DevR–DevS is a bona fide two-component system of *Mycobacterium tuberculosis* that is hypoxia-responsive in the absence of the DNA-binding domain of DevR

Deepak Kumar Saini,1 Vandana Malhotra,1 Deepanwita Dey,1 Neha Pant,1 Taposh K. Das2 and Jaya Sivaswami Tyagi1

Departments of Biotechnology1 and Anatomy2, All India Institute of Medical Sciences, New Delhi 110029, India

Two-component systems play a central role in the adaptation of pathogenic bacteria to the environment prevailing within host tissues. The genes encoding the response regulator DevR (Rv3133c/DosR) and the cytoplasmic portion (DevS201) of the histidine kinase DevS (Rv3132c/DosS), a putative two-component system of *Mycobacterium tuberculosis*, were cloned and the protein products were overexpressed, purified and refolded as N-terminally His6-tagged proteins from *Escherichia coli*. DevS201 underwent autophosphorylation and participated in rapid phosphotransfer to DevR in a Mg2+-dependent manner. Chemical stability analysis and site-directed mutagenesis implicated the highly conserved residues His395 and Asp54 as the sites of phosphorylation in DevS and DevR, respectively. Mutations in Asp8 and Asp9 residues, postulated to form the acidic Mg2+-binding pocket, and the invariant Lys104 of DevR, abrogated phosphoryl transfer from DevS201 to DevR. DevR–DevS was thus established as a typical two-component regulatory system based on His-to-Asp phosphoryl transfer. Expression of the *Rv3134c–devR–devS* operon was induced at the RNA level in hypoxic cultures of *M. tuberculosis* H37Rv and was associated with an increase in the level of DevR protein. However, in a devR mutant strain expressing the N-terminal domain of DevR, induction was observed at the level of RNA expression but not at that of protein. DevS was translated independently of DevR and induction of devS transcripts was not associated with an increase in protein level in either wild-type or mutant strains, reflecting differential regulation of this locus during hypoxia.

**INTRODUCTION**

Intracellular signal transfer in bacteria is dominated by phosphoryl transfer between conserved transmitter and receiver domains in regulatory proteins of two-component systems. *Mycobacterium tuberculosis* contains eleven complete systems and seven orphan proteins belonging to sensor kinase and response regulator protein families. They are expected to modulate gene expression and direct the adaptation of tubercle bacilli to environmental challenges. In the first step, a sensor, which is usually a membrane-associated histidine protein kinase, is autophosphorylated at a conserved histidine residue in an ATP-dependent manner in response to an environmental stimulus such as O2 tension, temperature, pH, ions, metabolites, etc. In the second step, the phosphoryl group is transferred to a highly conserved aspartic acid residue in the N-terminal domain of the response regulator (Parkinson & Kofoid, 1992; Stock *et al.*, 1995). Two-component regulatory proteins dephosphorylate response regulators in three different ways: through the phosphatase activity of sensor kinases; through reverse phosphorylation, which involves transfer of the phosphoryl group from response regulators back to sensor kinases; or by autodephosphorylation. Regulation may therefore take place by modulation of either the kinase activity or the phosphatase activity of these proteins. A change in the phosphorylation status of the response regulator protein in turn alters its affinity for DNA motifs present in the vicinity of target genes. These unique DNA–protein interactions typically modulate gene transcription, culminating in an appropriate adaptive response (Stock *et al.*, 1995, 2000).

The devR–devS genetic system was identified among genes differentially expressed in the virulent strain of *M. tuberculosis* (Kinger & Tyagi, 1993; Dasgupta *et al.*, 2000). DevR (Rv3133c) demonstrated homology to response regulator proteins of the NarL/UhpA/FixJ subfamily, and DevS (Rv3132c) to histidine sensor kinases. devR and devS are cotranscribed and conserved in *M. tuberculosis* and *Mycobacterium bovis* BCG (Dasgupta *et al.*, 2000); their nucleotide sequences are identical in *M. tuberculosis* (Cole *et al.*, 2000).
1998) and M. bovis except for a single amino acid replacement at codon 283 (Ile to Thr in M. bovis; D. Sharma & J. S. Tyagi, unpublished observation) in the putative transmembrane domain TM2 of DevS (DosS). Recent reports from our and other laboratories have indicated devR–devS expression to be hypoxia-responsive in pathogenic and nonpathogenic mycobacteria including M. tuberculosis (Sherman et al., 2001), M. bovis BCG (Boon et al., 2001) and Mycobacterium smegmatis (Mayuri et al., 2002). DevR was recently designated DosR (dormancy survival regulator) on account of its function in dormancy survival and gene regulation in M. bovis BCG (Boon & Dick, 2002).

In vitro phosphorylation studies have established the functionality of three two-component systems of M. tuberculosis, TrcR–TrcS (Haydel et al., 1999), RegX3–SenX3 (Himpens et al., 2000) and MprA–MprB (Zahrt et al., 2003). Here we report the functional in vitro reconstitution of the DevR–DevS two-component system of M. tuberculosis and identify His\(^{356}\) of DevS and Asp\(^{34}\) of DevR as the sites of phosphorylation. We further show that the DevR–DevS system is transcriptionally induced in hypoxic cultures of M. tuberculosis expressing the N-terminal domain of DevR. RNA and protein expression analysis of devR–devS under aerobic and hypoxic conditions in wild-type and devR-disrupted strains of M. tuberculosis highlight differential regulation of this locus at the levels of transcription and translation.

