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

Vinayak Singh, Deepak Chandra, Brahm S. Srivastava and Ranjana Srivastava

1Microbiology Division, Central Drug Research Institute, CSIR, Lucknow 226001, India
2Department of Biochemistry, University of Lucknow, Lucknow 226001, India

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) ΔilvD 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.

INTRODUCTION

Tuberculosis (TB), caused by Mycobacterium tuberculosis, remains a major public health concern due to 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...
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 (2R)-2,3-dihydroxyisovalerade and (2R,3R)-2,3-dihydroxy-3-methylvalerate to 2-ketoisovalerade and (3S)-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 Rv1089c 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 DH5α, 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 µg ml⁻¹), ampicillin (100 µg ml⁻¹) and chloramphenicol (25 µg ml⁻¹), were added as required during the growth of E. coli. The amino acids isoleucine, leucine and valine (ILV) were used at 100 µg 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::r:v0189c) 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 OD₉₀₀ 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 Ni³⁺–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 Mtb_IlvD (500 µg) 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 µg purified rMtb_IlvD protein was added to a preincubated 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-dihydroxyisovalerate was synthesized according to Cioffi et al. (1980).

Construction of an ilvD mutant of E. coli BL21(DE3). The TargetTron gene knockout system (Sigma) was used to create a mutation in ilvD gene of E. coli BL21(DE3). Thereafter we used an Eco_IlvD for the ilvD gene of E. coli. The Eco_IlvD gene sequence was analysed to locate possible intron insertion sites on the TargetTron design site (http://www.sigma-genosys.com/targettron/). 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 LLLtbr 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 HindIII and BsrGI 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 &
Complementation of the E. coli BL21(DE3) ilvD mutant. The recombinant plasmid pET32a::r0189c and control plasmid pET32a, with an ampicillin-resistance selection marker, were introduced into the E. coli BL21(DE3) 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) ΔilvD and E. coli BL21(DE3) ΔilvDpET32a::r0189c 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) ΔilvD, E. coli BL21(DE3) ΔilvD(pET32a) and E. coli BL21(DE3) ΔilvD(pET32a::r0189c) was performed as described above using 10.0 μg 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<sub>600</sub> 0.6–0.8) were diluted to OD<sub>600</sub> 0.05–0.1. The cultures were then incubated in 400 μl volumes in 100-well plates for 4 h at 37 °C in the presence or absence of 50 μM 2,2′-(hydroxynitrosodihydrazino)bis-ethanamine (DETA-NO; Sigma) as a NO donor, and the OD<sub>600</sub> 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<sub>600</sub> 0.8–1.0) were diluted to A<sub>540</sub> 0.05–0.1 in Middlebrook 7H9 medium or Middlebrook 7H9 medium supplemented with either ILV or ADC, and incubated in 400 μl 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 transcripational control of phsp-60, a strong constitutive promoter (Hickey et al., 1996). The orientation of the construct was confirmed by EcoRI 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<sub>600</sub> ~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<sub>600</sub>. 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<sup>5</sup> 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<sup>7</sup> bacilli of M. tuberculosis H37Rv. After 21 days infection, mice were sacrificed and their lungs were immediately suspended in 3 ml RNAprotect 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 RNAprotect 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 μl TE buffer containing 1 mg lysozyme ml<sup>−1</sup> and incubated for 5 min at room temperature. The RNA isolation was performed by using an RNAeasy 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 PCRAs 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 (rMb_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 rMb_IlvD protein was light brown in colour. In the enzymic assay of DHAD, the specific activity of purified rMb_IlvD was 24 μM mg<sup>−1</sup>. The K<sub>m</sub> for the purified rMb_IlvD using the racemic substrate DL-2,3-dihydroxyisovalerate at pH 8.0 was 3.7 mM.
Generation of \textit{E. coli} BL21(DE3) \textit{ΔilvD} 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\textit{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 \textit{ΔilvD} mutant of \textit{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\textit{ilvD} function in an \textit{E. coli} BL21(DE3) \textit{ΔilvD} mutant by complementation with \textit{Rv0189c}

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

DHAD encoded by \textit{Rv0189c} is inactivated by NO in \textit{E. coli}

The growth of \textit{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 \textit{E. coli} BL21(DE3) parent strain and the isogenic \textit{ΔilvD} insertion mutant and complemented strain \textit{ΔilvD}(pET32a::\textit{rv0189c}) in MM. However, growth inhibition was suppressed when ILV was supplemented to MM. The results suggested that the \textit{Rv0189c} protein behaved similarly to IlvD of \textit{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 \textit{μM}) prevents replication without reducing viability of \textit{M. tuberculosis}

Addition of DETA-NO at a moderate concentration (50 \textit{μM}) to \textit{M. tuberculosis} cultures growing in Middlebrook 7H9 medium without supplements blocked growth (Fig. 3). Lower concentrations (5 and 25 \textit{μM}) of DETA–NO allowed growth, and higher concentrations (100 and 500 \textit{μM}) 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 \textit{Rv0189c} on \textit{M. tuberculosis}

Transcript levels of \textit{Rv0189c} in sense (MS) and antisense (MAS) transformants of \textit{M. tuberculosis} (see Methods for strain construction) were determined by quantitative
Real-time PCR (qRT-PCR). Real-time PCR analysis showed that the expression of \textit{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 \textit{Rv0189c} was upregulated in the sense and downregulated in antisense constructs.

Since DHAD, the product of \textit{Rv0189c}, is involved in the synthesis of all three BCAAs, the nutritional requirement of sense and antisense constructs of \textit{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 \textit{Rv0189c} resulted in partial BCAA auxotrophy of \textit{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 \textit{in vitro}, ruling out the possibility of a reversion to prototrophy under \textit{in vivo} conditions.

