Biochemical and transcription analysis of acetohydroxyacid synthase isoforms in *Mycobacterium tuberculosis* identifies these enzymes as potential targets for drug development

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Acetohydroxyacid synthase (AHAS) is a biosynthetic enzyme essential for *de novo* synthesis of branched-chain amino acids. The genome sequence of *Mycobacterium tuberculosis* revealed genes encoding four catalytic subunits, *ilvB1* (Rv3003c), *ilvB2* (Rv3470c), *ilvG* (Rv1820) and *ilvX* (Rv3509c), and one regulatory subunit, *ilvN* (Rv3002c), of AHAS. All these genes were found to be expressed in *M. tuberculosis* growing *in vitro*. Each AHAS subunit gene was cloned and expressed in *Escherichia coli*. AHAS activity of IlvB1 and IlvG was found in cell-free lysates and with recombinant purified proteins. Kinetic studies with purified IlvG revealed positive cooperativity towards substrate and cofactors. To understand the role of the catalytic subunits in the biology of *M. tuberculosis*, expression of AHAS genes was analysed in different physiological conditions. *ilvB1*, *ilvB2* and *ilvG* were differentially expressed. The role of *ilvB1* in persistence is known, but the upregulation of *ilvB2* and *ilvG* in extended stationary phase, *ex vivo*, and in acid stress and hypoxic environments, suggests the relevance of AHAS enzymes in the metabolism and survival of *M. tuberculosis* by functioning as catabolic AHAS. These enzymes are therefore potential targets for drug development.

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INTRODUCTION

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* is estimated to have killed 1.3 million people in 2008 (WHO, 2009). Multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains of *M. tuberculosis* have been clinically isolated which do not respond to front-line antibiotics (Velayati *et al.*, 2009). New antibiotics acting on new targets are required to overcome the challenge posed by resistance to front-line drugs (Centers for Disease Control and Prevention, 2006). The availability of the complete genome sequence of *M. tuberculosis* and its annotation has made it possible to predict new targets which are essential for growth of tubercle bacilli (Cole *et al.*, 1998). A possible selective target, lacking in animal hosts, is branched-chain amino acid (BCAA) biosynthesis. In mycobacteria, BCAA auxotrophs were unable to grow *in vitro* or *in vivo* (Guleria *et al.*, 1996; McAdam *et al.*, 1995),

suggesting that the BCAA biosynthesis pathway could be a good target for new anti-TB drugs (Grandoni *et al.*, 1998).

Acetohydroxyacid synthase (AHAS; EC 2.2.1.6) is the first enzyme in the biosynthetic pathway leading to synthesis of BCAA and has two principal biological roles. It catalyses the first step in the synthesis of BCAA (isoleucine, leucine and valine: ILV), and in some bacteria it also functions as catabolic AHAS in the fermentation pathway in which pyruvate is channelled through acetolactate into the production of 2,3-butanediol (Fig. 1). Catabolic AHASs are known in other organisms such as *Aerobacter aerogenes*, *Enterobacter*, some *Klebsiella* and *Serratia* species, certain lactic acid bacteria (*Lactococcus* sp. and *Leuconostoc* sp.) and *Bacillus subtilis* (Blomqvist *et al.*, 1993; Johansen *et al.*, 1975; Mayer *et al.*, 1995; Renna *et al.*, 1993; Störmer, 1968b; Tsau *et al.*, 1992); they are induced by conditions such as presence of an excess of acetate (Blomqvist *et al.*, 1993; Johansen *et al.*, 1975; Mayer *et al.*, 1995; Störmer, 1968b; Störmer, 1977) and/or pyruvate (Tsau *et al.*, 1992), by low external pH (5.5–6.5) and low oxygen levels, and during the stationary phase (Renna *et al.*, 1993).

Genes encoding four catalytic subunits of AHAS, namely *ilvB1* (Rv3003c), *ilvB2* (Rv3470c), *ilvG* (Rv1820) and *ilvX*

Abbreviations: AHAS, acetohydroxyacid synthase; BCAA, branched-chain amino acids; FCS, fetal calf serum; ILV, isoleucine, leucine and valine.

Two supplementary tables and a supplementary figure are available with the online version of this paper.

Fig. 1. The BCAA biosynthesis pathway coupled with the butanediol fermentation pathway (boxed).