**METHODS**

**Construction of recombinant expression plasmids.** Recombinant DNA techniques were performed as described by Sambrook & Russell (2001). Template DNA for PCR for cloning was prepared by boiling M. tuberculosis H37Rv cells in 0.1 % Triton X-100, devR and truncated devS\(^{201}\) genes were amplified using Pfu DNA polymerase and gene-specific primers (underlined bases represent mutations introduced in primer sequences) containing engineered restriction enzyme sites to facilitate cloning. The devR gene was amplified using primers DevRNe (5’-GGCCCATATGGTTAAACGTCGACAGG-3’) and DevRSal (5’-CAGGCTTCTTGTCGACAGG-3’) and the DNA segment encoding the cytosolic portion of DevS (from amino acids 378 to 578) was amplified using DevSpl (5’-CAACGTGGATTCCGGGATCAG-3’) and DevSBamR (5’-GGCCGCGGTGATCGTGGCATAGG-3’). The devR PCR product was digested with NdeI and SalI treated with T4 DNA polymerase and cloned in the blunted EcoRI site of the proEX-HTa plasmid expression vector (Invitrogen) to generate pDSR217. The devS\(^{201}\) PCR product was digested with BamHI and cloned in the BamHI site of pPROEx-HTc to generate plasmid pSCS201.

**Overexpression, purification and refolding of recombinant proteins.** All recombinant plasmids were freshly transformed into Escherichia coli BL23(DE3) and grown at 37°C in 2 x YT medium containing 100 μg ampicillin ml\(^{-1}\) to an OD\(_{600}\) of 0.4–0.6. The production of recombinant proteins was induced by the addition of IPTG to a final concentration of 1 mM for 5–6 h. Cell pellets were suspended in phosphate-buffered saline (PBS), pH 7.4, and disrupted by sonication (5 pulses of 2 min each, Branson Sonifier 450). The insoluble protein fraction containing recombinant proteins was solubilized using denaturation buffer (20 mM Tris/HCl, pH 7.9, 10 %, v/v, glycerol, 500 mM NaCl and 20 mM imidazole containing 6 M guanidinium hydrochloride or 8 M urea) and proteins refolded on a solid matrix as described by Saini et al. (2002).

**Site-directed mutagenesis.** Point mutations were introduced into pDSR217 and pSCS201 expressing DevR and DevS\(^{201}\), respectively, using the QuikChange site-directed mutagenesis kit (Stratagene). All the wild-type and mutant protein expression constructs were verified by DNA sequence analysis using an ABI Prism 377 DNA sequencer.

**In vitro phosphorylation assays.** For autophosphorylation, DevS\(^{201}\) (15 μM) was incubated with 5 μCi \(^{32}\)P-ATP (1.85 × 10\(^7\) Bq mmol\(^{-1}\); 5000 Ci mmol\(^{-1}\); BRIT, India) in 10 μl reaction buffer (50 mM Tris/HCl, pH 8.0, 50 mM KCl, 25 mM MgCl\(_2\), 50 μM ATP) at 25°C for various time periods, after which aliquots were withdrawn and stored at −70°C until analysis. To study phosphotransfer, DevS\(^{201}\) was phosphorylated for 60 min as described above. Subsequently DevR protein (20 μM) was added, the reaction mixture was incubated at 25°C, and at indicated time points, aliquots were removed and stored at −70°C until analysis. Assays with mutant proteins were performed under identical conditions.

Experiments involving the simultaneous use of DevS\(^{201}\) and DevR were performed with His\(_6\)-DevS\(^{201}\) and DevR free of His\(_6\) tag. The latter was generated by digesting His\(_6\)-DevR with tREV protease (Invitrogen) at 30°C for 6 h followed by Ni\(^{2+}\)–NTA affinity chromatography. To assess the stability of DevR \(^{201}\) in the absence of DevS\(^{201}\), the latter was phosphorylated with \(^{32}\)P-ATP as described above and immobilized on Ni\(^{2+}\)–NTA agarose beads. The beads were washed free of unbound DevS and incubated with DevR for 2 min in reaction buffer, centrifuged at low speed for 30 s and the supernatant fraction containing purified DevR \(^{32}\)P was incubated in aliquots for up to 30 min at 25°C and subsequently analysed by SDS-PAGE and autoradiography.

**Analysis of chemical stability of DevR–P and DevS\(^{201}\)–P.** Phosphorylated DevR and DevS\(^{201}\) proteins were prepared as described above and treated in the presence of 2 % SDS with 1 M HCl, 0.1 M HCl, 1 M NaOH, 0.1 M NaOH or 0.1 M Tris/HCl, pH 8.0, for 30 min at 37°C. The reactions were neutralized with 0.25 vol. 2 M Tris/HCl, pH 8.0, and analysed by SDS-PAGE. All reactions were terminated with 5 μl 3 x stop buffer (250 mM Tris/HCl, pH 6.8, 10 %, v/v, glycerol, 1 % SDS, 280 mM β-mercaptoethanol, 0.01 % bromophenol blue) and analysed by 15 % SDS-PAGE and autoradiography.

