Downregulation of *Rv0189c*, encoding a dihydroxyacid dehydratase, affects growth of *Mycobacterium tuberculosis* *in vitro* and in mice

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Dihydroxyacid dehydratase (DHAD), a key enzyme involved in branched-chain amino acid (BCAA) biosynthesis, catalyses the synthesis of 2-ketoacids from dihydroxyacids. In *Mycobacterium tuberculosis*, DHAD is encoded by gene *Rv0189c*, and it shares 40% amino acid sequence identity and conserved motifs with DHAD of *Escherichia coli* encoded by *ilvD*. In this study, *Rv0189c* was overexpressed in *E. coli* and the resultant protein was characterized as a homodimer (~155 kDa). Functional characterization of *Rv0189c* was established by biochemical testing and by genetic complementation of an intron-disrupted *ilvD*-auxotrophic mutant of *E. coli* to prototrophy. Growth of *M. tuberculosis*, *E. coli* BL21(DE3) and recombinant *E. coli* BL21(DE3) Δ *ilvD* carrying *Rv0189c* was inhibited by transient nitric oxide (NO) exposure in minimal medium but growth was restored if the medium was supplemented with BCAA (isoleucine, leucine and valine). This suggested that inactivation of *Rv0189c* by NO probably inhibited bacterial growth. The role of *Rv0189c* in *M. tuberculosis* was elucidated by antisense and sense RNA constructs. Growth of *M. tuberculosis* transformed with a plasmid encoding antisense mRNA was markedly poor in the lungs of infected mice and in Middlebrook 7H9 broth compared to that of sense and vector-alone transformants, but growth was normal when the medium was supplemented with BCAA. Upregulation of *Rv0189c* was observed during the early exponential phase of growth, under acid stress and *ex vivo*, suggesting that *Rv0189c* has a role in the survival of *M. tuberculosis* during normal and stress conditions. It may be concluded that the DHAD encoded by *Rv0189c* is essential for the survival of *M. tuberculosis* and could be a potential drug/vaccine target, as it is absent in mammals.

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INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, remains a major public health concern despite the availability of combined chemotherapy, and is the leading cause of infectious-disease-related deaths after HIV/AIDS. The long and complex chemotherapy for TB has been further complicated by the emergence of multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis and by pandemics of HIV/AIDS (Pablos-Méndez *et al.*, 1998). The antibiotics used as first- and second-line drugs for TB therapy target only a small

number of crucial functions in the organism, hence identification of further essential biochemical pathways that are required for bacterial growth can be a good source of targets for novel drug design (Cole *et al.*, 1998). Auxotrophs of *M. tuberculosis* that are attenuated in mouse infection models have been described, namely mutants in the biosynthesis of lysine (*lysA*) (Pavelka *et al.*, 2003), proline (*proC*), tryptophan (*trpD*) and leucine (*leuD*) (Smith *et al.*, 2001). Amino acid biosynthesis should yield important antibiotic targets if the organism is unable to scavenge nutrients from the human host.

The branched-chain amino acid (BCAA) biosynthesis pathway could provide novel targets for the development of new anti-TB drugs, as BCAA auxotrophs of mycobacteria failed to grow in the host (Grandoni *et al.*, 1998; Guleria *et al.*, 1996; McAdam *et al.*, 1995). The relevance of leucine auxotrophy in the survival and persistence of *M. tuberculosis* and *Bacillus Calmette-Guérin* (BCG) *in vivo* and *ex vivo* has been demonstrated previously (Bange *et al.*, 1996; McAdam

Abbreviations: BCAA, branched-chain amino acids; DETA-NO, 2,2'-(hydroxynitrosohydrazino)bis-ethanamine; DHAD, dihydroxyacid dehydratase; ILV, isoleucine, leucine and valine; TB, tuberculosis.

A supplementary figure showing the alignment of the predicted amino acid sequence of *ilvD* proteins from mycobacteria and *E. coli* and a supplementary table of primers used in the study are available with the online version of this paper.

et al., 1995). A *leuD* mutant failed to grow *in vivo* in either immunocompetent BALB/c mice or immunodeficient SCID mice, and was cleared within a few months (Hondalus *et al.*, 2000). Acetohydroxyacid synthase (AHAS), keto-acid reductoisomerase (KARI) and dihydroxyacid dehydratase (DHAD) appear to be interesting targets because they are required for the synthesis of all three BCAA. AHAS is encoded by five ORFs, four encoding catalytic subunits and one encoding a regulatory subunit, while other enzymes (KARI and DHAD) are encoded by a single ORF in *M. tuberculosis* (<http://genolist.pasteur.fr/TubercuList/>). DHAD (EC 4.2.1.9) is an essential enzyme of bacteria for biosynthesis of BCAA and pantothenate (coenzyme A). In *M. tuberculosis*, *Rv0189c* (*ilvD*) has been annotated to encode DHAD. DHAD dehydrates the vicinal diols (2*R*)-2,3-dihydroxyisovalerate and (2*R*,3*R*)-2,3-dihydroxy-3-methylvalerate to 2-ketoisovalerate and (3*S*)-2-keto-3-methylvalerate, respectively, involving an enol intermediate (Arfin, 1969).

