

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

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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.

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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.*,

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 His³⁹⁵ of DevS and Asp⁵⁴ 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 reactions 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 DevRNde (5'-GCCCATATGGTAAAGGTCTTCTTGG-3') and DevRSal (5'-CCGGCTTTTCGTCGACGAGG-3') and the DNA segment encoding the cytosolic portion of DevS (from amino acids 378 to 578) was amplified using DevScytoF (5'-CAACGTCCGAT-CCGCGAACTCGACG-3') and DevSBamR (5'-GGCGCCGGGATC-CTGGCACTAGG-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 pPROEx-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 × YT medium containing 100 µg ampicillin ml⁻¹ to an OD₆₀₀ 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 resuspended 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 DevS₂₀₁, 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, DevS₂₀₁ (15 µM) was incubated with 5 µCi [γ -³²P]ATP (1.85 × 10⁵ 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 MgCl₂, 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, DevS₂₀₁ 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 DevS₂₀₁ and DevR were performed with His₆-DevS₂₀₁ and DevR free of His₆ tag. The latter was generated by digesting His₆-DevR with rTEV protease (Invitrogen) at 30 °C for 6 h followed by Ni²⁺-NTA affinity chromatography. To assess the stability of DevR~³²P in the absence of DevS₂₀₁, the latter was phosphorylated with [γ -³²P]ATP as described above and immobilized on Ni²⁺-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~³²P 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 DevS₂₀₁~P.

Phosphorylated DevR and DevS₂₀₁ 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 × 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 OD₅₉₀ 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 OD₅₉₀ 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 r.p.m. at 37 °C (the flasks were plugged with cotton to maintain aeration) to an OD₅₉₀ 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 OD₅₉₀ 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 MgCl_2 , 10 mM DTT, 0.5 mM dNTPs, 40 U ribonuclease inhibitor and 200 U Mo-MuLV RT enzyme (Invitrogen) at 25°C for 10 min and subsequently at 42°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 ChemImager densitometric software (Alpha Innotech) and plotted as arbitrary densitometric units (mean \pm SD) after normalizing against 23S rRNA. *Rv3134c* expression was analysed once each with two RNA preparations and SD was not plotted in this case. For each target, expression level is depicted relative to the H37Rv aerobic value (fixed at 100).

***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 Dasgupta *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 \pm SD of eight experiments from four cultures and the DevS value is the mean \pm 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;