(*Rv3509c*), and one regulatory subunit, *ilvN* (*Rv3002c*), have been annotated in the *M. tuberculosis* genome. Only *ilvB1* has been experimentally demonstrated to encode active AHAS (Choi *et al.*, 2005). Inactivation of *ilvB1* resulted in auxotrophy for ILV *in vitro* and attenuation in virulence, but the mutant grew in macrophages and persisted in infected mice without causing disease (Awasthy *et al.*, 2009). To our knowledge there have been no reports of AHAS activity encoded by *ilvB2*, *ilvG* and *ilvX* or their role in biology of *M. tuberculosis*. This study was undertaken to investigate if any of the AHAS subunits (*IlvB2*, *IlvG*, *IlvX*) of *M. tuberculosis* has AHAS activity or can function as a catabolic AHAS. All five annotated AHAS genes were cloned and overexpressed in *Escherichia coli* for biochemical characterization of AHAS activity. The expression of AHAS genes was examined in different physiological conditions by qRT-PCR to understand their role in *M. tuberculosis*.

METHODS

Bacterial strains, plasmids and growth conditions. The *E. coli* strains and expression vector were purchased from Novagen and propagated at 37 °C in Luria-Bertani (LB) medium. The *M. tuberculosis* H37Rv TMC102 strain was cultivated at 37 °C in Middlebrook 7H9 medium (Difco) supplemented with 0.2% (v/v) glycerol, 10% (v/v) albumin-dextrose-catalase (ADC; BD Biosciences) and 0.05% (v/v) Tween 80. The J774.1 murine macrophage cell line 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 and purification of recombinant AHAS proteins.

The AHAS genes were PCR-amplified using genomic DNA of *M. tuberculosis* H37Rv and gene-specific primers (Table 1). The amplified PCR products were cloned in pGEM-T vector, subcloned in pET28a vector for producing fusion proteins with an N-terminal His tag and transformed into *E. coli* expression host BL21(DE3). *E. coli* BL21(DE3) cells harbouring the desired plasmid were grown at 37 °C in LB broth containing 50 µg kanamycin ml⁻¹ to OD₆₀₀ 0.7–0.8. The expression of protein was induced by addition of 0.1–0.5 mM IPTG followed by incubation for 3–20 h at 18–37 °C (for details of the conditions see Supplementary Table S1, available with the online version of this paper). The cells were harvested, lysed and affinity purified on a Ni²⁺-charged HiTrap chelating column (Amersham) pre-equilibrated with binding buffer. The column was washed thoroughly with the same buffer and the bound proteins were eluted by applying a linear gradient of 50–500 mM imidazole in binding buffer (see Supplementary Table S2 for details). 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. Protein samples were analysed by SDS-PAGE on 12–15% gels (Laemmli, 1970) and protein concentration was determined by the Bradford method (Bradford *et al.*, 1976), using BSA as the standard.

Biochemical characterization of recombinant proteins. Sonicated crude cell extracts containing recombinant proteins were

Table 1. Sequences of oligonucleotide primers used in the study

Primer*	Sequence (5'–3')	Restriction site
For PCR amplification of genes for cloning		
ilvB1F	CGGGATCCGTGAGCGCACCAACCAAG	<i>Bam</i> HI
ilvB1R	CGGGATCCTCAGGCTGGCCTTCGGT	<i>Bam</i> HI
ilvB2F	CCGCTCGAGGGGTGACCGTCGGGGATC	<i>Xho</i> I
ilvB2R	CCGCTCGAGCTATCGACCCAGTCCAATG	<i>Xho</i> I
ilvGF	GGAATTCGATATGAGCACCGACAC	<i>Eco</i> RI
ilvGR	GGAATTCCTCAAGCCAGGTTGGATC	<i>Eco</i> RI
ilvNF	CGGGATCCATGAGCCCCAAGACGCAC	<i>Bam</i> HI
ilvNR	CGGGATCCCTACTTGGCGGTGCCGATG	<i>Bam</i> HI
ilvXF	CCGCTCGAGGGGTGAACGGGTGCTCAGGCCCTG	<i>Xho</i> I
ilvXR	CCGCTCGAGCTACCCACAAGCGATGGCAC	<i>Xho</i> I
For real-time PCR		
sigAU	CCGGGGCATGGCGTTTCTC	
sigAL	ATGCGGCCAGCTTGTTGAT	
16SRNAU	TCCCGGGCCTTGACACA	
16SRNAL	CCACTGGCTTCGGGTGTTA	
ilvB1U	GCTGCGCCACCACACATT	
ilvB1L	TGCGCGGCCACATCTG	
ilvB2U	GGCTGCCGGCTCACCAAC	
ilvB2L	AGTCCCGCCTTCTCGCAAAAC	
ilvGU	CACGGGCGCGGTCAAAA	
ilvGL	GGCGGTCAGATCCCCATAGAG	
ilvNU	GTGGCGGCGCTGTTCTCC	
ilvNL	AGCGCCAATTCCCGTGACA	
ilvXU	GTGCCGATGGTGGTGGTTGTC	
ilvXL	ATCCGCGGGCAGAATCAGC	