**Culture conditions.** Primary cultures of M. tuberculosis H37Rv and devR mutant strains were grown in 10 ml Dubos-Tween-Albumin (DTA) medium without any antibiotics or in the presence of kanamycin (20 μg ml\(^{-1}\)), respectively, at 37°C in 50 ml conical tubes to an OD\(_{590}\) of about 0.8–1.0. For expression analysis, cultures were set up as described below.

**Setup 1.** The primary culture was diluted to an OD\(_{590}\) of 0.025–0.05 in 300 ml (or 100 ml) fresh DTA media in a 1 litre (or 500 ml) flask and grown with shaking at 190 c.p.m. at 37°C (the flasks were plugged with cotton to maintain aeration) to an OD\(_{590}\) of 0.5–0.8. A 100 ml (or 30 ml) portion of the culture was immediately harvested by centrifugation (‘aerobic culture’). For hypoxia, 10 ml or 13 ml cultures were dispensed into 15 ml screw-capped tubes and kept static for 48 h (‘hypoxic culture’), after which the culture was pelleted in the same tube. In one of the culture tubes, methylene blue, added to monitor hypoxia generation, faded at 48 h, indicating that the cultures were hypoxic (but not the extent of hypoxia).

**Setup 2.** Alternatively, secondary cultures were set up at a very low initial OD\(_{590}\) of 0.005 in DTA medium (160 ml in 180 ml bottles) and allowed to grow under sealed static conditions for up to
40 days. RNA was isolated from 30 day and 40 day hypoxic cultures (which were anoxic/hypoxic as assessed by decolorization/fading of methylene blue in wild-type/mutant cultures). In this setup, cultures grew and adapted to hypoxia over a considerably longer period than in setup 1, where exponential-phase cultures were shifted to static conditions for 48 h, during which hypoxia was generated by consumption of oxygen.

**RT-PCR analysis.** The cell pellets were either immediately processed or stored at −70°C for a maximum of 3 days before RNA isolation. RNA was isolated using an RNaseasy mini kit (Qiagen). DNase-treated RNA and 200 ng random hexamer primers were mixed and denatured by heating at 70°C for 10 min followed by quenching on ice. RT reactions (20 μl volume) were set up in 50 mM Tris/HCl, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.5 mM dNTPs, 40 U RNase inhibitor and 200 U Mo-MuLV RT enzyme (Invitrogen) at 25°C for 50 min. The absence of DNA contamination in RNA was confirmed by checking for 23S rDNA in RNA preparations. RT reactions were heated at 70°C for 15 min and stored at −70°C until further use in PCR assays using gene-specific primers. RNA expression analysis was performed with two separate RNA preparations and two to four times with the same RNA preparation (i.e. four to eight analyses in total) in setup 1 cultures. In setup 2, analysis was performed with one RNA preparation three times. RT-PCR gels were scanned and quantified using ChemiImager densitometric software (Alpha Innotech) and plotted as arbitrary densitometric units relative to the H37Rv aerobic value (fixed at 100). SD was calculated from eight experiments from four cultures.

**M. tuberculosis protein lysate preparation.** Setup 1 cultures harvested as indicated above were gamma-irradiated and frozen at −20°C. Cell pellets were thawed, washed with 3 vols buffer (PBS containing protease inhibitor cocktail (Roche), 1 mM DTT, 0.5 mM EDTA and 0.1 mM PMSF), resuspended in 1 ml buffer and subjected to bead beating using an equal volume of 0.5 mM zirconium-silica beads for 5 × 1 min in a mini-bead-beater (Biospec). The cleared lysate was obtained by centrifugation at 20 000 g for 10 min at 4°C and stored at −20°C for use in immunoblot analysis.

**Immunoblotting.** The protein concentration in lysates was estimated by the BCA assay system (Pierce Biologicals). About 3 and 6 μg protein equivalent of lysates were subjected to Western blot analysis as described by Saini et al. (2002). Briefly, proteins were resolved by 12.5% SDS-PAGE and transferred to nitrocellulose membrane. Anti-DevR and anti-DevS201 rabbit polyclonal sera were cleaned of anti-E. coli antibodies as described by Das Gupta et al. (2000) and used at a dilution of 1:4000. Anti-rabbit horseradish peroxidase conjugate (Bangalore Genie) was used at 1:2000 dilution. M. tuberculosis whole-cell lysate (25 mg protein ml⁻¹), obtained from Dr John Belisle, Colorado State University, Fort Collins, CO, USA, under NIH contract AI-75320 and provided by Dr H. K. Prasad, AIIMS) was used as a positive control in all experiments. Blots were scanned and signals were quantified and plotted as arbitrary densitometric units relative to protein expression in H37Rv aerobic cultures (fixed at 100). The DevR value is the mean ± SD of eight experiments from four cultures and the DevS value is the mean ± SD of four experiments from four cultures.

**RESULTS**

**Overexpression, purification and refolding of His6-tagged proteins.**

A schematic representation of DevR and DevS proteins and their mutant derivatives is shown in Fig. 1(a). The C-terminal region of DevS is conserved among two-component sensors and predicted to function as a kinase;
this region was overexpressed as DevS201. DevR and DevS201 were purified from inclusion bodies by Ni2+-NTA affinity chromatography as N-terminally His6-tagged proteins of approximate molecular mass 25.5 kDa and 26.5 kDa, respectively (Fig. 1b). Soluble proteins were obtained by on-column protein refolding as described by Saini et al. (2002). The yields of DevR and DevS201 were 25 mg l$^{-1}$ and 40 mg l$^{-1}$, respectively. The mutant proteins were also purified by the same protocol with similar purity and yields. All the proteins were judged to be >90% pure by Coomassie staining after SDS-PAGE.

