Inactivation of the *ilvB1* gene in *Mycobacterium tuberculosis* leads to branched-chain amino acid auxotrophy and attenuation of virulence in mice

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Acetohydroxyacid synthase (AHAS) is the first enzyme in the branched-chain amino acid biosynthesis pathway in bacteria. Bioinformatics analysis revealed that the *Mycobacterium tuberculosis* genome contains four genes (*ilvB1*, *ilvB2*, *ilvG* and *ilvX*) coding for the large catalytic subunit of AHAS, whereas only one gene (*ilvN* or *ilvH*) coding for the smaller regulatory subunit of this enzyme was found. In order to understand the physiological role of AHAS in survival of the organism *in vitro* and *in vivo*, we inactivated the *ilvB1* gene of *M. tuberculosis*. The mutant strain was found to be auxotrophic for all of the three branched-chain amino acids (isoleucine, leucine and valine), when grown with either C₆ or C₂ carbon sources, suggesting that the *ilvB1* gene product is the major AHAS in *M. tuberculosis*. Depletion of these branched chain amino acids in the medium led to loss of viability of the Δ *ilvB1* strain *in vitro*, resulting in a 4-log reduction in colony-forming units after 10 days. Survival kinetics of the mutant strain cultured in macrophages maintained with sub-optimal concentrations of the branched-chain amino acids did not show any loss of viability, indicating either that the intracellular environment was rich in these amino acids or that the other AHAS catalytic subunits were functional under these conditions. Furthermore, the growth kinetics of the Δ *ilvB1* strain in mice indicated that although this mutant strain showed defective growth *in vivo*, it could persist in the infected mice for a long time, and therefore could be a potential vaccine candidate.

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INTRODUCTION

Mycobacterium tuberculosis, the causative agent of tuberculosis, infects approximately one-third of the world population and is responsible for a significant number of deaths (Hoft, 2008). Even though the disease can be controlled with a combination of drugs, the long duration of treatment results in non-compliance, which leads to relapse of the disease and evolution of drug resistance in the organism. The presence of drug-resistant bacteria poses a serious challenge to the treatment of tuberculosis, since a

significant number of bacteria are resistant to single and multiple drugs all over the world (Chan & Iseman, 2008). The availability of good genetic tools and the publication of the genome sequence of *M. tuberculosis* (Cole *et al.*, 1998) have led to rapid advances in understanding the biology of this important pathogen. Although a number of new inhibitors of *M. tuberculosis* have been reported, none has been advanced enough to be used clinically (Rivers & Mancera, 2008). Similarly, efforts to design new vaccines have also met with limited success (Hoft, 2008). Thus, identification and validation of new *M. tuberculosis* proteins as drug or vaccine candidates will help with the development of novel therapeutic approaches to control tuberculosis.

Acetohydroxyacid synthase (AHAS), also known as acetolactate synthase (ALS), is the key enzyme involved in biosynthesis of branched-chain amino acids (BCAA) in bacteria. AHAS catalyses the conversion of two pyruvate molecules into acetolactate, and is also involved in condensing pyruvate and 2-ketobutyrate to form 2-aceto-

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Abbreviations: AHAS, acetohydroxyacid synthase; BCAA, branched-chain amino acids; BMDM, bone-marrow-derived macrophages; DCO, double crossover; ILV, isoleucine, leucine and valine; SCO, single crossover.

Three supplementary figures showing genomic organization of *ilv* genes, multiple sequence alignment of AHAS large subunits, and PCR analysis of single-crossover recombinants are available with the online version of this paper.