*F, U: Forward direction; R, L: reverse direction.

prepared. A control extract was prepared in the same way from *E. coli* BL21(DE3) transformed with pET28a. Sonicates were cleared by centrifugation and cleared lysates were stored in 50 % (v/v) glycerol at -20°C . The AHAS activity was measured according to Choi *et al.* (2005) with the following modifications. Reaction buffer (160 μl), containing 100 mM potassium phosphate pH 7.5, 1 mM ThPP, 10 mM MgCl_2 , 50 μM FAD and 1.0 μg recombinant protein, was pre-incubated at 37°C for 10 min. The reaction was initiated by adding 40 μl 375 mM pyruvate (final concentration 75 mM) in a final reaction volume of 200 μl . After incubation at 37°C for 1 h, the reaction was terminated by the addition of 30 μl 4 M H_2SO_4 , followed by further incubation at 65°C for 15 min to decarboxylate the reaction product, acetolactate, into acetoin. One hundred microlitres of reaction product was mixed with 90 μl 0.5 % (w/v) creatine and 90 μl 5 % (w/v) α -naphthol solution in 2.5 M NaOH (freshly prepared), and incubated at 65°C for 15 min. The absorbance of the red-coloured complex of unknown structure ($\epsilon=20\,000\text{ M}^{-1}\text{ cm}^{-1}$) was measured at 525 nm with a Beckman spectrophotometer. The absorbance of the samples was compared with standard curve of acetoin concentration versus A_{525} . One unit (U) of activity is defined as the amount of enzyme required for the production of 1 μmol acetolactate min^{-1} under the assay conditions described above. For catabolic AHAS activity measurement, acetate (20 mM), which acted as an inducer of catabolic AHAS, was added to the reaction buffer. The kinetic parameters of AHAS were estimated under optimal conditions in which all the cofactors, FAD, ThPP and Mg^{2+} , were present at saturating concentrations. Kinetic data were analysed by non-linear regression using GraphPad Prism version 5.0 for Windows. The Michaelis–Menten equation (equation 1 where

$n=1$) was fitted to substrate and cofactor saturation curves, where v and S represent the initial velocity and substrate (or cofactor) concentration respectively. K_m and $V_{\max}$ represent Michaelis constant and maximal velocity respectively. Equation 1 determined the Hill coefficient (n) for substrate and cofactor binding:

$$v = V_{\max} \times S^n / [(K_m)^n + S^n] \quad (1)$$

For the analysis of optimum temperature and pH, the recombinant AHAS proteins were assayed at different temperatures and pH values using three different buffer systems: MOPS (pH 5.5–7.5), phosphate (pH 6.0–8.0) and Tris/HCl (pH 7.0–9.0).

***M. tuberculosis* infection in mice.** Eight-week-old BALB/c mice (18–20 g) were injected with 5×10^7 bacilli via the intravenous route. At 21 days post-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 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 bacteria. The bacteria were treated with RNeasy Protect for 5 min at room temperature for RNA isolation.

***M. tuberculosis* infection of the J774.1 murine macrophage cell line.** Macrophage infection was performed as described previously (Srivastava *et al.*, 2007). Briefly, the J774.1 mouse macrophage cell line was grown in DMEM supplemented with 10 % FCS at 37°C in a 5 % CO_2 humidified atmosphere. The macrophages were infected with *M. tuberculosis* at an m.o.i. of 5. After 4 h of infection, the

macrophages were washed twice with serum-free DMEM and then incubated in DMEM containing FCS and gentamicin ($50 \mu\text{g ml}^{-1}$). At different time points (0, 24, 48 and 72 h), the cells were harvested, washed with serum-free DMEM and then lysed with normal saline containing 0.1 % (v/v) Tween 80 and 0.1 % (w/v) SDS. The debris of lysed macrophages was cleared by centrifugation at 200 g and the supernatant containing bacteria was mixed with two volumes of RNeasy Protect, followed by centrifugation at 9000 g for 7 min to pellet bacteria for RNA isolation.