Dihydroxyacid → 2-ketoacid + H₂O

The *ilvD* gene of *Escherichia coli*, encoding DHAD activity, shows similarity to *Rv0189c* and shares some conserved regions. The *ilvD* gene of *E. coli* is an essential enzyme for BCAA biogenesis (Flint *et al.*, 1993a, b) and a mutation in *ilvD* resulted in auxotrophy for all the three BCAAs: isoleucine, leucine and valine (ILV). Recent studies in *E. coli* have shown that NO arrests bacterial growth via inhibition of the BCAA biosynthesis enzyme DHAD (Hyduke *et al.*, 2007; Ren *et al.*, 2008).

In this study, we have confirmed that DHAD is encoded by *Rv0189c* in *M. tuberculosis*. We have shown that the enzyme is inactivated by NO, resulting in bacteriostasis, and that it is an essential factor in the survival of bacilli *in vitro*, *ex vivo* and *in vivo*. *Rv0189c* was cloned and overexpressed in *E. coli*. The DHAD activity of *Rv0189c* was established by biochemical testing and functional complementation of an intron-disrupted *ilvD* mutant of *E. coli*. The response of *M. tuberculosis* to NO was examined, and the role of *Rv0189c* in *M. tuberculosis* was elucidated by using an antisense approach and transcription profiling under different stress conditions.

METHODS

Bacterial strains, plasmids and growth conditions. The *E. coli* strains and pET32a vector were purchased from Novagen. *E. coli* strains DH5 α , BL21(DE3) and OrigamiBL21(DE3) were propagated at 37 °C in Luria–Bertani medium (LB) or M9 minimal salts medium (MM; Sigma) containing 0.2 % glucose as carbon source. Antibiotics, kanamycin (50 μ g ml⁻¹), ampicillin (100 μ g ml⁻¹) and chloramphenicol (25 μ g ml⁻¹), were added as required during the growth of *E. coli*. The amino acids isoleucine, leucine and valine (ILV) were used at 100 μ g ml⁻¹ each. *M. tuberculosis* strain H37Rv was cultivated at 37 °C in Middlebrook 7H9 medium (Difco) supplemented with 0.2 % glycerol and 10 % (v/v) albumin-dextrose-catalase (ADC; BD Biosciences) and 0.05 % (v/v) Tween 80. The murine macrophage cell line J774.1 was maintained at 37 °C in a 5 % CO₂ atmosphere in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL) containing

10 % (v/v) fetal calf serum (FCS; Gibco). All chemicals were purchased from Sigma-Aldrich unless stated otherwise. The polymerase, restriction endonucleases and ligase were procured from MBI Fermentas. Optical density of cultures was measured with a DU 640 B spectrophotometer (Beckman).

Cloning, expression, purification and activity assays for Rv0189c protein. The *Rv0189c* gene was PCR amplified from genomic DNA of *M. tuberculosis* H37Rv using gene-specific primers RvDF and RvDR (for sequences see Supplementary Table S1, available with the online version of this paper). The amplified DNA fragment was cloned in pGEMT-easy vector (Promega) and subcloned into pET32a vector, producing a fusion protein with an N-terminal His tag for purification and a thioredoxin tag for increased solubility. The recombinant expression vector (pET32a:: *rv0189c*) was transformed into *E. coli* OrigamiBL21(DE3). A single colony was picked for protein expression. The bacterial culture was grown at 37 °C in LB medium containing ampicillin to OD₆₀₀ 0.7–0.8. Expression of the recombinant protein, designated rMtb_IlvD, was induced by the addition of 0.05 mM IPTG and additional incubation at 18 °C for 16 h. The cells were harvested and lysed, and the recombinant protein was affinity purified on a Ni²⁺-charged HiTrap chelating column (Amersham) pre-equilibrated with buffer I (20 mM sodium phosphate pH 8.0, 300 mM NaCl and 20 mM imidazole). The column was washed and the bound proteins were eluted by applying a linear gradient of 50–500 mM imidazole in buffer I. Eluted fractions containing the expressed protein were concentrated and dialysed against dialysis buffer [100 mM Tris-HCl (pH 7.8), 5 mM EDTA, 1 mM DTT and 20 % (v/v) glycerol] and stored at –80 °C until use. The molecular mass of the native rMtb_IlvD was determined by size-exclusion chromatography. Purified rMtb_IlvD (500 μ g) was chromatographed at a flow rate of 0.5 ml min⁻¹ on a HiPrep 16/60 Sephacryl S-200 column using gel filtration molecular mass markers (Sigma) on an FPLC system (Amersham).

