Mtb ΔdosSΔdosT (DKO). Mtb transformants denoted by DevS WT, G406A and L406A were selected on 7H11 agar containing hygromycin and kanamycin (Table S1). All the mutant strains of Mtb were confirmed to express DevS protein (data not shown).

**Overexpression and purification of recombinant DevR-GST and DevS WT proteins.** The recombinant expression plasmids (Table S2) were used to overexpress and purify N-terminally GST-tagged DevR protein and N-terminally His6-tagged proteins of DevS WT/mutant derivatives from *E. coli* BL21 as described earlier [15, 16]. The purified proteins were judged to be pure by Coomassie Brilliant Blue staining and were apparently free of other contaminating protein(s) that may contribute to kinase or phosphatase activity in *in vitro* assays. The purified proteins were used in autophosphorylation, phosphotransfer and phosphatase assays.

**In vitro kinase assay.** The DevS WT and its mutant derivative proteins were subjected to autophosphorylation using γ\(^{32}\)P ATP (5000 Ci mmol\(^{-1}\), BRIT, India) as described [4]. Briefly, protein was incubated with 5 μCi of γ\(^{32}\)P (5000 Ci mmol\(^{-1}\), BRIT, India) in 10 μl autophosphorylation reaction buffer (50 mM Tris.HCl, pH 8.0, 50 mM KCl, 10 mM MgCl\(_2\), 50 μM ATP) at 25 °C for 30 min, 60 min and 120 min. The autophosphorylation reactions were terminated with 10 μl 2x stop buffer (100 mM Tris.HCl, pH 6.8, 20 % glycerol, 2 % SDS, 280 mM β-mercaptoethanol, 0.01 % bromophenol blue), the samples were resolved by SDS-PAGE and the gels were subjected to phororimager analysis.
**Phosphotransfer assay.** To assess phosphotransfer from DevS to DevR, DevS WT/mutant derivatives were phosphorylated for 60 minutes as described above. Purified DevR protein (20 μM) was added to the phosphorylated DevS mix, and the reaction incubated at room temperature. Sample aliquots were removed at different time points (upto 64 min) and the reactions were terminated with 10 μl of 100 mM Tris.HCl, pH 6.8, 20 % glycerol, 2 % SDS, 280 mM β-mercaptoethanol, 0.01 % bromophenol blue as described [10]. The samples were resolved by SDS-PAGE and the gels were subjected to phosphorimaging analysis.

**In vitro phosphatase assay.** $^{32}$P acetyl phosphate was synthesized using acetate kinase and $\gamma^{32}$P ATP as described [17]. Briefly, 1 unit of acetate kinase (Sigma) was incubated in 10 μl reaction mix containing 25 mM Tris.HCl, pH 7.5, 60 mM CH$_3$COOK, 10 mM MgCl$_2$ and 15 μCi of $\gamma^{32}$P ATP at 25°C for 20 minutes. The phosphorylation of DevR with $^{32}$P acetyl phosphate was performed in a buffer containing 40 mM Tris.HCl, pH 8.0, 200 mM NaCl, 0.2 mM DTT, and 0.2 mM EDTA which was mixed with 10 μl of $^{32}$P acetyl phosphate and DevR (20 μM) in a 10 μl reaction mix and incubated at room temperature for 2 hours as described [10]. The dephosphorylation of DevR by DevS WT/mutant proteins was performed to assess in vitro phosphatase activity. Briefly, DevR~P was filtered through a 10 kDa filter device (Amicon Ultra, Millipore, Germany) to remove ATP, MgCl$_2$ and NaCl, washed twice with buffer containing 50 mM Tris.HCl, pH 8.0, 50 mM KCl, 1 mM EDTA and wash buffer lacking EDTA. This DevR~P free of ATP and acetyl phosphate was used in in vitro phosphatase assays; DevS WT/mutant protein (15 μM) was added to purified DevR~P and the reaction mix was incubated at room temperature. The reaction was terminated at the indicated time points, the samples were resolved by 15 % SDS-PAGE and were subjected to phosphorimager analysis. Phosphorimaging and
densitometric analysis of gels from *in vitro* assays were performed using Phosphorimager (PMI, Bio-Rad laboratories, USA) and compatible Quantity One® software (Bio-Rad laboratories, USA) to calculate the relative quantity of labelled proteins.