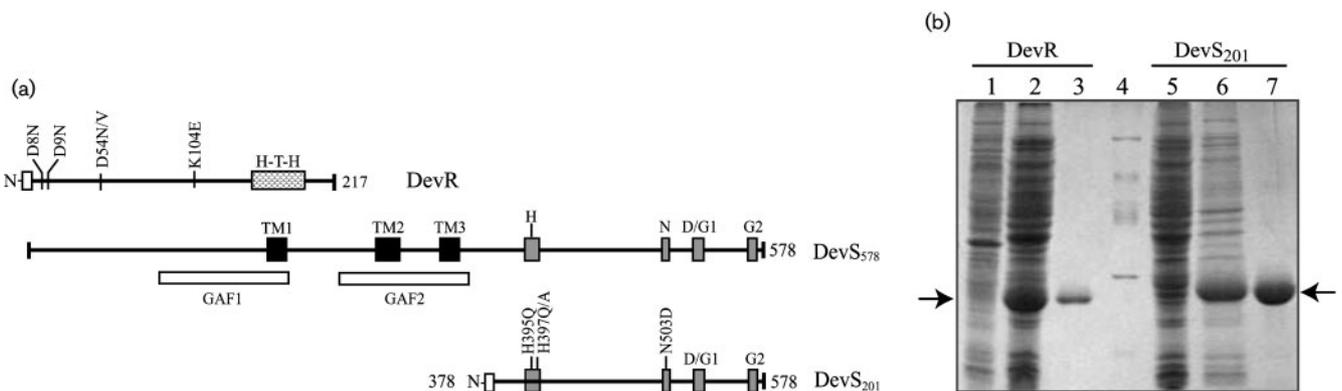


Fig. 1. (a) Features of DevR, DevS and mutant derivative proteins of *M. tuberculosis*. The white boxes at the N-termini of the overexpressed proteins represent the His₆-tag (approx. 3 kDa). H-T-H represents the helix–turn–helix motif (predicted by PROSITE). TM 1, 2 and 3 refer to transmembrane domains (predicted by TMpred). The white boxes below DevS₅₇₈ show GAF1 (residues 63–205) and GAF2 (residues 231–377) domains identified by InterProScan search. PROSITE, TMpred and InterProScan searches were performed at www.expasy.ch. The grey boxes indicate the H, N, D/G1 and G2 conserved sequence motifs of sensor histidine kinases (Parkinson & Kofoed, 1992). D8N, etc., represent mutated amino acid residues in single-letter code. DevS₂₀₁ refers to the 201 amino acid cytoplasmic domain of DevS (residues 378–578 inclusive) used in the phosphorylation experiments described in this study. (b) SDS-PAGE analysis of *E. coli* lysates and purified proteins. Lanes 1 and 5, *E. coli* lysate of uninduced cells carrying recombinant expression constructs pDSR217 and pSCS201, respectively; lanes 2 and 6, *E. coli* lysate of IPTG-induced cells carrying these constructs; lanes 3 and 7, purified DevR and DevS₂₀₁, respectively; lane 4, protein molecular mass marker.