The kinetic study of the DHAD enzyme was performed using the activity assay as described by Flint *et al.* (1993a). In the enzyme assay reaction, 1.0 μ g purified rMtb_IlvD protein was added to a pre-incubated reaction mixture containing 50 mM Tris/HCl (pH 8.0), 10 mM MgCl₂ and 10 mM DL-2,3-dihydroxyisovalerate. The reaction product (keto acids) was measured at 240 nm using a molar absorption coefficient of 0.19 mM⁻¹ cm⁻¹. The substrate DL-2,3-dihydroxy-isovalerate was synthesized according to Cioffi *et al.* (1980).

Construction of an Δ *ilvD* mutant of *E. coli* BL21(DE3). The TargeTron gene knockout system (Sigma) was used to create a mutation in *ilvD* gene of *E. coli* BL21(DE3). Thereafter we used a *Eco_ilvD* for the *ilvD* gene of *E. coli*. The *Eco_ilvD* gene sequence was analysed to locate possible intron insertion sites on the TargeTron design site (<http://www.sigma-genosys.com/targetron/>). The program predicted 10 intron insertion sites across the 1851 bp of the *Eco_ilvD* gene. For optimal gene interruption and stable insertion, a target site with an e-value <0.5 in the antisense strand at position 486/487 from the initial ATG was chosen for intron modification. Three unique primers (Supplementary Table S1) for retargeting the LL.LtrB intron, suggested by the software, and one universal primer provided in the kit were used to mutate the intron of 350 bp by using JumpStart REDTaq ReadyMix (Perutka *et al.*, 2004). The mutated PCR product was treated with restriction enzymes *Hind*III and *Bsr*GI to produce cohesive ends and then ligated to the linearized pACD4K-C vector provided in the kit. The ligation product was transformed into *E. coli* BL21(DE3) and transformants were selected on LB agar containing kanamycin and chloramphenicol as described in the manufacturer's protocol. The transformants thus selected were checked for *Eco_ilvD* knockouts by colony PCR using primers for the *Eco_ilvD* gene (Supplementary Table S1) that flank the target gene (Hofmann &

Brian, 1991). The transformants were screened for BCAA auxotrophy by replica plating on MM, MM containing ILV and LB.

Complementation of the *E. coli* BL21(DE3) Δ ilvD mutant. The recombinant plasmid pET32a::rv0189c and control plasmid pET32a, with an ampicillin-resistance selection marker, were introduced into the *E. coli* BL21(DE3) Δ ilvD mutant. The colony-forming ability of transformants was examined on MM, MM supplemented with ILV, and LB. Growth of *E. coli* BL21(DE3), *E. coli* BL21(DE3) Δ ilvD and *E. coli* BL21(DE3) Δ ilvD(pET32a::rv0189c) was compared in MM and ILV-supplemented MM with a Bioscreen C growth reader (Oy Growth Curves). The DHAD activity assay for *E. coli* BL21(DE3), *E. coli* BL21(DE3) Δ ilvD, *E. coli* BL21(DE3) Δ ilvD(pET32a) and *E. coli* BL21(DE3) Δ ilvD(pET32a::rv0189c) was performed as described above using 10.0 μ g crude extract as enzyme source.

Nitric oxide (NO) exposure. The *E. coli* strains were cultivated in MM with and without ILV. Mid-exponential-phase cultures (OD_{600} 0.6–0.8) were diluted to OD_{600} 0.05–0.1. The cultures were then incubated in 400 μ l volumes in 100-well plates for 4 h at 37 °C in the presence or absence of 50 μ M 2,2'-(hydroxynitrosodihydroxy)bis-ethanamine (DETA-NO; Sigma) as a NO donor, and the OD_{600} was monitored in a Bioscreen C growth reader.

M. tuberculosis was grown in Middlebrook 7H9 medium with glycerol and ADC. Mid-exponential-phase cells of *M. tuberculosis* (OD_{600} 0.8–1.0) were diluted to A_{600} 0.05–0.1 in Middlebrook 7H9 medium or Middlebrook 7H9 medium supplemented with either ILV or ADC, and incubated in 400 μ l volumes in 96-well plates for 4 days in the presence or absence of DETA-NO. After serial dilution in PBS (pH 7.2) with 0.02 % Tween 80, cells were plated for c.f.u. determination on Middlebrook 7H11 agar plates containing 10 % OADC.