Preparation of *M. tuberculosis* cultures at different growth phases. Bacteria were grown to the late-exponential phase without shaking and diluted 1:500 in 100 ml Middlebrook 7H9 broth with the supplements mentioned above at 37 °C. Samples were withdrawn after 7 days (early exponential phase, $\text{OD}_{600} \sim 0.2$), 15 days (mid exponential phase, $\text{OD}_{600} \sim 0.5$), 40 days (stationary phase, $\text{OD}_{600} \sim 1.5$) and 8 months (extended stationary or dormant phase). The cells were centrifuged, treated with RNeasy Protect and processed for RNA isolation.

Acid-stress and nutrient starvation. For acid stress, *M. tuberculosis* culture ($\text{OD}_{600} \sim 0.6$) grown in Middlebrook 7H9 supplemented with ADC was harvested, washed twice with PBS (pH 7.2) and suspended in 60 ml PBS. The suspended culture was divided into three parts of 20 ml. These cultures were centrifuged and resuspended in PBS at pH 4.5, 5.5 and 7.2. After 4 h incubation with shaking (50 r.p.m.) at 37 °C, the cells were harvested by centrifugation, treated with RNeasy Protect Bacteria reagent and processed for RNA isolation. The nutrient-starved cells were prepared as described by Betts *et al.* (2002). A 7-day-old *M. tuberculosis* culture was washed twice with PBS (pH 7.2), then suspended in 150 ml PBS and starved for nutrients at 37 °C. Aliquots of the 30 ml culture were taken after 0, 4, 12, 24 and 96 h of starvation, and the pellet of bacteria was treated with RNeasy Protect Bacteria reagent and used for RNA isolation.

Cultivation of *M. tuberculosis* in an O_2 -depleted environment. The O_2 -stressed cultures, microaerophilic (NRP-1) and anaerobic (NRP-2), were generated according to the Wayne model (Wayne & Hayes, 1996) with minor modification. Briefly, 17 ml Dubos Tween-albumin medium was inoculated with 170 μl of a mid-exponential phase *M. tuberculosis* culture in a 20 mm screw-cap tube in 0.5 head space ratio. Methylene blue ($1.5 \mu\text{g ml}^{-1}$) was added as an indicator of oxygen depletion. Cultures were harvested after 12 days (NRP-1), 26 days (NRP-2) and 40 days (extended NRP-2 or NRP-3). Cell viability was determined by c.f.u. counts of 8, 12, 20, 30, and 40 day cultures. For hypoxic conditions with a different carbon source, Dubos Tween-albumin medium containing 0.5 % sodium acetate was used.

RNA isolation, cDNA synthesis and real-time PCR. The bacterial culture was centrifuged at 3000 g in a clinical centrifuge (Beckman) at 4 °C for 15 min and the pellet was suspended in 300 μl TE (Tris-EDTA) buffer containing 1 mg lysozyme ml^{-1} . The suspension was incubated for 5 min and 750 μl RLT buffer (Qiagen) containing 0.1 % β -mercaptoethanol was added. The mixture was transferred to a 2 ml screw-cap tube containing 0.4 ml 0.1 mm diameter zirconia/silica beads (Biospec Products). Three 30 s pulses in a bead beater disrupted the cells. Cell debris was separated and the supernatant was transferred to a 2 ml tube (Eppendorf) containing 250 μl RLT buffer to which 500 μl 96 % ethanol was added. The mixture was transferred to an RNeasy column and processed according to the manufacturer's instructions (Qiagen). The isolated RNA was quantified with the Quan-iT RNA Assay kit (Invitrogen), treated with DNase I (Takara) to remove any residual contamination of genomic DNA and confirmed by PCR. The cDNA was then synthesized from 0.5 μg DNase I-treated total RNA in a total reaction volume of 20 μl using RevertAid M-MuLV Reverse Transcriptase