**Autophosphorylation of DevS201 and stability of DevS201 $\sim$ P**

DevS201 was rapidly labelled with [γ-32P]ATP in an autophosphorylation reaction; about 80% and 98% of the maximum radioactivity incorporated in the protein was detected at 1 h and 4 h, respectively (Fig. 2a). The protein was not labelled when [γ-32P]ATP was used in place of [γ-32P]ATP, indicating that phosphorylation of DevS201 occurred only through transfer of a γ-phosphate group from ATP and not from non-specific binding of ATP to the protein (data not shown). The radioactivity associated with DevS201 did not decrease rapidly when DevS201 $\sim$ 32P purified free from ATP was incubated further; the protein retained >90% and about 30% of the radioactivity at 4 h and 24 h, respectively (Fig. 2b). Further, the radiolabelled phosphoryl moiety incorporated in DevS201 $\sim$ 32P was not replaced by the addition of a 100-fold molar excess of unlabelled ATP. Likewise, [γ-32P]ATP could not replace the phosphoryl moiety in DevS201 $\sim$ P phosphorylated previously with 500 nM unlabelled ATP (Fig. 2c). These experiments suggest DevS201 $\sim$ P to be a stable species under the conditions tested. Autophosphorylation was optimal in the presence of 25 mM MgCl$_2$ (50 μM to 100 mM range tested; data not shown). MnCl$_2$ could replace MgCl$_2$ to some extent (approx. 60% efficiency) while CaCl$_2$ was unsuitable as cofactor in the phosphorylation reaction (Fig. 2d). Among two-component systems, Mg$^{2+}$ has been reported to be the most commonly used divalent ion in the reaction (Emmerich et al., 1999; Walker & DeMoss, 1993; Mukai et al., 1990); Mn$^{2+}$ and Ca$^{2+}$ have been used as optimal ions in place of Mg$^{2+}$ in certain instances, such as for TrcS (Haydel et al., 1999).

**Phosphotransfer from DevS201 $\sim$ P to DevR**

To assess phosphotransfer, DevS201 was autophosphorylated with [γ-32P]ATP for 60 min before the addition of DevR. Phosphotransfer occurred rapidly and the maximum signal in DevR $\sim$ 32P was obtained within 2 min of DevR addition. In the reaction containing both DevR and DevS201, dephosphorylation of DevR was not accompanied by a reverse phosphotransfer to DevS. Rather, a rapid net loss of protein-bound radiolabelled phosphate was noted and dephosphorylation of both proteins was nearly complete within 10 min (Fig. 3a). Thus while DevS201 $\sim$ P was of intrinsically high stability, it was labile in the presence of DevR and rapidly transferred its phosphoryl group to

![Fig. 2. Autophosphorylation of DevS201. (a) Time-dependent autophosphorylation of DevS201. DevS201 was incubated in reaction buffer as described and samples were removed at indicated time points, chilled on ice and analysed by SDS-PAGE. Gel slices corresponding to the labelled protein were excised and radioactivity incorporated was quantified by liquid scintillation counting. (b) Stability of DevS201 $\sim$ 32P. DevS201 $\sim$ 32P was prepared as described, unincorporated [γ-32P]ATP was removed by filtration through a 10 k Nanosep device and stability of DevS $\sim$ P was analysed by incubation at 25 °C for up to 24 h. (c) Stability of DevS201 $\sim$ P in the presence of competitor. DevS201 $\sim$ P was phosphorylated with either [γ-32P]ATP or unlabelled ATP. An excess of competitor ATP was added, [γ-32P]ATP to unlabelled DevS201 $\sim$ P and unlabelled ATP to DevS201 $\sim$ 32P, respectively. Samples were removed at indicated time points and analysed by SDS-PAGE. (d) Ionic requirement for autophosphorylation. DevS201 was autophosphorylated in reaction buffer containing the divalent ions indicated (25 mM each, as chloride salts): lane 1, Mg$^{2+}$; lane 2, Mn$^{2+}$; lane 3, Ca$^{2+}$; lane 4, 25 mM EDTA.](image-url)
generate DevR~P. Purified DevR~^{32}P was stable for longer in the absence (half-life approx. 20 min) than in the presence of DevS~P (half-life approx. 3 min, earliest time point evaluated), implying the presence of phosphatase activity in DevS_{201} (data not shown). Furthermore, in the presence of increasing concentrations of DevR (2·5–40 μM), there was a proportionate increase in the extent of dephosphorylation of DevS_{201}~^{32}P within a 2·5 min period (data not shown). To examine the requirement for Mg^{2+} in the phosphotransfer reaction, labelled DevS_{201}~^{32}P was synthesized and purified by filtration through a 10 k Nanosep device (Pall Life Sciences) to remove Mg^{2+} and ATP. Phosphotransfer from DevS_{201}~P to DevR occurred only in the presence of MgCl₂ (Fig. 3b), not that of MnCl₂ or CaCl₂ (not shown). Formation of DevR~P from DevS~P was not dependent on the presence of ATP (Fig. 3b).