**Transcript profile of \textit{Rv0189c} in \textit{M. tuberculosis} under different conditions**

We determined the transcript level of \textit{Rv0189c} in cells grown \textit{in vitro} in different conditions as well as \textit{ex vivo} and \textit{in vivo}. For transcript analysis, RNA was isolated from three independent experimental setups. When cells were harvested from mid-exponential-phase cultures growing in...
supplemented Middlebrook 7H9 medium, the level of \( \text{Rv0189c} \) mRNA was \( 0.75 \pm 0.05 \)-fold greater relative to \( \sigma\text{A} \) transcript, whereas in \( \text{M. tuberculosis} \) harvested from lungs of mice after 21 days of infection it was \( 1.4 \pm 0.1 \)-fold greater relative to \( \sigma\text{A} \). Hence, it appears that transcription of \( \text{Rv0189c} \) is 2-fold greater in \( \text{M. tuberculosis} \) in vivo compared to in vitro. It was also found that the \( \text{Rv0189c} \) 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, \( \text{Rv0189c} \) expression was upregulated by \( 1.72 \pm 0.2 \)-fold at pH 4.5 and \( 1.42 \pm 0.06 \) fold at pH 5.5, compared to that at pH 7.2 (Fig. 5b). The relative gene expression profiles of \( \text{Rv0189c} \) starved for 0, 4, 12, 24, 48, 72 and 96 h are shown in Fig. 5(c). It appears that nutrient starvation causes downregulation of \( \text{Rv0189c} \).

The expression profile of \( \text{Rv0189c} \) in \( \text{M. tuberculosis} \) in the J774.1 cell line at 0, 24, 48 and 72 h, compared with \( \text{M. tuberculosis} \) H37Rv grown in vitro and sampled at the same time intervals, demonstrated upregulation of \( \text{Rv0189c} \) mRNA expression by \( 1.5 \pm 0.2 \)-, \( 1.97 \pm 0.15 \)- and \( 2.3 \pm 0.2 \)-fold at 24, 48 and 72 h, respectively (Fig. 5d). However, the expression levels of other genes involved in BCAA biosynthesis, \( \text{ilvB1}, \text{ilvN} \) and \( \text{ilvC} \), remained unchanged in \( \text{M. tuberculosis} \) 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 \( \text{Rv0189c} \) gene has been annotated in the \( \text{M. tuberculosis} \) 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 (Sassetti et al., 2001, 2003). Thus if \( \text{Rv0189c} \) indeed codes for DHAD activity, as in \( \text{E. coli} \), mutations in \( \text{Rv0189c} \) 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 \( \text{rMtb}_\text{IlvD} \) protein from the soluble fraction showed comparable DHAD activity with the earlier reported activity of \( \text{E. coli} \) 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 \( \text{E. coli} \) 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 \( \text{Rv0189c} \). The \( \text{E. coli BL21(DE3)} \) \( \text{ΔilvD} \) mutant was unable to grow on MM unless supplemented with all three BCAAs. Introduction of the recombinant pET32a vector expressing \( \text{Rv0189c} \) into \( \text{E. coli BL21(DE3)} \) \( \text{ΔilvD} \) resulted in reversion to prototrophy. No reversion to prototrophy was observed upon introduction of a control plasmid; hence, the effect was specific to \( \text{Rv0189c} \). This was also supported by DHAD activity in crude cell extracts of the parent strain and \( \text{Rv0189c-complemented} \) mutant, whereas in the mutant strain...
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) ΔilvD. 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.

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 (**).

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 transcriptionsal study demonstrated that \textit{Rv0189c} is expressed both in vitro and in vivo. The higher mRNA level of \textit{Rv0189c} in mice as compared to exponential-phase cultures grown in Middlebrook 7H9 medium suggests the importance of \textit{Rv0189c} during growth in vivo. The variations in \textit{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 \textit{Rv0189c} transcription in \textit{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 \textit{Rv0189c} was observed during nutrient starvation, because many other genes involved in the BCAA biosynthetic pathway, namely \textit{ilvB1}, \textit{ilvN}, \textit{ilvC}, \textit{leuA}, \textit{leuC} and \textit{leuD}, have also been reported to be downregulated under similar conditions, suggesting metabolic slowdown (Betts et al., 2002). The fact that \textit{Rv0189c} was upregulated in \textit{M. tuberculosis} inside murine macrophages while no change in expression was seen for other BCAA biosynthetic pathway genes (\textit{ilvB1}, \textit{ilvN} and \textit{ilvC}) suggests that this function may be related to the survival of \textit{M. tuberculosis} ex vivo. The interaction of NO with the IlvD protein has previously been demonstrated in \textit{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 \textit{M. tuberculosis} is also inhibited by NO via the inactivation of \textit{Rv0189c}.

In conclusion, the present study demonstrates that \textit{Rv0189c} encodes DHAD, which is functionally equivalent to the \textit{ilvD} gene of \textit{E. coli}. Antisense studies of \textit{Rv0189c} suggest that it is an essential gene for the growth of \textit{M. tuberculosis} in vitro. \textit{Rv0189c} is probably required for intracellular growth, as is evident by its upregulation \textit{ex vivo} and \textit{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.

**ACKNOWLEDGEMENTS**

We thank the director of the institute for providing facilities and support. The study was supported by CSIR grant SIP0026. V. S. is the recipient of a Senior Research Fellowship (SRF) of the Council of Scientific and Industrial Research (CSIR), India. The communication number from CDRI is 7832.

**REFERENCES**


Edited by: W. Bitter