Cloning and construction of antisense and sense Rv0189c expression vectors. The Rv0189c gene was amplified from genomic DNA of *M. tuberculosis* H37Rv by PCR using the primers Rv0189cF (forward) and Rv0189cR (reverse) (Supplementary Table S1) and cloned in mycobacterial integrative expression vector pMV361 to make sense and antisense constructs. PCR was carried out using *Taq* DNA polymerase and the following protocol: denaturation at 95 °C for 5 min, followed by 30 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C for 2 min, with a final extension step at 72 °C for 5 min. The amplicon was cloned in pGEMT-easy vector and subcloned in pMV361 under the transcriptional control of phsp-60, a strong constitutive promoter (Hickey *et al.*, 1996). The orientation of the construct was confirmed by *Eco*RI restriction digestion. The fragments generated in the sense construct were 4579 and 1614 bp in size whereas for the antisense construct the fragments generated were 6077 and 116 bp in size. The plasmids containing Rv0189c in the sense and antisense orientations were designated pMV361-0189s and pMV361-0189as, respectively. The recombinant constructs were introduced into *M. tuberculosis* H37Rv via electroporation using a cell porator (Bio-Rad) to obtain strains expressing the sense and antisense mRNA of Rv0189c. The following recombinant strains were derived from *M. tuberculosis* H37Rv: H37Rv harbouring vector pMV361 alone (MV); H37Rv harbouring pMV361-0189s (MS); and H37Rv harbouring pMV361-0189as (MAS).

Growth kinetics of antisense and sense transformants *in vitro* and *in vivo* in mice. The MV, MS and MAS cultures were grown in Middlebrook 7H9 in the presence of ILV to OD_{600} ~1. Cultures were washed with and resuspended in fresh Middlebrook 7H9, with and without ILV. The cultures were incubated at 37 °C for 4 weeks and growth was monitored by measuring OD_{600} . Growth of these strains was measured *in vivo* in the lungs of 6- to 8-week-old (18–20 g) BALB/c mice. Mice were inoculated intravenously with *M. tuberculosis* H37Rv bacilli suspended in PBS via the tail vein. Each animal

received approximately 10^5 c.f.u. Mice were sacrificed at weekly intervals to plate lung homogenates on Middlebrook 7H10 supplemented with OADC.

Transcript analysis of Rv0189c in mice. Eight-week-old (18–20 g) BALB/c mice were infected intravenously with 5×10^7 bacilli of *M. tuberculosis* H37Rv. After 21 days infection, mice were sacrificed and their lungs were immediately suspended in 3 ml RNeasy Protect Bacteria reagent (Qiagen) to stabilize RNA. The lungs were homogenized and the resulting suspension was filtered through a muslin cloth followed by centrifugation at 300 g for 7 min to pellet the resulting debris. The supernatant was then centrifuged at 9000 g for 10 min to pellet the bacteria. The bacteria were again stabilized with RNeasy Protect for 5 min at room temperature for RNA isolation. The experimental protocols were approved by the animal ethics committee of the institute.

Preparation of *M. tuberculosis* cultures at different growth phases and under acid stress or nutrient starvation. The procedures were as described in the accompanying paper (Singh *et al.*, 2011).

***M. tuberculosis* H37Rv infection of the J774.1 murine macrophage cell line.** This was done as described in the accompanying paper (Singh *et al.*, 2011).

RNA isolation, cDNA synthesis and real-time PCR. Pellets of bacterial cultures were suspended in 300 μ l TE buffer containing 1 mg lysozyme ml^{-1} and incubated for 5 min at room temperature. The RNA isolation was performed by using an RNeasy kit, according to the manufacturer's protocol (Qiagen). The isolated RNA was quantified with a Quant-iT RNA Assay kit (Invitrogen), treated with DNase I (Takara) to remove any residual contamination of genomic DNA and confirmed by PCR amplification. The procedures for cDNA synthesis and real-time PCR were as described by Singh *et al.* (2011), except that the PCRs were set up at room temperature instead of on ice before starting the reactions. In comparing relative expression ratios, $P < 0.05$ was considered significant, and $P < 0.01$ highly significant.