**Interaction ELISA.** Protein-protein interaction between DevR and DevS WT/mutant proteins was assessed in an Enzyme linked immunosorbent assay (ELISA) format. Briefly, a 96-well microtiter plate was coated with 180 pmol of DevS WT/mutant proteins in coating buffer (sodium carbonate bicarbonate buffer, 0.05 M, pH 9.6) and was incubated overnight at 4°C in a humidified chamber. The plate was blocked for 2 h at 37°C with 4% BSA made in 1x phosphate buffer saline (PBS) and 0.25% Tween-20. DevR (90 pmol) was added to the wells and incubated at 25°C for 2 h. The plate was washed thrice with 1x PBS and 0.1% Tween-20 and twice with 1x PBS; polyclonal anti-DevR antibody (generated in rabbit) was then added to each well at a dilution of 1:5000 and the plate was incubated for 2 h at 25°C. The plate was again washed thrice with 1x PBS and 0.1% Tween-20 and twice with 1x PBS, followed by the addition of goat anti-rabbit IgG-HRP conjugate secondary antibody (1:5000, GeNei™, India) for 1 h at 25°C. The plate was again washed, developed with 3,3', 5,5' tetramethylbenzidine substrate and absorbance was measured at 450 nm.

**In vivo kinase and phosphatase function assays.** To assess *in vivo* kinase activity, Mtb was cultured in 100 ml of DTA medium to an OD\textsubscript{595} of ~0.2–0.3. A 50 ml aliquot (10 ml in 50-ml tubes in triplicate for RNA isolation and in duplicate for urea lysate preparation) was centrifuged immediately for ‘aerobic’ condition sample. The remaining 50 ml culture was distributed in 10 ml aliquots as described above and kept standing for 5 days to generate ‘hypoxic’ cultures for RNA isolation and urea lysate preparation as described [10].
For *in vivo* phosphatase assay, DevS WT/mutant Mtb strains were aerobically cultured in 7H9 acetate or 7H9 glucose medium (10 ml culture in 50-ml tubes) and the cells were harvested at an OD$_{595}$ ~0.1–0.2 [10]. For RNA isolation, the cells were harvested from triplicate cultures under different conditions, each pellet was resuspended in 1 ml of TRI reagent (Sigma-Aldrich®, USA) and processed as described earlier [18]. Western blotting of whole cell urea lysates prepared from duplicate cultures for the respective conditions was performed as described [19].

**Western blot analysis.** Immunoblot analysis of α-crystallin (HspX) and RNA polymerase sigma factor A (SigA) was performed under different culture conditions (15 µg of protein per lane, two biological replicate cultures, each in duplicate) in various Mtb strains using polyclonal antibodies that were raised in rabbits as described earlier [15].

**RT-qPCR analysis.** Mtb RNA was isolated as described earlier [15] from cultures grown in different media under aerobic or hypoxic conditions. Briefly, 200 ng of RNA was reverse transcribed to cDNA using High capacity cDNA reverse transcription kit (Applied Biosystems®, Life Technologies™ USA). The cDNA was analysed by qPCR using gene-specific primers (Table S4) and iQ™ SYBR® Green Supermix (in CFX96 Real-Time PCR-detection system, Bio-Rad). The transcript levels of individual genes in various RNA samples were normalized with reference to 16S rRNA and *clpC*. Relative fold expression of selected DevR regulon genes in various mutant strains was examined with respect to (w.r.t) aerobic or non-inducing conditions of DevS WT (i) under hypoxia of mutant strains relative to DevS WT aerobic (Hypoxia/DevS-WT-0 day) and (ii) in aerobic 7H9 acetate and glucose medium of mutant strains relative to DevS WT glucose (Ace/DevS WT-Aer- Glu).
Results

DHp α1 helix of HisKA_3 family harbours residues crucial for phosphatase function.

Several residues of the α1 helix in the DHp domain have been implicated in kinase positive phosphatase negative (K⁺P⁻) functions in NarX SK of the HisKA_3 family [11, 12]. DevS belongs to the same family of proteins and to identify residues that confer a K⁺P⁻ phenotype, we analysed selected residues located distal to the DxxxQ motif in the α1 helix of the DHp domain in DevS. The selected amino acid residues, Phe-403, Gly-406, Leu-407, Gly-411, His-415, are marked on DevS structure modelled on that of DesK structure using PyMOL software (Fig. 1).