**Phosphorylation sites in DevS_{201} and DevR proteins**

To analyse the nature and type of amino acids phosphorylated in DevS_{201} and DevR proteins, chemical stability analysis of the phosphorylated proteins was performed. Phosphoramidates, which are generated by phosphorylation of the conserved histidine residue in sensor kinases, are stable under alkaline conditions but sensitive to acidic pH, whereas the opposite is true for phosphoaspartic acid, which is generated by phosphorylation of the conserved aspartate residue in response regulator proteins (Stock et al., 1995, 2000). The phosphatidyl bond of DevS_{201}~P was acid-labile and base-stable, while the acyl phosphate bond in DevR~P was stable in mildly acidic conditions and labile under basic conditions (Fig. 3c). The pH stabilities of the phosphorylated proteins are consistent with the existence of a histidyl-phosphate group for DevS and an aspartyl-phosphate for DevR.

By sequence homology with known proteins belonging to two-component systems, the conserved His residue at position 395 in DevS and the conserved Asp residue at position 54 in DevR were predicted to be the sites of

---

**Fig. 3.** (a) Phosphotransfer from DevS_{201}~P to DevR. DevS_{201}~^{32}P was prepared as described, DevR was added and the tubes were incubated for up to 30 min. (b) Mg^{2+}-dependent phosphotransfer from DevS_{201}~P to DevR. DevS_{201}~^{32}P was prepared as described, filtered through a 10 k Nanosep device and washed to remove MgCl₂ and ATP. DevR (20 μM) and Mg^{2+} were added as indicated and the tubes incubated for 3 min. (c) Chemical stability of DevS_{201}~P and DevR~P. DevS_{201}~^{32}P and DevR~^{32}P were prepared as described. Lane 1, control reaction containing 2% SDS; lane 2, plus 1 M HCl; lane 3, plus 0·1 M HCl; lane 4, plus 1 M NaOH; lane 5, plus 0·1 M NaOH. (d) Phosphorylation of DevS_{201} mutant proteins. DevS_{201} protein (WT, wild-type) and its mutants were phosphorylated as described. (e) Phosphotransfer to DevR mutant proteins. DevS_{201}~^{32}P was prepared as described, incubated with different mutants for 3 min and analysed. DevR-D54V behaved like DevR-D54N in this assay (not shown). The lower panels in (d) and (e) show the Coomassie-stained profile of the radioactive panel.
phosphorylation (Dasgupta et al., 2000). Their involvement in the phosphorylation reactions was confirmed by the analysis of mutant proteins. His^{395} and Asp^{54} residues in DevS_{201} and DevR proteins, respectively, were altered by mutagenesis to create mutant proteins DevS_{201}-H395Q and DevR-D54V. The mutant proteins were overproduced, purified as His_{6}-tagged proteins, refolded and analysed in phosphorylation reactions. The DevS_{201}-H395Q mutant protein was defective in autophosphorylation (Fig. 3d). In contrast, the mutation of His^{397} to Ala or Gln in DevS_{201} did not affect its phosphorylation, confirming that phosphorylation occurs only at the conserved His^{395} residue within the H box. Likewise, mutation of the Asp^{54} residue in DevR to Val rendered it unable to accept the phosphoryl moiety from DevS_{201}~P (Fig. 3e).

The pH stability and mutagenesis experiments collectively confirmed that His^{395} of DevS_{201} and Asp^{54} of DevR participate in the auto- and transphosphorylation reactions, respectively, as is typical of proteins belonging to two-component systems.

**Mutational analysis of other conserved residues in DevS_{201} and DevR**

The mutation of the Asn^{503} residue located in the N box of DevS (Fig. 1a) to Asp also blocked the phosphorylation of DevS_{201} (Fig. 3d). The N box residue, together with the D/G1 and G2 boxes, is implicated in the formation of the ATP-binding region of DevS. Likewise, Asp^{8} and Asp^{9} in DevR were predicted to correspond to acidic residues at analogous positions in other response regulator proteins that together with Asp^{54} form the acidic pocket and coordinate Mg^{2+} required for the phosphorylation reaction (Stock et al., 2000). Mutant DevR proteins were created wherein these two residues were singly mutated to Asn (DevR-D8N and DevR-D9N) and the invariant Lys^{104} was mutated to Gln (DevR-K104E). All the mutant proteins were defective in phosphotransfer from wild-type DevS_{201}~P protein, confirming the essentiality of the Mg^{2+}-binding pocket and Lys^{104} in the phosphotransfer reaction (Fig. 3e).

**Full-length DevR is necessary for normal basal expression under aerobic conditions**

The effect of devR disruption on its own transcription was analysed in a *M. tuberculosis* devR mutant constructed by allelic exchange (Malhotra et al., 2004). Briefly, a kanamycin-resistance cassette encoding AphI was cloned into a unique *PpuM* site within the devR gene. DNA sequence analysis of the devR::*kan* locus in the mutant strain indicated the disruption of the devR ORF accompanied by the creation of an ORF potentially encoding a putative 406 amino acid fusion polypeptide composed of 145 amino acids corresponding to the N-terminal receiver domain of DevR and the AphI protein (designated DevRN-Aph, Fig. 4) and lacking the DNA-binding domain in the C-terminal region.