RESULTS

Overexpression, purification and enzymic activity of Rv0189c

The Rv0189c gene was cloned and the Rv0189c (rMtb_IlvD) protein overexpressed in *E. coli* OrigamiBL21(DE3). A single band of purified protein appeared on SDS-PAGE with an apparent molecular mass of 78.3 kDa (Fig. 1a), which was close to the calculated molecular mass of the recombinant protein [59.3 kDa (DHAD) + 19 kDa (Thioredoxin-His-tag) = 78.3]. The apparent molecular mass of the native protein was determined as 155 ± 5 kDa by size-exclusion chromatography (Fig. 1b), indicating a homodimeric structure similar to that previously reported for the DHAD from other organisms (Arfin, 1969; Armstrong *et al.*, 1977; Flint *et al.*, 1993a; Kanamori & Wixom, 1963; Kim & Lee, 2006; Xing & Whitman, 1991). The purified rMtb_IlvD protein was light brown in colour. In the enzymic assay of DHAD, the specific activity of purified rMtb_IlvD was 24 mU mg^{-1} . The K_m for the purified rMtb_IlvD using the racemic substrate DL-2,3-dihydroxyisovalerate at pH 8.0 was 3.7 mM.

Fig. 1. Analysis of recombinant protein obtained by expression of pET32a::rv0189c plasmid in *E. coli* OrigamiBL21(DE3). (a) SDS-PAGE analysis was performed using a 12% polyacrylamide gel and the proteins were stained with Coomassie blue R-250. Lanes: 1, protein marker (#0671; MBI Fermentas); 2, induced *E. coli*; 3, induced *E. coli*(pET32a); lane 4, un-induced *E. coli*(pET32a::rv0189c); 5, induced *E. coli* (pET32a::rv0189c); 6, soluble fraction of *E. coli* (pET32a::rv0189c); 7, insoluble fraction of *E. coli* (pET32a::rv0189c); lane 8, purified rMtb_Ilvd. (b) Size-exclusion chromatogram showing dimeric form (molecular mass 155 ± 5 kDa) of rMtb_Ilvd.

Generation of *E. coli* BL21(DE3) Δ ilvD mutant

In the PCR of the colony with the intron insertion, an amplification fragment of about 3851 bp was obtained, which corresponded to the 1851 bp length of Eco_ilvD and the 2000 bp of the inserted intron. About 97% mutation efficiency was observed using the TargeTron system. The growth study of DHAD mutants revealed lack of growth on MM whereas full growth was observed in ILV-supplemented MM and in LB medium, indicating that the Δ ilvD mutant of *E. coli* BL21(DE3) exhibited auxotrophy for ILV. Growth did not occur when isoleucine, leucine and valine were supplemented individually or in any combination of any two of the three amino acids (data not shown).

Restoration of Eco_ilvD function in an *E. coli* BL21(DE3) Δ ilvD mutant by complementation with Rv0189c

As the *E. coli* BL21(DE3) Δ ilvD mutant was unable to grow on MM, it was possible to test for DHAD activity that was encoded by Rv0189c by genetic complementation. We therefore introduced pET32a::rv0189c into the *E. coli* BL21(DE3) Δ ilvD mutant. The Δ ilvD mutant grew very well in MM when supplemented with ILV or complemented with Rv0189c, whereas the mutant carrying the empty plasmid pET32a failed to grow in MM. DHAD activity was found in crude cell extracts of *E. coli* BL21(DE3) Δ ilvD(pET32a::rv0189c) and *E. coli* BL21(DE3), whereas enzyme activity was absent in *E. coli* BL21(DE3) Δ ilvD and *E. coli* BL21(DE3) Δ ilvD(pET32a). Therefore, Δ ilvD cells grew only on supplementation of all three amino acids (ILV) or when they contained a recombinant plasmid that expressed rMtb_Ilvd.

DHAD encoded by Rv0189c is inactivated by NO in *E. coli*

The growth of *E. coli* strains in MM was inhibited by bacteriostatic fluxes of NO arising from the decomposition of DETA-NO, while in MM containing ILV, bacterial growth was unaffected (Fig. 2). The growth-inhibiting effect of NO was clearly evident for the *E. coli* BL21(DE3) parent strain and the isogenic Δ ilvD insertion mutant and complemented strain Δ ilvD(pET32a::rv0189c) in MM. However, growth inhibition was suppressed when ILV was supplemented to MM. The results suggested that the Rv0189c protein behaved similarly to IlvD of *E. coli* in response to NO, as the NO released by DETA-NO became bacteriostatic in MM and the addition of ILV restored growth.

DETA-NO (50 μ M) prevents replication without reducing viability of *M. tuberculosis*

Addition of DETA-NO at a moderate concentration (50 μ M) to *M. tuberculosis* cultures growing in Middlebrook 7H9 medium without supplements blocked growth (Fig. 3). Lower concentrations (5 and 25 μ M) of DETA-NO allowed growth, and higher concentrations (100 and 500 μ M) caused a reduction of c.f.u. (data not shown). Presence of ILV and ADC supplements in Middlebrook 7H9 medium neutralized the inhibiting effect of NO on growth.