(Fermentas). The reaction mixture contained random hexamer primer (0.2 μg), reaction buffer, 20 U RiboLock RNase inhibitor and 1 mM dNTPs. The reaction was performed according to the manufacturer's instructions (Fermentas). Real-time PCR was performed in a LightCycler 480 II (Roche), using LightCycler 480 SYBR Green I Master (Roche). Reactions containing 1 $\times$ SYBR Green I Master, 1 μl cDNA product and 0.5 μM of each primer in 20 μl were set up on ice. Samples were heated to 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. Fluorescence was captured at the end of each cycle after heating to 80 °C to ensure the denaturation of primer-dimers. Melting-curve analysis was done to confirm the primer specificity. The 16S rRNA and *sigA* genes were used as reference genes for normalization. All the data analysis was done using LightCycler 480 II software version 1.5. The experiments were performed in triplicate and the relative expression ratio was calculated from triplicate normalized ratio values for each gene, with SD. A paired *t* test was performed using GraphPad Prism version 5.00 for Windows (GraphPad Software) to determine the significance of differences in relative expression ratio; $P < 0.05$ was considered significant, and $P < 0.01$ highly significant.

RESULTS

All five AHAS genes are expressed *in vitro*

Expression levels of the AHAS genes were determined in cells from mid-exponential-phase cultures in Middlebrook 7H9 broth. Transcription levels of the AHAS genes were normalized to those of *sigA* mRNA, and calculated based on the RNA used for reverse transcription. The expression levels of the AHAS genes were 0.16 ± 0.07 (*ilvB1*), 0.53 ± 0.08 (*ilvB2*), 0.63 ± 0.05 (*ilvG*), 0.8 ± 0.4 (*ilvN*) and 0.6 ± 0.09 (*ilvX*) relative to the *sigA* mRNA, suggesting that these genes may have a functional role in the life cycle of *M. tuberculosis*.

Overexpression and purification of recombinant proteins

Overexpression of AHAS proteins in the expression host was confirmed by SDS-PAGE followed by Western blotting with anti-His antibodies. Single bands on SDS-PAGE confirmed the homogeneity of the purified recombinant proteins, with apparent molecular masses close to the calculated molecular masses of the recombinant proteins (Supplementary Fig. S1).

IlvG possess AHAS activity

The AHAS activity in crude extracts of recombinant AHAS proteins is shown in Table 2. The background AHAS activity of *E. coli* carrying plasmid pET28a was very low in comparison to the recombinant AHAS constructs. It was reported previously that acetate induced the activity of the degradative acetolactate synthase of *Bacillus subtilis* (Holtzclaw & Chapman, 1975). Therefore, AHAS activity in all four crude extracts was assayed in the presence of 20 mM acetate. We observed ~1.5-fold increase in specific activity of IlvG in crude extracts when acetate was added. The AHAS activity of purified recombinant IlvG was

Table 2. AHAS specific activity in crude extracts

The results are means $\pm$ SD of three determinations; ND, not determined. The AHAS specific activity for vector-only cells was 1.4 ± 0.3 mU mg⁻¹.

Recombinant protein	AHAS specific activity in crude extracts (mU mg ⁻¹)	
	Without acetate	With 20 mM acetate
Rv3003c (IlvB1)	42 (± 1.5)	36 (± 2.3)
Rv3470c (IlvB2)	8 (± 1.1)	11 (± 1.4)
Rv1820c (IlvG)	11 (± 1.7)	18 (± 3.7)
Rv3509c (IlvX)	0*	0
Rv3002c (IlvN)	ND	ND

*No activity detected.

focussed on because AHAS activity of IlvB1 has been reported previously (Choi *et al.*, 2005), IlvX did not show AHAS activity, and IlvB2 showed activity only in crude extracts, not as a purified protein. The kinetic parameters of IlvG were measured by fitting the data to equation (1) and are compared to published results for IlvB1 in Table 3. The results presented in Fig. 2 suggest that the pyruvate saturation curve does not follow simple Michaelis–Menten kinetics. The $V_{\max}$ was $1.39 (\pm 0.04)$ U mg⁻¹ and the K_m for pyruvate was 11.89 mM (± 0.86). The Hill coefficient (n) was $1.66 (\pm 0.15)$, indicating positive cooperativity for the binding of pyruvate. The apparent affinity for ThPP and MgCl₂, determined from the cofactor concentration required to support half-saturation of enzymic activity ($K_{0.5}$), was 41.65 μ M (± 2.8 ; $n=1.8 \pm 0.15$) and 183.1 μ M (± 18.69 ; $n=1.9 \pm 0.25$) respectively. The AHAS activity of IlvG protein was not changed on addition of IlvN protein, suggesting that IlvN is not a regulatory subunit for IlvG (data not shown). The maximal activity for IlvB1 and IlvG was in the temperature range 35–40 °C. The optimum pH for IlvG and IlvB1 was in the range 6–7 and 7–8 respectively.