Since devR and devS are cotranscribed with the upstream *Rv3134c* gene (encoding a protein containing two Usp domains) in the wild-type strain (Dasgupta et al., 2000), it is likely that devS is transcribed from the promoter(s) located upstream of *Rv3134c* and/or devR. Further, insertion of the kanamycin-resistance cassette into the devR ORF could cause premature termination and exert a polar effect on devS. Using RNA from aerobic cultures, basal levels of devR, devS and *Rv3134c* transcripts in the devR mutant were compared to those in the wild-type strain. The basal expression level was consistently lower in mutant vs wild-type strains cultured in both setup 1 and setup 2 (Fig. 4a, b).

This suggested that (1) full-length DevR was necessary for basal expression of this locus, and (2) the lower level of devS transcripts in the mutant strain (Fig. 4c; compare lanes 1 and 3, devS panel) was not due to polar effects as the expression of the upstream *Rv3134c* gene was also lower (Fig. 4a, b). Our results are not in agreement with those of Sherman et al. (2001), who reported the abrogation of transcription from this locus in an *Rv3134c* mutant. The possible reasons for the discrepancy in our observations are discussed later.

**The DevR N-terminal domain is sufficient for hypoxic induction of RNA from the *Rv3134c-devR-devS* locus**

Next we compared transcript levels of this locus in the devR mutant and wild-type strains in hypoxic cultures. A modest but consistent increase in the expression of the locus was noted during hypoxia in wild-type cultures (Fig. 4a, b). In the devR mutant strain, wild-type devR transcripts were obviously not detected (Fig. 4a, b, second panel), but transcripts from the flanking regions were induced during hypoxia (Fig. 4a, b). Furthermore, aphI transcripts from the kanamycin-resistance cassette were also induced, which indicated *Rv3134c/devR* promoter activation since the cloned cassette lacks its own promoter. In hypoxic cultures we consistently observed higher devR transcript levels 3' to the cloned cassette and of the cassette itself, for which we have no explanation. But we found 12/18 and 11/20 matches in a sequence stretch (in *devR* upstream of the cassette) to the DNA motif proposed by Florczyk et al. (2003) and Park et al. (2003) to bind DevR. Thus the induction of these genes occurred reproducibly in the mutant and the fold-induction matched that in the wild-type strain. Importantly, hypoxic induction occurred in the absence of the DNA-binding C-terminal domain of DevR.

**Levels of DevR and DevS proteins in aerobic cultures of wild-type and devR mutant strains**

The devR and devS genes overlap by 1 bp and are cotranscribed as mentioned earlier (Dasgupta et al., 2000). A classical Shine–Dalgarno sequence was not apparent in sequences immediately upstream of the devS ORF, although a TGATGGGA sequence having a 4/7 match with a proposed mycobacterial consensus sequence (Dale & Patki, 1990).
was present 2 bp upstream of its AUG initiation codon. Therefore DevS may be made either independently or by cotranslation with DevR. In the mutant strain, the latter possibility is discarded since stop codons were present in all three frames downstream of the AphI-coding region. So while the presence of devS and devR–aphI transcripts was not in doubt it was imperative to detect DevS and DevRN–Aph proteins in the mutant strain.

Aerobic expression of DevR and DevS was confirmed by Western blotting. Using anti-DevR sera, an approximately 23 kDa protein was detected in wild-type lysates, while an approximately 45 kDa protein of the expected size was detected in mutant lysates (Fig. 5a, lanes 1 and 2, upper panels). In mutant lysates, labelling resulted from immuno-reactivity of the N-terminal two-thirds of DevR in the DevRN–Aph fusion protein (Fig. 4). The DevR protein level was modestly but consistently higher in wild-type cultures as compared to mutant cultures (Fig. 5a, upper panels) and reflected the differences observed at the RNA level.

Fig. 4. RT-PCR analysis of M. tuberculosis cultures in response to hypoxia. (a) Analysis of RNA from setup 1; (b) Analysis of RNA from setup 2. Culture conditions are described in Methods. The primers used for RT-PCR analysis are indicated below the genes in the diagram at the top. Positive and negative controls were included for all PCR reactions. All PCR assays involved 35 cycles of amplification except for devS (45 cycles), devRf2/devRf3 (30 cycles) and 23S rRNA (17 cycles). devR transcripts were not detected in mutant cultures using the f2r2 primer pair that flanks the kan cassette and fails to give an amplicon owing to its large size. A and H refer to aerated and hypoxic cultures, respectively and 30dH and 40dH to hypoxic cultures grown under sealed static conditions for 30 days and 40 days, respectively.
However, it is possible that the difference could also be due to differences in seroreactivity of DevR vs DevRN–Aph. With anti-DevS sera, an approximately 62 kDa protein corresponding to DevS was detected in both wild-type and mutant bacteria (Fig. 5a, lanes 1 and 2, lower panels). DevS protein expression in the mutant strain confirmed that DevS was translated independently of DevR.

**Dichotomy of DevR and DevS protein expression in response to hypoxia**

To confirm the observation of RNA induction, DevR and DevS protein levels were assessed in hypoxic cultures also. Hypoxia-adapted wild-type cultures showed a modest but consistent increase in the level of DevR protein compared to aerobic cultures (Fig. 5a, upper left panel, lanes 3 and 4). This was in agreement with earlier observations made in *M. bovis* BCG (Boon *et al.*, 2001). However, the level of DevRN–Aph protein was not elevated in hypoxic mutant bacteria (Fig. 5a, upper right panel, lanes 3 and 4). The possibility that the fusion protein is inherently less stable was excluded, as discrete immunoreactive bands were detected in hypoxic mutant lysates. In contrast, the levels of DevS protein remained unchanged in hypoxia in both wild-type and mutant cultures (Fig. 5a, lower panels, lane 4 vs lane 2; Fig. 5b). Thus the transcriptional induction of devS is not associated with an increase in the level of DevS protein under hypoxic conditions.