2-hydroxybutyrate. The absence of this enzyme from higher eukaryotes makes it an attractive target for development of anti-mycobacterials. In fact, inhibitors of AHAS, sulfonylureas, are a well-known group of herbicides that are also capable of inhibiting the growth of a number of bacteria, including mycobacteria (LaRossa & Schloss, 1984; Grandoni *et al.*, 1998; Zohar *et al.*, 2003). AHAS has been well characterized in enterobacteria, and three isozymes of AHAS (type I IlvBN, type II IlvGM and type III IlvIH) have been reported from *Escherichia coli* and *Salmonella typhimurium* (Guardiola *et al.*, 1974; Shaw *et al.*, 1980). The three species of AHAS differ from each other in certain biochemical characteristics, such as sensitivity to valine and inhibition by sulfonylureas (Barak *et al.*, 1987). In terms of their role in different physiological conditions, it has been postulated that the three isoforms of AHAS are critical for growth on different carbon sources, with AHAS I playing a major role during growth on acetate or palmitate, while AHAS II is required for growth in the presence of glucose (Barak *et al.*, 1987; Dailey *et al.*, 1987). It has been proposed that unlike Gram-negative bacteria, the Gram-positive bacteria possess a single species of AHAS. The biochemical characterization of recombinant AHAS from *Bacillus stearothermophilus*, which has been designated IlvBN, revealed that it is a functional equivalent of the type III AHAS (IlvIH) of enterobacteria (Porat *et al.*, 2004). Similarly, *Corynebacterium glutamicum* was shown to possess a single species of AHAS (IlvBN), which is responsible for the synthesis of BCAA (Keilhauer *et al.*, 1993). Biochemical characterization of a recombinant AHAS (IlvBN) from two mycobacterial species, *Mycobacterium avium* and *M. tuberculosis*, has been reported (Zohar *et al.*, 2003; Choi *et al.*, 2005). Both of these enzymes could be inhibited by sulfonylureas and were shown to be sensitive to inhibition by valine, to varying extents.

Auxotrophic mutants of a number of bacteria with varied nutritional requirements, such as aromatic amino acids, co-factors, purines and pyrimidines, have been shown to be attenuated for virulence in mice. This suggests that the *in vivo* environment does not provide sufficient amounts of the various amino acids and co-factors required for growth (Hoiseth & Stocker, 1981; Alexander *et al.*, 1993; Hondalus *et al.*, 2000; Atkins *et al.*, 2002). As these mutants can persist in animals for long periods of time without causing disease, a number of them have been evaluated as vaccine candidates. In particular, an AHAS mutant of *Burkholderia pseudomallei*, which requires isoleucine, leucine and valine (ILV) for growth, was found to be attenuated for virulence in mice. Animals vaccinated with the mutant strain were protected against challenge by virulent bacteria (Atkins *et al.*, 2002). As the current vaccine, *M. bovis* BCG, does not offer good protection against tuberculosis, a number of approaches to generate better vaccines have been tried (Martin, 2006). Amongst them, use of auxotrophic mutants as vaccine candidates has been attempted by a number of investigators with varying degrees of success

(Jackson *et al.*, 1999; Hondalus *et al.*, 2000; Smith *et al.*, 2001; Sambandamurthy & Jacobs, 2005). A *purC* mutant of *M. tuberculosis* was shown to offer protection against virulent *M. tuberculosis* in a guinea pig model (Jackson *et al.*, 1999). Mutants exhibiting double auxotrophy (lysine and pantothenate or leucine and pantothenate) have been found to persist in animals for long periods of time, and were effective in reducing organ pathology and bacterial loads when mice and guinea pigs were challenged with virulent *M. tuberculosis* (Sambandamurthy & Jacobs, 2005). The availability of *M. tuberculosis* mutants requiring BCAA for growth would provide an opportunity for assessing their protective ability against tuberculosis.

The absence of AHAS enzymes from humans, and their specific inhibition by sulfonylureas, have led to the idea of using AHAS as a possible antibacterial drug target. Although wild-type *S. typhimurium*, carrying genes coding for all three species of AHAS, were found to be resistant to sulfonylureas, mutants lacking IlvBN were sensitive to these herbicides, pointing to the possibility of using sulfonylureas for therapeutic purposes in bacteria lacking homologues of IlvBN (LaRossa & Schloss, 1984; LaRossa & Smulski, 1984). It has also been reported that the growth of *Brucella abortus* inside macrophages could be inhibited by sulfonylureas, raising the possibility of using AHAS as a drug target for intracellular pathogens (Boigegrain *et al.*, 2005). Although sulfonylureas have been shown to inhibit the growth of *M. tuberculosis* (Grandoni *et al.*, 1998), the redundancy of genes coding for the larger subunit of AHAS in this organism makes it difficult to conclude whether the effects of sulfonylureas result from inhibition of single or multiple isozymes of AHAS. In order to understand the role of AHAS in the growth and survival of mycobacteria *in vitro* and *in vivo*, and to validate AHAS as a drug target or vaccine candidate, we inactivated the *ilvB* gene of *M. tuberculosis* and studied the mutant phenotype *in vitro*, and in macrophages and mice. Our data suggest that IlvB of *M. tuberculosis* is the major AHAS required for BCAA synthesis. The survival kinetics of the mutant in mice suggests that the $\Delta ilvB1$ strain of *M. tuberculosis* might be a suitable vaccine candidate.