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 ~ P

DevS₂₀₁ was rapidly labelled with [γ -³²P]ATP in an auto-phosphorylation 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 [α -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 ~ 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 ~ 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 ~ P phosphorylated previously with 500 nM unlabelled ATP (Fig. 2c). These experiments suggest DevS₂₀₁ ~ P to be a stable species under the conditions tested. Autophosphorylation was optimal in the presence of 25 mM MgCl₂ (50 μ M to 100 mM range tested; data not shown). MnCl₂ could replace MgCl₂ to some extent (approx. 60% efficiency) while CaCl₂ was unsuitable as cofactor in the phosphorylation reaction (Fig. 2d). Among two-component systems, Mg²⁺ 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); Mn²⁺ and Ca²⁺ have been used as optimal ions in place of Mg²⁺ in certain instances, such as for TrcS (Haydel *et al.*, 1999).

Phosphotransfer from DevS₂₀₁ ~ 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 ~ 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 ~ P was of intrinsically high stability, it was labile in the presence of DevR and rapidly transferred its phosphoryl group to

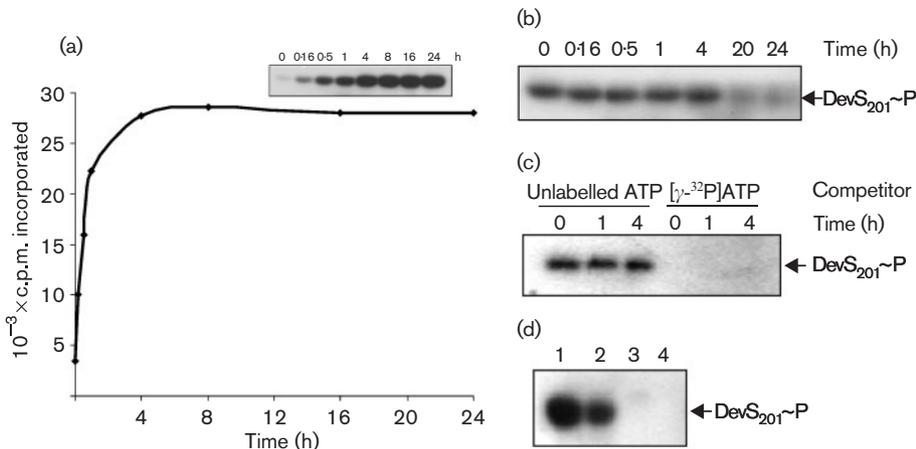
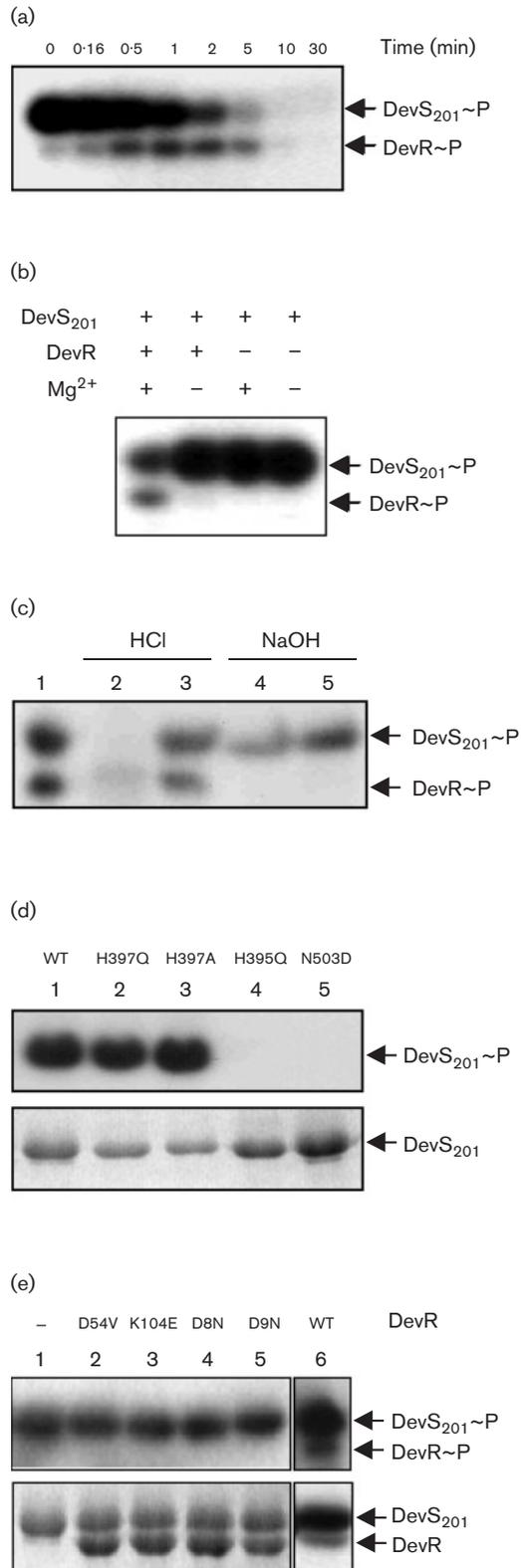