Expression of *ilvG* and other AHAS isoforms varies in different physiological conditions

Expression of the AHAS genes was examined in cells grown in various conditions by measuring the mRNA transcripts

of each gene. As shown in Fig. 3(a), the mRNA levels of all AHAS genes except *ilvG* were significantly downregulated in mouse lungs at 21 days post-infection. The transcript profiles of the AHAS genes of *M. tuberculosis* infecting the J774.1 cell line at the 0, 24, 48 and 72 h time points revealed that the transcript levels of *ilvB2* and *ilvG* mRNA were upregulated whereas those of *ilvB1* and *ilvN* were repressed, and no significant change in expression was observed in the mRNA level of *ilvX* (Fig. 3b).

The effect of growth phase, pH and nutrient starvation on expression of AHAS genes was studied (Fig. 4). The level of transcription of the AHAS genes varied with growth phase (Fig. 4a). *ilvB1* was transcribed more in mid-exponential phase and its transcription level was low during the extended stationary phase. The expression of *ilvB2* was upregulated in the mid-exponential and extended stationary phase. The degree of upregulation of *ilvG* was almost identical during the mid-exponential and extended stationary phases. The level of *ilvN* mRNA gradually decreased as growth progressed. The mRNA level of the *ilvX* gene was found to be very similar during different growth phases. The transcript profiles of all the AHAS genes of *M. tuberculosis* H37Rv in acidic conditions (Fig. 4b) indicated that in mildly acidic conditions (pH 5.5), *ilvB2* and *ilvG* had increased expression, but in highly acidic conditions (pH 4.5) only *ilvB2* expression was significantly increased. The expression of *ilvB1* was repressed under

Table 3. Kinetic parameters for AHAS from mycobacteria

Error terms indicate SD; see cited papers for details.

Enzyme	C/H*	Specific activity (U mg ⁻¹)	K_m for pyruvate (mM)	K_s for ThPP (μ M)	K_s for Mg ²⁺ (μ M)	Reference
IlvG (<i>M. tuberculosis</i>)	C	1.39 ± 0.04	11.89 ± 0.86	41.65 ± 2.8	183.1 ± 18.69	This study
IlvB1 (<i>M. tuberculosis</i>)	C	2.8	2.76 ± 0.12	51.23 ± 2.3	270 ± 20.8	Choi <i>et al.</i> (2005)
	H	4.6	1.56 ± 0.39	1.40 ± 0.3		Choi <i>et al.</i> (2005)
<i>M. avium</i> AHAS	H	4	2.0 ± 0.2	7.5 ± 0.4		Zohar <i>et al.</i> (2003)

*C and H indicate catalytic subunit and holoenzyme, respectively.

Fig. 2. Saturation curves for substrate and cofactors with purified recombinant IlvG. The AHAS assay was conducted under the standard reaction conditions as described in Methods. The concentration of substrate pyruvate (a), cofactor ThPP (b) and cofactor MgCl₂ (c) was varied as indicated. The saturation curves did not follow simple Michaelis-Menten kinetics ($n=1$ in the equation $v = V_{\max} \times S^n / [(K_m)^n + S^n]$; broken lines). Similar results were obtained in three independent experiments, and the figure shows the results of one of these experiments. Error bars indicate SEM.

acidic conditions, but that of the other AHAS genes was not significantly affected. The relative gene expression profiles of *M. tuberculosis* starved in PBS for 0, 4, 12, 24 and

Fig. 3. Transcript profiles of AHAS genes in mouse lungs and in murine macrophage J774.1 cell line. (a) All AHAS genes except *ilvG* were significantly downregulated in the lungs of mice at 21 days post-infection relative to *in vitro*-grown cells. (b) Macrophages were infected with *M. tuberculosis* at an m.o.i. of 5, and RNA of *M. tuberculosis* was isolated at 0, 24, 48 and 72 h. The expression level of AHAS genes was normalized to *sigA* and compared with RNA isolated at 0 h. *ilvB2* and *ilvG* were upregulated, whereas *ilvB1* and *ilvN* were downregulated, and no change was found for *ilvX*. The results represent mean relative expression values of triplicate experiments with SD values (error bars) from one of the three experiments. $P < 0.05$ was considered significant (*), and $P < 0.01$ highly significant (**).