In vitro activity of DevS mutants: kinase, phosphotransfer and phosphatase activities.

The TM of DevS contains the DHp and CA domains and includes residues from 378 to 578 (DevSTM). DevSTM is proficient in in vitro autophosphorylation, phosphotransfer to DevR and dephosphorylation of DevR~P [4, 10]. Alanine substitutions were generated at Phe-403 (F403A), Gly-406 (G406A), Leu-407 (L407A), Gly-411 (G411A) and His-415 (H415A) in DevSTM Wild Type (henceforth mentioned as DevS WT, Tables S2 and S3) and the purified WT and mutant TM proteins were used for initial in vitro screening of mutant proteins.

Autokinase activity.

DevS WT and its alanine substitution mutant proteins were tested for autokinase activity using γ³²P ATP which leads to phosphorylation at H395 residue [4] (Fig. 2A). Relative to DevS~P level of WT protein at 60 min (which was taken as 100%), G411A mutant exhibited hyperkinase activity (~188%), F403A showed a major decrease in autokinase activity (~86% decrease), whereas
G406A, L407A and H415A exhibited a near-normal or weak kinase activity (~10%, 43% and 46% decrease, respectively, Fig. 2B and C, Table 1).

**Phosphatase activity.**

We previously showed [10] that DevR harbours a feeble intrinsic phosphatase activity compared to that of DevS (t$_{1/2}$ of DevR$\sim$P ~46 min vs. ~3 min in the presence of DevS WT). The phosphatase activity of DevS WT and its mutant proteins was assayed next by measuring the dephosphorylation of DevR$\sim$32P (Fig. 2D). DevS WT mediated a rapid and efficient dephosphorylation of DevR$\sim$32P (t$_{1/2}$ of ~3 min, Fig. 2E and F), F403A, G406A and L407A exhibited a significant decrease in phosphatase activity (t$_{1/2}$ of ~16 to 36 min, Fig. 2E and F), whereas G411A and H415A mutants retained their phosphatase activity (t$_{1/2}$ of ~0.8 to 3.2 min, Fig. 2E and F). From autokinase and phosphatase analysis of these DevS mutant proteins, we conclude that F403A exhibits a substantial decrease in both kinase (partial, abbreviated as ‘par’) and phosphatase function, whereas G406A, L407A G411A and H415A preserved their kinase activity to varying extents, and of these, two mutant proteins namely G406A and L407A, exhibited a substantial decrease in phosphatase activity (K$^+$P$^-$, Table 1).

**Phosphotransfer activity.**

The phosphotransfer assay is a coupled assay wherein we monitored the ability of DevS mutant protein to transfer the phosphosignal to DevR and also dephosphorylate DevR$\sim$P (Fig. 3A). The G406A and L407A K$^+$P$^-$ mutants exhibited a delay in phosphotransfer activity as compared to DevS WT (Fig. 3B, C). Importantly, the radioactive signal in DevR$\sim$P was retained for a longer time duration in the presence of G406A and L407A proteins which was attributed to a substantial
decrease in the phosphatase activity of these mutants. In contrast, the G411A and H415A mutants were active in phosphotransfer and phosphatase function (Fig. 3B, C) and the F403A mutant poorly transferred phosphosignal to DevR (Fig. 3B, C).

Interaction between DevR and DevS.

The interaction between purified DevR and DevS WT/mutant derivatives was monitored next using ELISA. A 96-well microtitre plate was coated with DevS WT/mutant proteins and interacted with GST-tagged DevR protein. Protein-protein interaction was monitored using anti-GST monoclonal antibody and HRP-conjugated Goat anti-Mouse IgG antibody. A reduction in interaction was observed for the K^+P^- mutants, G406A and L407A and for K^parP^-F403A mutant which might underlie the defect in their phosphatase activity (Fig. 3D). H415A also showed an interaction defect although it did not hamper its phosphatase activity. The G411A mutant protein showed a 2-fold enhanced interaction with DevR as compared to DevS WT which may contribute to its hyperkinase activity (Fig. 3D). The observed defects in the phosphotransfer and phosphatase activities of F403A, G406A and L407A mutant proteins could be attributed to a defect in interaction between DevR and DevS proteins as a consequence of mutation.