Fig. 2. Autophosphorylation of DevS₂₀₁. (a) Time-dependent autophosphorylation of DevS₂₀₁. DevS₂₀₁ was incubated in reaction buffer as described and samples were removed at indicated time points, chilled on ice and analysed by SDS-PAGE. Gel slices corresponding to the labelled protein were excised and radioactivity incorporated was quantified by liquid scintillation counting. (b) Stability of DevS₂₀₁ ~ ³²P. DevS₂₀₁ ~ ³²P was prepared as described, unincorporated [γ -³²P]ATP was removed by filtration through a 10 k Nanosep device and stability of DevS ~ P was analysed by incubation at 25 °C for up to 24 h. (c) Stability of DevS₂₀₁ ~ P in the presence of competitor. DevS₂₀₁ ~ P was phosphorylated with either [γ -³²P]ATP or unlabelled ATP. An excess of competitor ATP was added, [γ -³²P]ATP to unlabelled DevS₂₀₁ ~ P and unlabelled ATP to DevS₂₀₁ ~ 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, Mg²⁺; lane 2, Mn²⁺, lane 3, Ca²⁺; lane 4, 25 mM EDTA.

generate DevR~P. Purified DevR~³²P 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 DevS₂₀₁ (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 DevS₂₀₁~³²P within a 2.5 min period (data not shown). To examine the requirement for Mg²⁺ in the phosphotransfer reaction, labelled DevS₂₀₁~³²P was synthesized and purified by filtration through a 10 k Nanosep device (Pall Life Sciences) to remove Mg²⁺ and ATP. Phosphotransfer from DevS₂₀₁~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 DevS₂₀₁ and DevR proteins

To analyse the nature and type of amino acids phosphorylated in DevS₂₀₁ 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 DevS₂₀₁~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 DevS₂₀₁~P to DevR. DevS₂₀₁~³²P was prepared as described, DevR was added and the tubes were incubated for up to 30 min. (b) Mg²⁺-dependent phosphotransfer from DevS₂₀₁~P to DevR. DevS₂₀₁~³²P was prepared as described, filtered through a 10 k Nanosep device and washed to remove MgCl₂ and ATP. DevR (20 μM) and Mg²⁺ were added as indicated and the tubes incubated for 3 min. (c) Chemical stability of DevS₂₀₁~P and DevR~P. DevS₂₀₁~³²P and DevR~³²P 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 DevS₂₀₁ mutant proteins. DevS₂₀₁ protein (WT, wild-type) and its mutants were phosphorylated as described. (e) Phosphotransfer to DevR mutant proteins. DevS₂₀₁~³²P 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. His³⁹⁵ and Asp⁵⁴ residues in DevS₂₀₁ and DevR proteins, respectively, were altered by mutagenesis to create mutant proteins DevS₂₀₁-H395Q and DevR-D54V. The mutant proteins were overproduced, purified as His₆-tagged proteins, refolded and analysed in phosphorylation reactions. The DevS₂₀₁-H395Q mutant protein was defective in autophosphorylation (Fig. 3d). In contrast, the mutation of His³⁹⁷ to Ala or Gln in DevS₂₀₁ did not affect its phosphorylation, confirming that phosphorylation occurs only at the conserved His³⁹⁵ residue within the H box. Likewise, mutation of the Asp⁵⁴ residue in DevR to Val rendered it unable to accept the phosphoryl moiety from DevS₂₀₁ ~ P (Fig. 3e).