Effect of downregulation of Rv0189c on *M. tuberculosis*

Transcript levels of Rv0189c in sense (MS) and antisense (MAS) transformants of *M. tuberculosis* (see Methods for strain construction) were determined by quantitative

Fig. 2. Effect of DETA-NO on growth of *E. coli* strains. Bacteria growing exponentially were transferred to MM (□), MM+DETA-NO (■), MM+ILV (△) or MM+ILV+DETA-NO (▲) and growth was monitored as OD₆₀₀ in a Bioscreen C growth reader. (a) *E. coli* BL21(DE3)(pET32a); (b) *E. coli* BL21(DE3) Δ ilvD; (c) *E. coli* BL21(DE3) Δ ilvD(pET32a::rv0189c). The *Rv0189c*-complemented *E. coli* BL21(DE3) Δ ilvD strain grew on MM but in the presence of DETA-NO the growth was inhibited similar to wild-type *E. coli*.

real-time PCR (qRT-PCR). Real-time PCR analysis showed that the expression of *Rv0189c* in strain MS was higher (8.5 ± 0.56 -fold), and in strain MAS was lower (0.26 ± 0.5 -fold), than the vector control (Fig. 4a). Thus the expression of *Rv0189c* was upregulated in the sense and downregulated in antisense constructs.

Since DHAD, the product of *Rv0189c*, is involved in the synthesis of all three BCAAs, the nutritional requirement of

Fig. 3. Effect of DETA-NO on growth of *M. tuberculosis*. Exponentially growing bacilli were inoculated in Middlebrook 7H9 medium, or Middlebrook 7H9 medium supplemented with ILV and ADC. The cultures were treated with 12.5, 25 or 50 μ M DETA-NO for 4 days at 37 °C. C.f.u. ml⁻¹ were determined by plating on 7H11 agar containing oleic acid-albumin-dextrose-catalase (OADC). The arrow indicates the initial inoculum. Similar results were obtained in duplicate experiments.

sense and antisense constructs of *M. tuberculosis* was analysed. Poor growth of the MAS construct was found in Middlebrook 7H9 medium, but the addition of ILV to this medium enhanced growth (Fig. 4b). The MS and vector-control transformants grew well in Middlebrook 7H9 medium (data not shown). To establish whether the MAS transformant was auxotrophic for all three BCAAs, cultures were grown in the presence of IL, IV or LV. The growth of this transformant was negligible in these conditions (data not shown). These experiments show that downregulation of *Rv0189c* resulted in partial BCAA auxotrophy of *M. tuberculosis*.

During infection in mice, the initial bacterial loads of the three strains (MV, MS and MAS) in the lungs varied slightly. In contrast to the MV and MS bacteria, which increased in numbers in the lungs of infected animals over a period of 4 weeks (from 5.9×10^4 to 4.5×10^5 and 3.8×10^4 to 9.2×10^5 for MV and MS, respectively), the MAS strain was found to be attenuated for growth in the lungs (Fig. 4c). MAS showed a slow reduction in c.f.u. from 5.2×10^4 to 9.5×10^3 . When recovered from lungs, it was confirmed that the MAS strain was partially auxotrophic for ILV when grown *in vitro*, ruling out the possibility of a reversion to prototrophy under *in vivo* conditions.

Transcript profile of *Rv0189c* in *M. tuberculosis* under different conditions

We determined the transcript level of *Rv0189c* in cells grown *in vitro* in different conditions as well as *ex vivo* and *in vivo*. For transcript analysis, RNA was isolated from three independent experimental setups. When cells were harvested from mid-exponential-phase cultures growing in

Fig. 4. Antisense mRNA of *Rv0189c* affects the growth of *M. tuberculosis*. (a) Transcript analysis of *Rv0189c* sense and antisense constructs with vector control. The results shown are the means of at least two independent experiments performed in triplicate. Error bars indicate SD. (b) *In vitro* growth of antisense transformant in Middlebrook 7H9 broth (○) and Middlebrook 7H9 + ILV (●) at 37 °C. A slight drop in OD₆₀₀ at the last time point is due to clumping of the culture. The experiments were repeated three times and results from a representative experiment are shown. Error bars represent SEM. (c) Growth of *M. tuberculosis* strains MV (▲), MS (■) and MAS (○) in lungs of mice at various time points. The data presented are the means ± SEM. The three strains were obtained from the lungs of three animals at each time point indicated. The experiment was repeated twice and was shown to be reproducible.