In vivo activity of Mtb DevS mutants.

In view of the limitation of studying truncated proteins lacking the regulatory sensory module (SM) in the in vitro assays described above, we proceeded to expand our understanding of DevS negative regulatory function by performing in vivo studies. For this we analyzed the two K^+P^- mutants, G406A and L407A, that were identified through in vitro analysis, by generating Mtb strains that constitutively express full-length versions of DevS WT/mutant protein in a double
knockout strain of devS and dosT (Table S1). The complemented strains were confirmed to express equivalent levels of DevS protein to ensure an unequivocal interpretation of the in vivo assay readouts (data not shown).

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Residues G406 and L407 do not contribute to DevS positive function in vivo.

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The standing 5-day hypoxia model [10] was used to assess the in vivo kinase activity of the K⁺P⁻ mutants (Fig. 4A). Herein, the expression of DevR regulon genes under hypoxia was used as a readout of positive (kinase) function of DevS. DevS WT has a very potent negative function (phosphatase) which suppresses aerobic expression of the regulon; where as it switches to a kinase predominant form (positive) under hypoxia to induce the regulon [10]. Aerobic expression of selected regulon genes, namely devR, hspX, Rv1738, fdxA and Rv3130c, was negligible in DevS WT as expected and was taken as the basal level to determine the relative expression of the regulon in the K⁺P⁻ mutants of Mtb under aerobic and hypoxic conditions. The mutant strains displayed a K⁺ phenotype as evident from the induction of the representative regulon genes under hypoxia (Fig. 4B). Notably, a K⁺ phenotype (regulon induction) was also observed under aerobic conditions in both the mutants, which was ascribed to a defect in the negative function of DevS (phosphatase). Western blot analysis confirmed the RNA expression pattern; HspX was induced in the G406A and L407A mutants under both aerobic and hypoxic conditions, in contrast to the absence of regulon expression in aerobic DevS WT cultures (Fig. 4C). Based on these in vitro and in vivo analysis, both G406A and L407A mutants were confirmed to retain their K⁺ function.

Residues G406 and L407 are required exclusively for negative function of DevS.

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To examine the in vivo phosphatase function of G406 and L407 residues, DevR regulon gene
expression was monitored in aerobic cultures of Mtb G406A and L407A mutants that were grown in 7H9 acetate medium (Fig. 4D). Briefly in this aerobic model, when Mtb is grown on acetate, DevR is phosphorylated at D54 by acetyl phosphate leading to regulon induction [20]; and the level of induction was compared to that in Mtb cultures grown in 7H9 glucose (non-inducing condition) as described earlier [10].

The expression of four representative regulon genes, namely hspX, fdxA, devR, and Rv3130, was examined in this aerobic model. None of these genes were induced in the DevS WT strain grown in either 7H9 acetate or 7H9 glucose media, which was ascribed to its potent phosphatase activity. The regulon expression in 7H9 glucose-grown DevS WT culture was used as a control to determine relative gene expression levels in the mutants. An induction of all the four regulon genes was observed (≥2 to 95-fold) in G406A and L407A Mtb mutant strains irrespective of the culture media that was used to grow the bacteria (Fig. 4E). The constitutive expression of HspX protein was consistent with the RNA expression pattern of their corresponding genes in these mutant strains (Fig. 4F). From this analysis, we conclude that G406 and L407 residues of DevS are individually sufficient for conferring negative (phosphatase) function without majorly influencing its kinase activity. This study has yielded two Mtb strains with K^+P^- phenotype that display a constitutive expression of the DevR regulon gene in a condition- (aerobic/hypoxic) and culture medium- (glucose/acetate) independent manner. The constitutive expression of the DevR regulon in these mutants is attributed to the absence of strong negative regulatory function that resides in G406 and L407 residues of DevS.
Discussion