96 h (Fig. 4c) showed downregulation of *ilvB1*, *ilvG* and *ilvN*, but little change in the expression level of *ilvX*. Only *ilvB2* was found to be upregulated, at each time point.

The expression of AHAS genes in hypoxic conditions was also examined. The hypoxic condition was characterized by sensitivity of cells to metronidazole and resistance to rifampicin, and by measuring expression levels of the hypoxia marker genes *hspX* and *icl1* (Saxena *et al.*, 2008; Wayne & Hayes 1996). As shown in Fig. 5(a), all the AHAS genes except *ilvX* were downregulated in the NRP-1 stage. In the NRP-2 stage, *ilvB1*, *ilvN* and *ilvX* expression was repressed, while *ilvB2* and *ilvG* were overexpressed. In NRP-3, the expression of *ilvB2* and *ilvG* was enhanced. Since an increase in AHAS activity was observed with IlvG protein in the presence of acetate, we reproduced the Wayne hypoxic model with the addition of 0.5 % acetate in

Fig. 4. Expression of AHAS isoforms during various growth phases, and under acid stress and nutrient starvation. (a) Expression at different phases of growth, represented relative to the early exponential phase. (b) Expression in response to acid stress, represented relative to pH 7.2. (c) Expression in response to nutrient starvation, represented relative to 0 h. The expression levels were normalized to *sigA*. The results represent mean relative expression values of triplicate experiments with SD values (error bars) from one of the three experiments. $P < 0.05$ was considered significant (*), and $P < 0.01$ highly significant (**).

the Dubois medium. Interestingly, the presence of acetate in the medium resulted in enhanced expression of *ilvB2* and *ilvG* in stages NRP-2 and NRP-3 (Fig. 5b).

DISCUSSION

The AHAS genes are distributed in different operons along the genome of *M. tuberculosis*, which suggests their differential regulation under different physiological condi-

Fig. 5. Effect of hypoxic conditions on the expression level of AHAS isoforms in the presence of (a) glucose and (b) acetate as carbon source. The hypoxic state was generated as described in Methods. The expression levels were normalized to 16S rRNA and compared with RNA isolated from mid-exponential-phase cultures. The results represent mean relative expression values of triplicate experiments with SD values (error bars) from one of the three experiments. $P < 0.05$ was considered significant (*), and $P < 0.01$ highly significant (**).

tions. Since AHAS genes are involved in the BCAA biosynthetic pathway, they may be important for survival of mycobacteria *in vivo* and under other environmental conditions, about which very little is known. All the AHAS genes were transcribed in *M. tuberculosis* growing normally *in vitro*, suggesting their function in growth and survival. The importance of *ilvB1* in survival and virulence of *M. tuberculosis* has been reported previously (Awasthy *et al.*, 2009; Choi *et al.*, 2005).

When individual catalytic subunits were expressed in *E. coli*, only IlvB1 and IlvG were found to display AHAS activity. IlvB2 expressed AHAS activity in crude extracts only. The reason for absence of activity by purified IlvB2 is not clear but it could be due to sensitivity of the enzyme to detergent or oxygen, buffer compatibility, change in protein configuration, the reaction components or lack of defined conditions. An interesting observation in this study was the enhanced AHAS activity of IlvG in the presence of acetate, a C_2 carbon source. The enzyme kinetics study of recombinant IlvG revealed positive cooperativity in the

saturation curves as found previously with *IlvB1* (Choi *et al.*, 2005) and AHAS from barley, *Neurospora crassa*, *Lactococcus lactis*, *Serratia marcescens*, *Methanococcus aeolicus*, *Aerobacter aerogenes* and *Haemophilus influenzae* (Choi *et al.*, 2007; Kuwana *et al.*, 1968; Mifflin, 1971; Snoep *et al.*, 1992; Störmer, 1968b; Xing & Whitman, 1994; Yang & Kim, 1993).