supplemented Middlebrook 7H9 medium, the level of *Rv0189c* mRNA was $.75 \pm 0.05$ -fold greater relative to *sigA* transcript, whereas in *M. tuberculosis* harvested from lungs of mice after 21 days of infection it was 1.4 ± 0.1 -fold

greater relative to *sigA*. Hence, it appears that transcription of *Rv0189c* is 2-fold greater in *M. tuberculosis* *in vivo* compared to *in vitro*. It was also found that the *Rv0189c* mRNA transcript level was increased during early exponential phase and reduced in the extended stationary phase or dormant phase compared to levels during the mid-exponential phase (Fig. 5a). During acid stress, *Rv0189c* expression was upregulated by 1.72 ± 0.2 -fold at pH 4.5 and 1.42 ± 0.06 fold at pH 5.5, compared to that at pH 7.2 (Fig. 5b). The relative gene expression profiles of *Rv0189c* starved in PBS for 0, 4, 12, 24, 48, 72 and 96 h are shown in Fig. 5(c). It appears that nutrient starvation causes downregulation of *Rv0189c*.

The expression profile of *Rv0189c* in *M. tuberculosis* in the J774.1 cell line at 0, 24, 48 and 72 h, compared with *M. tuberculosis* H37Rv grown *in vitro* and sampled at the same time intervals, demonstrated upregulation of *Rv0189c* mRNA expression by 1.5 ± 0.2 -, 1.97 ± 0.15 - and 2.3 ± 0.2 -fold at 24, 48 and 72 h, respectively (Fig. 5d). However, the expression levels of other genes involved in BCAA biosynthesis, *ilvB1*, *ilvN* and *ilvC*, remained unchanged in *M. tuberculosis* in macrophages.

DISCUSSION

DHAD is considered as an essential enzyme that plays an important role in the biosynthesis of the BCAAs in organisms ranging from archaea and bacteria to eukarya. The *Rv0189c* gene has been annotated in the *M. tuberculosis* genome database (<http://genolist.pasteur.fr/TubercuList/>) as coding for DHAD activity. It has no homologue in the mammalian genome and is identified as an essential gene (Sasseti *et al.*, 2001, 2003). Thus if *Rv0189c* indeed codes for DHAD activity, as in *E. coli*, mutations in *Rv0189c* should arrest the synthesis of ILV and bacterial proliferation by inhibiting translation without inducing a similar effect in the mammalian system.

The enzymic assay with rMtb_IlvD protein from the soluble fraction showed comparable DHAD activity with the earlier reported activity of *E. coli* DHAD (Flint *et al.*, 1993a). The reason for low activity might be due to differences in protein stability, its affinity towards substrate and possibly its sensitivity to oxygen. Similar studies with *E. coli* DHAD revealed its sensitivity to inactivation by oxidants (Flint *et al.*, 1993a, b). Based on the similarity, a strategy of genetic complementation was adopted for functional characterization of *Rv0189c*. The *E. coli* BL21(DE3) $\Delta ilvD$ mutant was unable to grow on MM unless supplemented with all three BCAAs. Introduction of the recombinant pET32a vector expressing *Rv0189c* into *E. coli* BL21(DE3) $\Delta ilvD$ resulted in reversion to prototrophy. No reversion to prototrophy was observed upon introduction of a control plasmid; hence, the effect was specific to *Rv0189c*. This was also supported by DHAD activity in crude cell extracts of the parent strain and *Rv0189c*-complemented mutant, whereas in the mutant strain

Fig. 5. Transcript profile of *Rv0189c* in various conditions. (a) *Rv0189c* transcript levels varied in different growth phases. Expression was high during early exponential phase (2.36 ± 0.15 -fold change) and was reduced in the dormant phase (0.24 ± 0.04 -fold). (b) Expression of *Rv0189c* was increased in acidic conditions. (c) Nutrient starvation caused the downregulation of *Rv0189c*. (d) Transcription profile of *Rv0189c* along with *ilvB1*, *ilvN* and *ilvC* in *M. tuberculosis* harvested from murine macrophage J774.1 cell line at different time points. *Rv0189c* expression was increased at 24, 48 and 72 h, whereas there was no significant change in the transcript levels of *ilvB1*, *ilvN* and *ilvC*. Both *sigA* and 16S rRNA was used for normalization of transcript levels. The results represent mean relative expression values of experiments performed in triplicate with SD (error bars) calculated from one of the three experiments. $P < 0.05$ was considered significant (*), and $P < 0.01$ highly significant (**).

DHAD activity was absent. This clearly demonstrated that the protein encoded by *Rv0189c* was biologically active as it restored the function of DHAD in *E. coli* BL21(DE3) Δ *ilvD*.