DevR mounts a relatively slower response in terms of gene regulation as compared to certain response regulators such as CheY and NtrC [21, 22]. The likely underlying reason is that DevR is activated (by deoxy DevS/ DosT) only when a dissolved O₂ concentration of \( \leq 65 \) μM is attained [23]. Furthermore, the timing of DevR response is consistent with the mode of hypoxia generation; it is relatively rapid (3 hrs) in the vitamin C model, wherein O₂ is depleted relatively rapidly [23], compared to 1 day in the standing model where hypoxia develops gradually due to a slow settling of the cells in culture [24]. Notably, DevR~P is a quite stable species (t½ ~46 min) compared to CheY~P and NtrC~P response regulators (t½ 23 seconds and 4 min, respectively) [21, 22] and justifies the presence of phosphatase activity in DevS [10] to calibrate down the DevR output response under aerobic conditions. A deeper understanding of DevS negative control (phosphatase) function that prevents DevR regulon expression under aerobic conditions was hampered by the unavailability of a phosphatase defective mutant. In the present study, using mutational analysis of selected residues that are located in the α1 helix of the DHp domain, we have obtained crucial insights into DevS phosphatase function.

G406 or L407 residues in the α1 helix of DHp domain are crucial for DevS negative function.

We have identified four classes of mutations on the basis of their net effect on DevS kinase and phosphatase functions. Alanine substitution mutants in either G406 or L407 represent the first class of mutants. They were assigned as K+P (Fig. 5A); mutation in either G406 or L407 residue in the α1 helix led to a near-complete abrogation of phosphatase function and enabled constitutive expression of the DevR regulon in Mtb (ON phenotype, Fig. 5B). This helix in DevS contains the highly conserved phosphorylatable H395 residue and the conserved DxxxQ (396 to
400 residues) motif that are central to the kinase and phosphatase activities of SKs [10, 12] and forms a major interaction interface between SK and RR [25-27]. Phosphatase activity in particular is governed by strong interaction between the TM of SK and phosphorylated RR [28]. In this context, the ~50% decrease in interaction between DevS G406A/ L407A and DevR may compromise the interaction interface between DevS and DevR. A similar K⁺P⁻ phenotype was reported for mutations in the corresponding residues of NarX, namely K410 and M411, where these residues are aligned on the same helical phase as Q404 of the DxxxQ motif in NarX [11]; whereas in DevS these residues are in close proximity but offset to Q400 in the DHp α1 helix. Further studies are required to obtain insights into the mechanisms by which these mutations impact DevS-DevR interaction and activity.

Notably, these phosphatase- and interaction-defective mutants have retained kinase function to varying extents, implying that the interaction defect does not majorly compromise kinase function. The constitutive ON phenotype under aerobic condition of Mtb DevS G406A/ L407A mutants confirms that the kinase: phosphatase balance is overwhelmingly tilted in favour of kinase and this is because of a severe defect in phosphatase function. The remaining mutants namely, F403A, G411A and H415A, lacked a clear K⁺P⁻ phenotype in in vitro assays (Fig. 5A) and were not followed up in the present study which probed the contribution of α1 helix in DevS phosphatase function.

**Constitutive ON expression of the DevR regulon in G406 and L407 DevS mutants.**

The generation of G406A and L407A DevS mutants that are constitutive for aerobic expression of the DevR regulon was a highlight of this study. Purified DevS WT protein is reported to exist in
an O$_2$-bound state \textit{in vitro} \cite{29}. Mtb wild type aerobic culture harbours a dominant phosphatase activity that precludes expression of the DevR regulon, while under hypoxia, the dominant kinase activity of DevS induces regulon expression \cite{10}. Although the O$_2$-binding status of DevS was not investigated in the present study, it is reasonable to assume that DevS mutant proteins were not altered in their O$_2$-binding properties (Fig. 5B) since the mutations were created in the TM, and not in the SM. The constitutive ON phenotype of DevS G406A and L407A mutants bears resemblance to the ON phenotype of truncated NarX SK which constitutively activated NarL RR in \textit{E. coli} \cite{30}. Likewise FixLC, deleted of its heme-binding domain, exhibited autokinase activity and activated FixJ RR, but no longer regulated these activities in response to O$_2$ in \textit{Rhizobium meliloti} \cite{31}. A similar kinase-dominant phenotype independent of the negative signal (unsaturated fatty acids) was reported for the cytoplasmic fragment of DesK in \textit{Bacillus subtilis} \cite{32}.