![Immunoblot analysis of DevR and DevS proteins expressed in *M. tuberculosis* in aerobic and anaerobic setup 1 cultures. Lanes 1 and 3, 3 μg total protein; lanes 2 and 4, 6 μg total protein. Lane 5, *M. tuberculosis* whole-cell lysate (see Methods). (b) Western blots were scanned and quantified as described.](image)
Immunoelectron microscopic analysis of these cultures using anti-DevS and anti-DevR sera showed labelling in the entire cellular compartment, with no indication of a specific pattern of intracellular localization in either aerobic or hypoxia-adapted cultures (Fig. 6).

**DISCUSSION**

DevR and DevS$_{201}$ proteins of *M. tuberculosis* were over-expressed and purified from *E. coli*. The refolded proteins exhibited properties typical of two-component system proteins, namely autophosphorylation of a conserved histidine residue (His$^{395}$) in the cytoplasmic domain of the histidine kinase DevS and phosphotransfer of phosphoryl moiety from DevS$_{201}$ to a conserved aspartic acid residue (Asp$^{35}$) in the response regulator DevR. DevR–DevS was thereby established to be a bona fide two-component system of *M. tuberculosis*.

The stability of DevS$_{201}$~P was modulated by DevR; the phosphorylated species had a very short half-life (<1 min) in the presence of DevR but was very stable in its absence (90% of radioactivity retained in DevS~P at 4 h). DevS$_{201}$~P stability was in contrast to that of some other sensor kinases such as RegS of *Bradyrhizobium japonicum* and NarX of *E. coli*, the half-lives of whose phosphorylated forms were reported to be 15 min and >1 h. Dephosphorylation of DevR was enhanced in the presence of DevS$_{201}$, as reported for the phosphorylated response regulators NarL and RegR in the presence of their cognate sensor kinases (Emmerich *et al.*, 1999; Walker & DeMoss, 1993). The phosphorylated species of *M. tuberculosis* two-component systems TrcR–TrcS and RegX3–SenX3, when present together, appeared to be more stable than those of DevR–DevS. At 10 min (only a single point was studied; Haydel *et al.*, 1999), TrcR~P and TrcS~P were markedly more stable than DevR~P and DevS~P; the phosphorylated species of both proteins all but disappeared in the latter system at the same time point. Likewise, the phosphorylated species of SenX3 and RegX3 were quite stable up to at least 30 min (last time point examined; Himpens *et al.*, 2000). The phosphorylation kinetics of DevR–DevS indicate a rapid rate of phosphotransfer between DevS$_{201}$ and DevR and a rapid rate of signal dissipation from DevR. In vivo, these kinetics would enable bacilli to respond quickly to hypoxia and also revert rapidly to the basal state upon hypoxia reversal. However, the *in vitro* phosphorylation properties of DevS$_{201}$ may not truly reflect its *in vivo* response as it lacks the N-terminal domain; the results therefore need to be viewed with caution.

Our results suggest DevS to be the cognate sensor kinase of DevR. Histidine kinases typically sense a specific signal by their N-terminal domains, and the N-terminal domain of DevS is predicted to contain two GAF domains that are present in many proteins implicated in sensory and signalling pathways (Ho *et al.*, 2000). The nature of the sensed signal and the precise role of DevS and its GAF domains in the hypoxia response are yet to be elucidated. The absence of a severe phenotype in a devS mutant with regard to hypoxic viability compared to a severe loss in hypoxic viability and expression of dormancy proteins such as Acr in a devR mutant of *M. bovis* BCG, along with

**Fig. 6.** Immunoelectron microscopy of DevR and DevS proteins. *M. tuberculosis* H37Rv and devR mutant bacteria (aerobic and hypoxic cultures from setup 1) were fixed with glutaraldehyde, developed with anti-DevR or anti-DevS$_{201}$ antibodies and gold-conjugated anti-rabbit second antibody and assessed by immunoelectron microscopy as described by Saini *et al.* (2002).
a lack of phenotype in Acr expression in a devS mutant of
_M. tuberculosis_, suggested the operation of a compensatory
pathway involving signalling from an alternative sensor
kinase (Boon & Dick, 2002; Sherman et al., 2001). We had
earlier highlighted the similarity between the putative
orphan histidine kinase Rv2027c and DevS (Dasgupta
et al., 2000). So Rv2027c could compensate for the lack of
DevS by generating phosphorylated DevR. DevR may also
be activated by a low-molecular-mass phosphate donor
such as acetyl phosphate. Another possibility is that DevR–
DevS is not the primary system to sense and respond to
hypoxia but part of a cascade involving other proteins. The
_M. tuberculosis_ genome does not have homologues to
bacterial proteins that sense oxygen such as FixL FNR, Dos
and HemAT (Patschkowski et al., 1996; Kiley & Bienert,
1998; Delgado-Nixon et al., 2000; Hou et al., 2001). Yet
another possibility is that DevS does not perceive oxygen
but some metabolite generated as a consequence of oxygen
depreivation, as does the _E. coli_ histidine kinase ArcB
(Georgellis et al., 1999). Cytochrome c oxidase was recently
suggested as the sensor for oxygen limitation and nitric
oxide in _M. tuberculosis_ (Voskuil et al., 2003).