The pH stability and mutagenesis experiments collectively confirmed that His³⁹⁵ of DevS₂₀₁ and Asp⁵⁴ 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 DevS₂₀₁ and DevR

The mutation of the Asn⁵⁰³ residue located in the N box of DevS (Fig. 1a) to Asp also blocked the phosphorylation of DevS₂₀₁ (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, Asp⁸ and Asp⁹ in DevR were predicted to correspond to acidic residues at analogous positions in other response regulator proteins that together with Asp⁵⁴ form the acidic pocket and coordinate Mg²⁺ 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 Lys¹⁰⁴ was mutated to Glu (DevR-K104E). All the mutant proteins were defective in phosphotransfer from wild-type DevS₂₀₁ ~ P protein, confirming the essentiality of the Mg²⁺-binding pocket and Lys¹⁰⁴ 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 *PpuMI* 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 aerobic 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. 4: 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 TGATGGA sequence having a 4/7 match with a proposed mycobacterial consensus sequence (Dale & Patki, 1990)

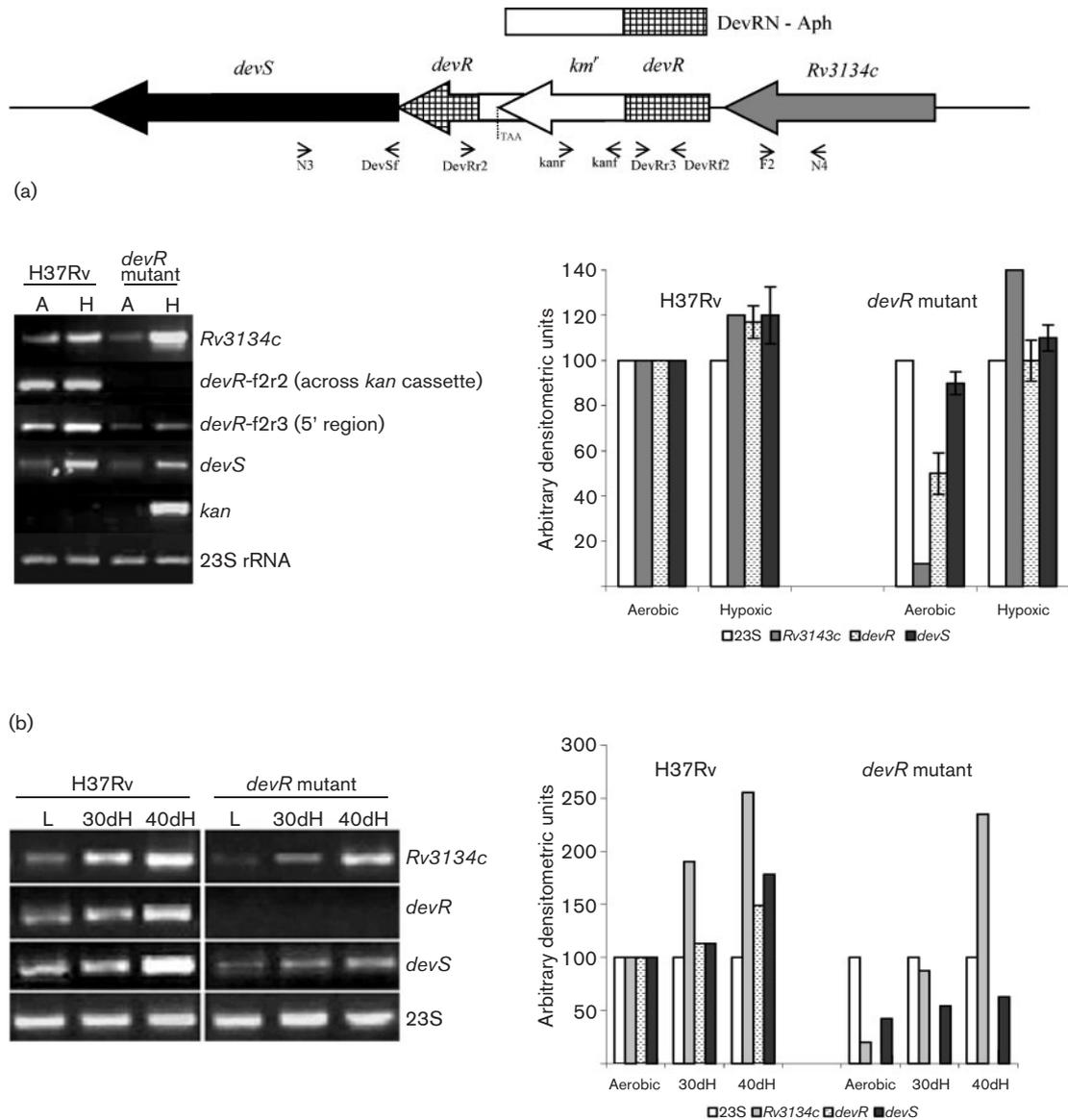


Fig. 4. RT-PCR analysis of *M. tuberculosis* cultures in response to hypoxia. (a) Analysis of RNA from setup 1; (b) Analysis of RNA from setup 2. Culture conditions are described in Methods. The primers used for RT-PCR analysis are indicated below the genes in the diagram at the top. Positive and negative 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.

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 immunoreactivity 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.

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.