Our findings and those of NarX, FixL and DesK SKs highlight the crucial role of the SM not only in signal sensing but also determining the balance in kinase: phosphatase activities which reside in the TM. Like other SKs, DevS WT protein is believed to undergo a conformational change under hypoxia to a kinase-active state in the absence of negative signal, O$_2$ (deoxy-DevS, Fig. 5B). The \textit{in vivo} phenotype of full-length DevS mutant proteins described in the present study resembles that of closely-related truncated DevS lacking its N-terminal SM from \textit{M. smegmatis} \cite{33}, indicating that the G406A/ L407A mutations bypass ligand-bound regulation of phosphatase activity. Interestingly, mutation of E87 residue of DevS, located in GAF-A of its SM, rendered the protein constitutively kinase active \textit{in vitro}, which highlighted the regulatory role of inter-domain communication that occurs upon ligand recognition \cite{34}. We demonstrate
for the first time that mutations in the TM can overlook O₂ regulation, produce a DevS that is
insensitive to O₂-bound regulation, and display a phenotype that resembles SM mutants of DevS
[34] which were defective in signal propagation from the SM to the TM.

Additional layers of DevR regulation have been proposed through post-translational mechanisms
that include T198/T205 phosphorylation [35] and K182 acetylation [36]. Phosphorylation of DevR
at D54 residue is absolutely essential for activation [37]. Importantly D54V mutant DevR protein
is not phosphorylated in the presence of DevS/acetyl phosphate [4, 18], demonstrating that
phosphorylation of DevR by DevS/acetyl phosphate occurs at D54 and not at alternative residues.
Since the DevR regulon is not aerobically induced in strains lacking DevS/DosT [10], therefore
phosphorylation and/ or acetylation of DevR is/ are ruled out as a primary contributor(s) to the
phosphatase mutant phenotype described here.

In summary, the generation of Mtb K⁺P⁻ DevS mutants in this study has revealed the significant
contribution of G406 and L407 in maintaining the kinase: phosphatase balance in favour of
phosphatase under aerobic conditions. The DevS-DevR two-component system is essential for
Mtb adaptation to hypoxia and this signalling system is considered as a relevant target for
developing anti-TB molecules that target persistent bacteria and can help in shortening the duration
of TB treatment [7, 38]. In this context, the constitutively ON Mtb strains described in the present
study provide a rapid whole-cell screening strategy for DevR antagonists directly in an aerobic
(not hypoxic) setup.

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Author Contribution

JST, PK and KK conceptualized the experiments, PK, KK and SK designed and performed the experiments, PK, KK, SK, UDG, SSB and JST analyzed the data and wrote the paper. All authors have critically analyzed the data and approve the final version of the manuscript.

Supporting Information

Table S1. List of Mycobacterium tuberculosis strains used in the study.
Table S2. List of plasmids used in the study.
Table S3. Primers used for site-directed mutagenesis.
Table S4. Primers used in the study for RT-qPCR analysis.
Table 1

Summary of *in vitro* activities of DevS WT and mutant proteins

| Mutant* | Relative autokinase activity at 60 min (%) | Phosphotransfer activity | Phosphatase activity t1/2 of DevR~P (min) | Interaction ELISA | Protein activity |
|---------|------------------------------------------|--------------------------|------------------------------------------|-------------------|-----------------|
| DevS WT | 100                                      | +                        | 3                                        | 2+                | K⁺                |
| F403A   | 14                                       | -                        | 32                                       | 1+                | K⁻ par P⁺         |
| G406A   | 90                                       | +                        | 36                                       | 1+                | K⁻ P⁺            |
| L407A   | 57                                       | +                        | 18                                       | 1+                | K⁺ P⁻            |
| G411A   | 188                                      | +                        | 1                                        | 3+                | K+++ P⁺          |
| H415A   | 54                                       | +                        | 3                                        | 1+                | K⁺ P⁻            |

* Refers to DevS WT and mutant proteins. Red-boxed residues were further investigated for *in vivo* kinase and phosphatase activities. DevS mutants were classified as kinase positive/negative/partial (K⁺/K⁻/Kpar) or phosphatase positive/negative (P⁺/P⁻).
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Figure Legends

**Fig. 1. Predicted structure of DevS superimposed on the structure of DesK.** Monomeric DHp and catalytic and ATP-binding (CA) domains of DevS were generated using PyMOL (https://www.pymol.org/) based on the structure of DesK (PDB ID 3EHH). His-395 phosphorylation site, Asp-396 and Gln-400 conserved residues of the DxxxQ motif and the residues in the α1 helix investigated in the present study are marked.