Rv3134c–devR–devS expression at the RNA level exhibited
two notable properties. First, it was expressed in the devR
mutant strain, albeit at a lower level compared to the
wild-type strain. Since the mutant strain expresses the
N-terminal domain of DevR we conclude that basal
transcription of the operon was not absolutely dependent
on the presence of full-length DevR. Secondly, hypoxic
induction occurred in both the wild-type and mutant
strains. Our observations regarding devR–devS expression
are at variance with those of Sherman et al. (2001) and Park
et al. (2003) with regard to (1) the induction noted in the
present study vs the loss of induction in their study in
mutant bacteria, and (2) the fold-induction in hypoxia
(less than twofold in this study vs tenfold in their study).
These discrepancies could be ascribed to differences in
mutant bacilli, the assays used (semi-quantitative RT-PCR
vs microarrays) and the conditions employed to develop
hypoxia. The devR mutant strain used by us expressed the
N-terminal domain of DevR while their mutant was deleted
of nearly all _Rv3134c_ sequences and also some upstream
sequences (Sherman et al., 2001) that possess promoter
activity which is hypoxia-responsive (Bagchi et al., 2003).
Our culture conditions involved the setting in of hypoxia
from utilization of oxygen by exponential-phase cultures
in sealed tubes during a 48 h period (setup 1) or by
adaptation of cultures of low starting density to hypoxia
in sealed bottles over a 40 day growth period (setup 2).
It is possible that our cultures (smaller headspace and no
stirring) may be in NRP2 rather than NRP1 (Wayne &
Hayes, 1996) and the peak of induction falls away so
that we see lower levels of induction. Such a peaking and
falling of _devR_ promoter activity was observed by us in
_M. smegmatis_ cultured under similar conditions (Bagchi
et al., 2003). The discrepancy in fold-induction can be
tested by generating hypoxia in cultures exposed to 0·2 %
oxygen for 2 h as described by Sherman et al. (2001). Thus
the differences in the rate and extent of hypoxia generation
and their effect on the physiological state of the bacilli
might be the root cause of the observed differences in
fold-induction. However, this does not change the observa-
tion that the _Rv3134c–devR–devS_ locus is induced during
hypoxia in the mutant strain.

In the mutant strain DevS can be phosphorylated at the
onset of hypoxia and then could rapidly transfer the
phosphoryl moiety to DevRN-Aph in the first step of
the signalling cascade. This is feasible, as phosphotransfer
from kinases to independent N-terminal domains of
response regulators was reported to occur in Spo0A, RegR and
TrcR (Fujita & Losick, 2003; Emmerich et al., 1999; Haydel
et al., 1999). However, the second step of phosphorylation-
mediated activation of DNA binding would not occur,
as mutant DevR protein lacks the DNA-binding gene-
activating domain. In this context, the hypoxic induction
of this locus in the mutant strain is striking and suggests
a role for the N-terminal domain of DevR. The absence of
an increase in DevS protein levels during hypoxia is also
striking. Since phosphosignal transfer from DevS to
DevR is extremely rapid and occurs at a rate that is
proportional to the concentration of the latter, perhaps
an enhanced amount of DevS protein is unnecessary for
efficient signalling during hypoxia. In the case of DevR,
however, the constitutive level of DevR protein may be
insufficient for hypoxic regulation of the _devR_ regulon
and a mechanism of protein amplification may be necessary
to elicit activation of the regulon. This may be very critical
after infection, when the bacteria would need to imple-
ment widespread changes in gene expression in response
to hypoxia.

In summary, the modest but reproducible hypoxic induc-
tion of _devR–devS_ expression in _M. tuberculosis_ expressing
the DevR N-terminal domain (this study) but not in a _devR_
deletion mutant (Park et al., 2003) suggests a critical role
for the DevR N-terminal domain and warrants detailed
studies of the receiver and activation domains in DevR. _In
vitro_ reconstitution of DevR–DevS and the availability of
a mutant strain expressing the N-terminal domain of DevR
provide an unique opportunity to decipher the regulatory
features of this locus in _M. tuberculosis_. The biochemical
properties of DevS and DevR proteins provide us with
valuable assays to screen for potential inhibitors that
interfere with their function and obstruct the initiation
and/or maintenance of the hypoxia response (US patent
pending). The study of the DevR–DevS signalling pathway
will improve our understanding of the dormancy response
in _M. tuberculosis_ and perhaps provide novel molecules for
the treatment of persistent tuberculosis.

**ACKNOWLEDGEMENTS**

Deepak Sharma is acknowledged for readily providing able assistance
in computer analysis. Illah Joshi is thanked for providing anti-DevR
proteins. Deepak Sharma is acknowledged for readily providing able assistance
in computer analysis. Illah Joshi is thanked for providing anti-DevR
rabbit polyclonal sera. D.K.S. is grateful to the University Grants Commission for a Senior Research Fellowship. Financial assistance provided to J.S.T. by the Council for Scientific and Industrial Research, Government of India is acknowledged.

REFERENCES