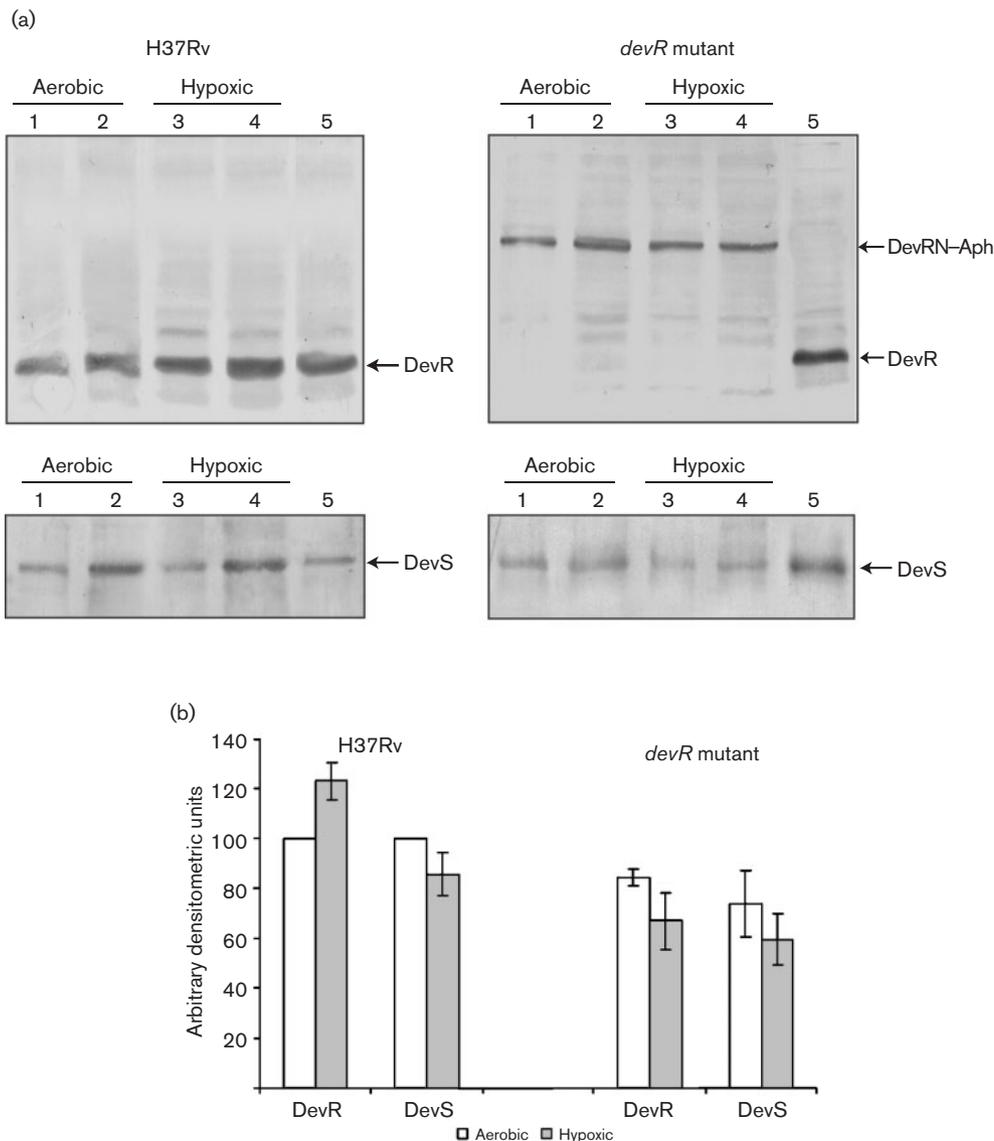


Fig. 5. Immunoblot analysis of DevR and DevS proteins expressed in *M. tuberculosis* in aerobic and anaerobic setup 1 cultures. Lanes 1 and 3, 3 μ g total protein; lanes 2 and 4, 6 μ g total protein. Lane 5, *M. tuberculosis* whole-cell lysate (see Methods). (b) Western blots were scanned and quantified as described.

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 DevS₂₀₁ 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 (His³⁹⁵) in the cytoplasmic domain of the histidine kinase DevS and phosphotransfer of phosphoryl moiety from DevS₂₀₁ to a conserved aspartic acid residue (Asp⁵⁴) in the response regulator DevR. DevR–DevS was thereby established to be a bona fide two-component system of *M. tuberculosis*.

The stability of DevS₂₀₁~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). DevS₂₀₁~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 DevS₂₀₁, 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 DevS₂₀₁ 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 DevS₂₀₁ 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

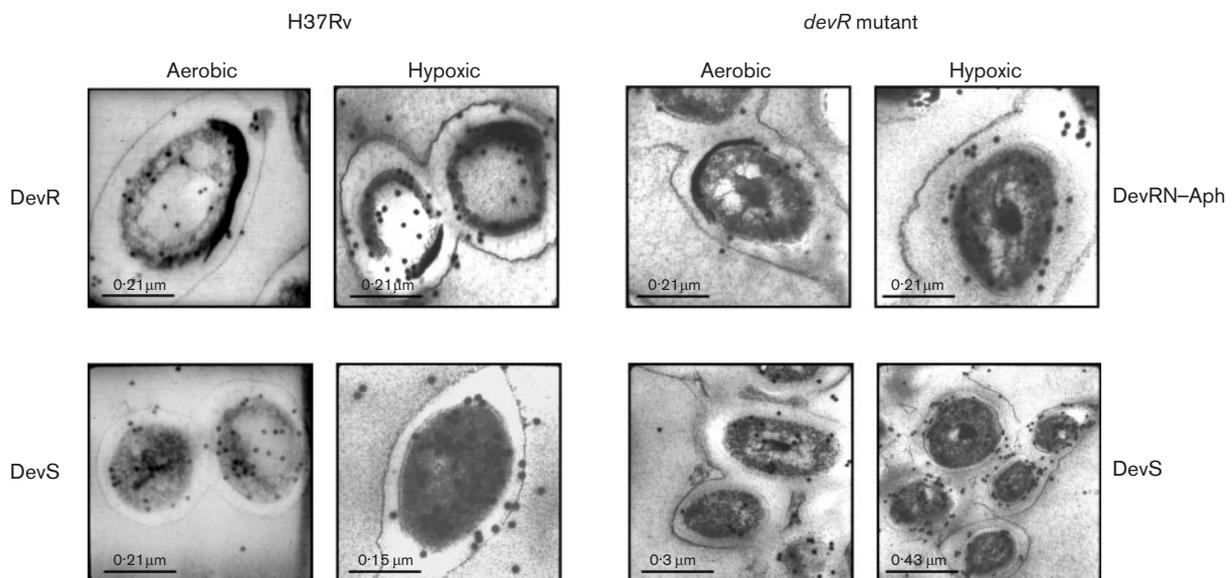


Fig. 6. Immunoelectron microscopy of DevR and DevS proteins. *M. tuberculosis* H37Rv and *devR* mutant bacteria (aerobic and hypoxic cultures from setup 1) were fixed with glutaraldehyde, developed with anti-DevR or anti-DevS₂₀₁ antibodies and gold-conjugated anti-rabbit second antibody and assessed 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 deprivation, 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 observation 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~P 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 implement widespread changes in gene expression in response to hypoxia.

In summary, the modest but reproducible hypoxic induction 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.

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