Proteins containing iron–sulphur clusters or mononuclear iron centres are susceptible to NO (Spiro, 2007). *In vivo* studies indicated that DHAD 4Fe–4S clusters are highly sensitive to NO (Hyduke *et al.*, 2007). The transient NO exposure effectively inhibits cell growth of *E. coli* in MM under anaerobic growth conditions and bacterial growth is restored when the NO-exposed cells are supplemented with the BCAAs (Ren *et al.*, 2008). Our observation that NO exposure inhibited the growth of the *Rv0189c*-complemented *E. coli* strain in MM but not in MM containing ILV suggested that NO probably binds with the Fe–S cluster of the *Rv0189c* protein, similar to IlvD of *E. coli*, and leads to inactivation of the protein, resulting in growth arrest. As NO released by murine macrophages plays an important role in combating *M. tuberculosis* (Chan *et al.*, 1992), we examined the effect of NO on *M. tuberculosis*. It was found that transient NO exposure effectively inhibits cell growth of *M. tuberculosis* in MM while there was no effect of NO on *M. tuberculosis* growing in MM supplemented with ILV.

These results are in agreement with those of earlier studies in *E. coli* (Hyduke *et al.*, 2007; Ren *et al.*, 2008). Earlier DETA-NO was used to create a nitro-oxidative stress that limits the replication of *M. tuberculosis* (Lin *et al.*, 2009).

In order to assess the role of *Rv0189c* in the growth of *M. tuberculosis*, expression of the gene was altered by using an antisense approach. Antisense RNA has been employed as a tool for reducing gene expression and has been successfully used in mycobacteria to establish the role of the alkyl hydroperoxide reductase (*ahpC*) (Wilson *et al.*, 1998), superoxide dismutase A (*sodA*) (Edwards *et al.*, 2001), *sigA* (Wu *et al.*, 2004) and *Rv3303c* (Akhtar *et al.*, 2006) genes. In this study, we used complete mRNA as an antisense molecule. This showed conclusively that downregulation of *Rv0189c* in *M. tuberculosis* resulted in partial auxotrophy for ILV and reduction of growth in Middlebrook 7H9 medium without ILV. The antisense-transformed (MAS) strain is attenuated for replication in mouse lungs, although it also persists in the lung, which suggests MAS might be able to synthesize suboptimal levels of BCAAs, allowing survival but not providing enough BCAA for growth. Further studies using a knockout mutant will help to resolve this issue.

The transcriptional study demonstrated that *Rv0189c* is expressed both *in vitro* and *in vivo*. The higher mRNA level of *Rv0189c* in mice as compared to exponential-phase cultures grown in Middlebrook 7H9 medium suggests the importance of *Rv0189c* during growth *in vivo*. The variations in *Rv0189c* transcript profiles at different growth phases, including increased transcript levels during early exponential phase and downregulation during dormant phase, suggest that upregulation of the pathway occurs in order to meet the amino acid requirement during rapid-growth phase. The increase in *Rv0189c* transcription in *M. tuberculosis* at low pH may mimic environmental signals encountered by phagocytosed mycobacteria, because an increase in transcription levels of the gene was also observed in the infected macrophage cell line. It is not surprising that downregulation of *Rv0189c* was observed during nutrient starvation, because many other genes involved in the BCAA biosynthetic pathway, namely *ilvB1*, *ilvN*, *ilvC*, *leuA*, *leuC* and *leuD*, have also been reported to be downregulated under similar conditions, suggesting metabolic slowdown (Betts *et al.*, 2002). The fact that *Rv0189c* was upregulated in *M. tuberculosis* inside murine macrophages while no change in expression was seen for other BCAA biosynthetic pathway genes (*ilvB1*, *ilvN* and *ilvC*) suggests that this function may be related to the survival of *M. tuberculosis ex vivo*. The interaction of NO with the IlvD protein has previously been demonstrated in *E. coli* (Hyduke *et al.*, 2007; Ren *et al.*, 2008) and it is known that macrophages, a key component of the host immune system, produce small diffusible reactive molecules such as NO to create an environment hostile to bacterial proliferation (Ignarro, 2000). The above results suggest that growth of *M. tuberculosis* is also inhibited by NO via the inactivation of *Rv0189c*.

In conclusion, the present study demonstrates that *Rv0189c* encodes DHAD, which is functionally equivalent to the *ilvD* gene of *E. coli*. Antisense studies of *Rv0189c* suggest that it is an essential gene for the growth of *M. tuberculosis in vitro*. *Rv0189c* is probably required for intracellular growth, as is evident by its upregulation *ex vivo* and *in vivo*, and in coping with the stress conditions. In the absence of a mammalian homologue, DHAD could be a potential drug or vaccine target.

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