**Fig. 2. In vitro phosphatase and autokinase activities of DevS.** (A) Schematic diagram of *in vitro* autokinase assay. (B) *In vitro* autokinase activity. DevS WT/mutant proteins were subjected to autophosphorylation. Phosphorimages of WT/mutants at 30, 60 and 120 min are shown. (C) The percentage of phosphorylated WT/mutant DevS at each time point is calculated relative to DevS~P WT at 60 min which is assigned as 100% during phosphorimaging analysis. (D) Schematic diagram of *in vitro* phosphatase assay. (E) *In vitro* phosphatase activity. DevR~32P was incubated with DevS WT/ mutant protein to monitor the decrease in the level of DevR~32P due to DevS phosphatase activity. Phosphorimage of DevR~32P (% DevR~P) over 120 min is shown. (F) Percentage of DevR~32P remaining at various time points from 0.5 min to 120 min is plotted relative to 0 min which is taken as 100%.

**Fig. 3. In vitro phosphotransfer activity of DevS.** (A) Schematic diagram of *in vitro* phosphotransfer assay. (B) *In vitro* phosphotransfer activity. Phosphotransfer from DevS~32P WT/mutant proteins to DevR was monitored from 0.25 to 64 min and phosphorimages are shown. (C) The percentage of DevR~32P remaining at each time point was plotted (DevS WT~32P at 0 min was taken as 100%). (D) Interaction between DevS and DevR. The extent of DevR-DevS protein interaction was measured in a 96-well ELISA format using anti-GST
monoclonal antibody (to detect GST-tagged DevR). Mean ± SD of A490 of test wells after subtraction of A490 control wells (no protein) is shown.

**Fig. 4. In vivo kinase and phosphatase activity of DevS.** (A) Schematic representation of in vivo DevS kinase function. (B) In vivo DevS kinase assay. Relative fold gene expression in hypoxic and aerobic Mtb cultures was assessed by RT-qPCR (after normalization with housekeeping controls, 16S rRNA and clpC) and are shown relative to that of DevS WT aerobic cultures (0 day) (shown as 1). The results are expressed as Mean ± SD of 4-6 replicates from 3 independent Mtb cultures. ∆∆Ct method was used for all the expression analysis by RT-qPCR. (C) Whole cell lysates of Mtb cultures grown in Dubos medium under aerobic (0 day) and hypoxic conditions (5 days) were subjected to immunoblotting with antibodies against HspX and SigA (normalizing control). Dotted arrow indicates cross-reacting protein in the lysates. (D) Schematic representation of in vivo DevS phosphatase function. (E) In vivo DevS phosphatase assay. Relative fold gene expression was determined by RT-qPCR analysis of aerobic Mtb cultures grown in 7H9 acetate or 7H9 glucose medium (Ace/Glu) after normalization with housekeeping controls (as above) relative to DevS WT cultures grown in glucose medium (shown as 1). Relative fold expression is expressed as Mean ± SD of triplicate (4-6 independent Mtb cultures). (F) Western blot analysis of whole cell lysates of Mtb aerobic cultures grown in acetate or glucose medium expressing DevS WT/mutant proteins. The immunoblots were developed with antibodies against HspX and SigA and a representative blot from four blots is shown.