The transcription profiles *in vitro* emphasize the role of *ilvB1*, *ilvG* and *ilvB2* in growth from the mid-exponential to the extended stationary phase, but apparently *ilvB1* was not required in the late stages of growth. In infected macrophages, *ilvG* and *ilvB2* were upregulated while *ilvB1* was down-regulated. This suggested a role for *ilvG* and *ilvB2* genes in macrophages and may explain the ability of an *ilvB1* mutant to survive in mice and grow in macrophages (Awasthy *et al.*, 2009). Earlier reports on amino acid auxotrophs of *M. tuberculosis* in animals suggest that sufficient amino acids are not available to the bacterium (Hondalus *et al.*, 2000; Sambandamurthy *et al.*, 2002; Smith *et al.*, 2001).

The upregulation of *ilvG* and *ilvB2* was also observed in response to low pH and to hypoxia; in the case of hypoxia, the upregulation was higher in the presence of acetate as carbon source. The transcription profile of AHAS isoforms in the hypoxic state showed that there is a distinct expression of AHAS genes as the cells adapt to the changing oxygen environment. In the NRP-1 state almost all the AHAS genes were downregulated, whereas during NRP-2 *ilvB2* and *ilvG* were upregulated. The upregulation of *ilvB2* during the NRP-2 stage has been reported previously (Cho *et al.*, 2006). The increased expression of *ilvG* in *in vitro* aerated cultures as well as in the NRP-2 and extended NRP-2 (NRP-3) stages of hypoxia in the presence of acetate suggested that *IlvG* behaves as a catabolic AHAS. It was reported earlier that low oxygen levels and the presence of excess acetate activated the catabolic AHAS, leading to activation of the butanediol fermentation pathway (Blomqvist *et al.*, 1993; Johansen *et al.*, 1975; Mayer *et al.*, 1995; Störmer, 1968a, b). The enhanced expression of *ilvB2* and *ilvG* *ex vivo* and in acidic conditions also supports their role as catabolic AHASs. Over-acidification of the intracellular environment and culture medium due to acidic product accumulation is prevented by the conversion of pyruvate to uncharged 3-hydroxy-2-butanone (HB) and/or 2,3-butanediol (BD) (Speck & Freese, 1973; Tsau *et al.*, 1992), which can be reutilized during the stationary growth phase when glucose has been depleted (Grundy *et al.*, 1993). Therefore, the HB biosynthesis pathway is also an energy-storing strategy (Johansen *et al.*, 1975; Mayer *et al.*, 1995) and could be employed by *M. tuberculosis* during persistence. Moreover, owing to the reversible transformation between HB and BD coupled with the NAD/NADH conversion, the pathway has been regarded as participating in regulation of the NAD/NADH ratio in bacteria (Johansen *et al.*, 1975; Magee & Kosaric, 1987).

During nutrient starvation, *ilvB2* was the only AHAS subunit gene that was notably upregulated. Nutrient starvation causes *M. tuberculosis* to arrest growth, minimize

aerobic metabolism and become resistant to isoniazid, rifampicin and metronidazole while maintaining viability. In this state, the transcription of all AHAS genes except *ilvB2* and *ilvX* was downregulated, indicating a general slowdown of metabolism. Although the metabolic role of *IlvB2* and *IlvX* could not be established, the induced genes may facilitate the survival of *M. tuberculosis* under these conditions. These proteins may perform specific functions in mycobacteria, and further investigation of their relevance in the latent state is warranted. The results of this investigation together with previous reports (Awasthy *et al.*, 2009; Choi *et al.*, 2005) strongly emphasize the role of *ilvB1*, *ilvB2* and *ilvG* in survival of *M. tuberculosis*. The regulatory role of *ilvN* has been suggested (Choi *et al.*, 2005) and the role of *ilvX* needs to be probed.

The findings of the present study suggest that *ilvG* encodes a functional AHAS and probably plays a major role during infection in macrophages. It is clear that AHAS enzymes are critical for *M. tuberculosis*. Because of their role in BCAA synthesis, inhibition of AHAS enzymes would result in auxotrophy for ILV, thus preventing growth either *in vitro* or intracellularly in a eukaryotic host. On the other hand, AHAS isoforms *IlvG* and *IlvB2* function as catabolic enzymes and thus may help *M. tuberculosis* survive in the persistent state, as their expression was upregulated during the extended stationary phase, in macrophages, and in acidic and low-oxygen conditions. It seems that multiple AHAS isozymes help in the survival of *M. tuberculosis*. Hence, AHAS enzymes should be considered as a potential target for therapeutic development.

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