**Fig. 5. α1 helix of DevS harbors residues required exclusively for its phosphatase function.** (A) The individual DevS mutants (in red) were classified as kinase positive/negative/partial (K+/K-
or phosphatase positive/negative (P+/P−) on the basis of in vitro and/ or in vivo kinase and phosphatase assays. The conserved H395 phosphorylation site and D396 and Q400 residues in the DxxxQ motif are indicated in bold. (B) Proposed mechanism underlying DevS phosphatase activity. Phosphorylated DevR is indicated in grey; DevS WT in O2-bound/ unliganded form is indicated in blue/pink (phosphatase dominant/ kinase dominant, respectively); DevS mutants (O2-bound) are shown in blue-red (kinase dominant); SM, sensory module; TM, transmitter module; REC, receiver domain; OUT, output domain. Left panel, interaction between DevS WT and DevR is facilitated by G406 and L407 residues of the α1 helix in the TM module of DevS, which in turn promotes hydrolysis of DevR-P in the phosphatase-dominant state of DevS to repress DevR regulon expression under normoxia (O2-bound DevS). Central panel, phosphorylation of H395 in DevS (unliganded DevS) WT and phosphotransfer to D54 in DevR leads to DevR regulon induction under hypoxia. Right panel, interaction between DevS and DevR is diminished in G406A/ L407A DevS mutants, which is associated with a defect in DevS phosphatase activity under normoxia (O2-bound DevS) leading to constitutive expression of the regulon. ‘?’ denotes O2 binding or not independently of regulation.
**A** *In vitro* autophosphorylation assay

\[ \text{DevS}^{\text{TM}} \text{WT/mutant protein} \]

\[ \xrightarrow{\text{ATP}} \]

\[ \xrightarrow{\text{ADP + Pi}} \]

\[ \text{DevS}^{\text{~32P}} \]

SDS-PAGE and phosphorimaging

---

**B**

| DevS WT | F403A | G406A |
|---------|-------|-------|
| 30 60 120 | 30 60 120 | 30 60 120 |

\[ \text{(Min)} \]

**C**

\[ \text{Time (min)} \]

\[ \% \text{DevR}^{\text{~32P}} \]

---

**D** *In vitro* phosphatase assay

\[ \text{\^{32}P Acetyl phosphate was prepared} \]

\[ \xrightarrow{\text{DevR}^{\text{~32P}}} \]

\[ \text{Added} \]

\[ \text{DevS}^{\text{TM WT/mutant protein}} \]

SDS-PAGE and phosphorimaging

---

**E**

| Added | DevS WT | F403A | G406A |
|-------|--------|-------|-------|
| 0 0.5 1 2 5 10 30 45 60 120 | | | |

\[ \text{(Min)} \]

---

**F**

| Added | DevS WT | F403A | G406A |
|-------|--------|-------|-------|
| 0 0.5 1 2 5 10 30 45 60 120 | | | |

\[ \% \text{DevR}^{\text{~32P}} \]

\[ \text{Time (min)} \]
**Fig. 3**

**A In vitro Phosphotransfer assay**

DevS<sub>WT</sub>/mutant protein

\[ \text{ATP} \]

\[ \text{ADP + Pi} \]

\[ \text{DevS} \sim^{32}\text{P} \]

Added DevR

SDS-PAGE and phosphorimaging

**B**

| Time (min) | 0.0625 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 |
|-----------|--------|-------|------|-----|---|---|---|---|----|----|----|
| DevS WT   |        |       |      |     |   |   |   |   |    |    |    |
| F403A     |        |       |      |     |   |   |   |   |    |    |    |
| G406A     |        |       |      |     |   |   |   |   |    |    |    |

**C**

- **DevS WT**
- **F403A**
- **G406A**

**D**

(DevS-DevR interaction)

- **DevS WT**
- **F403A**
- **G406A**
- **L407A**
- **G411A**
- **H415A**
**Fig. 4**

**A. In vivo DevS kinase assay**
Mtb strains cultured aerobically in Dubos medium

- Aerobic condition (0 day)
- Hypoxic condition (5 day)

RNA expression analysis and western blotting

**D. In vivo DevS phosphatase assay**
Mtb strains cultured aerobically

- 0.2% Glucose (control)
- 0.2% Acetate

RNA expression analysis and western blotting

**B.**

**C.**

**E.**

**F.**

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**Fig. 5**

**Normoxia**

- **DevR regulation OFF**
- **Phosphatase dominant**

**Hypoxia**

- **DevR regulation ON**
- **K+P+**
- **Kinase dominant**

**Loss of Negative regulation**

- **Normoxia**
- **DevS WT**
- **G406A or L407A**
- **Constitutive DevR regulon ON**

**DevR regulon OFF**

**DevR regulon ON**

**Constitutive DevR regulon ON**