METHODS

Bacterial strains. The strains used in this study are listed in Table 1.

Materials. Amino acids (isoleucine, leucine and valine) and pantothenic acid were obtained from Sigma. Hygromycin B was purchased from Roche.

Bioinformatics analysis. AHAS large subunits from *E. coli* K-12 and *M. tuberculosis* were collated from Colibri (<http://genolist.pasteur.fr/Colibri/>) and TubercuList (<http://genolist.pasteur.fr/TubercuList/>), respectively. The respective amino acid sequences of *E. coli* and *M. tuberculosis* proteins were retrieved from Swiss-Prot and TrEMBL, aligned using PILEUP, and the multiple sequence alignment was generated using Jalview. Sequences selected for alignment were as follows: *E. coli* IlvB (b3671), IlvG (b3767) and IlvI (b0077); *M. tuberculosis* IlvB1 (Rv3003c), IlvB2 (Rv3470c), IlvG (Rv1820) and IlvX

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> DH5 α	<i>endA1 hsdR17 supE44 recA1 relA1 Δ(lacZYA-argF)</i>	Lab stock
<i>M. tuberculosis</i> H37Rv (ATCC 27294)	Virulent strain of <i>M. tuberculosis</i>	Lab stock
pGOAL19	<i>E. coli</i> ori, <i>sacB</i> , <i>lacZ</i> , Hyg ^r	Parish & Stoker (2000)
pMV261	Mycobacterial expression vector	Stover <i>et al.</i> (1991)
pYUB178	Integrating vector for mycobacteria	Pascopeella <i>et al.</i> (1994)
pAZI272	Derived from pMV261 by replacing oriM with <i>att</i> and <i>int</i> regions from pYUB178	This study
pAZI0235	Δ <i>ilvB1</i> with upstream and downstream sequences cloned into <i>ScaI</i> site of pGOAL19	This study
pAZI0259	<i>ilvB1</i> cloned into <i>EcoRI</i> – <i>HindIII</i> sites of pAZI272	This study

(Rv3509c). The identity and similarity matrix of AHAS large subunit protein sequences from *E. coli* and *M. tuberculosis* was generated using MatGAT1.8 (Matrix Global Alignment Tool).

***M. tuberculosis* culture conditions.** *M. tuberculosis* H37Rv cultures were routinely grown in 7H9 or 7H11 medium (Difco) containing albumin dextrose complex (ADC). Isoleucine, leucine and valine (100 μ g ml⁻¹ each), pantothenic acid (50 μ g ml⁻¹), hygromycin (50 μ g ml⁻¹) or kanamycin (10 μ g ml⁻¹) were added to 7H9 + ADC culture medium when required.

DNA amplification by PCR. Screening of single-crossover (SCO) and double-crossover (DCO) recombinants in *M. tuberculosis* was done by PCR using Taq DNA polymerase. Single colonies were picked from plates, resuspended in 50 μ l TE (10 mM Tris, 1 mM EDTA) and boiled for 10 min. Five microlitres of this cell lysate was used for PCR in a 25 μ l volume. The denaturation and extension reactions were performed at 94 °C and 72 °C respectively. The annealing temperature and extension time for each PCR was based on the *T_m* of the primer pair and length of the PCR product. The sequences of the primers used in this study are given in Table 2.

Plasmid constructs. The plasmids used in this study are described in Table 1. The construction of the knockout construct was outsourced to Bangalore Genei. The recombination substrate for creating a deletion in the *ilvB1* gene of *M. tuberculosis* consisted of the mutant gene with flanking sequences, cloned into a suicide vector, pGOAL19. The 205 bp deletion in the *ilvB1* gene (base pairs 258–463) was generated by overlapping PCR in such a way that the deleted region was flanked by 817 bp on the 5' side and 864 bp on the 3' side of the deletion. The 1693 bp PCR-amplified DNA fragment was first cloned into a TA cloning vector (pDK101) and was subsequently

moved into the *ScaI* site of pGOAL19, to generate the knockout construct pAZI0235. In order to convert pMV261 (Stover *et al.*, 1991) into an integrating vector, a *MluI*–*XbaI* fragment from pYUB178 (Pascopeella *et al.*, 1994) containing *att* and *int* sites was used to replace a *MluI*–*XbaI* fragment (encompassing oriM) from pMV261. The resulting plasmid was designated pAZI272. To make the complementation construct, the *ilvB1* gene was amplified from *M. tuberculosis* genomic DNA and cloned into the *EcoRI*–*HindIII* sites of the mycobacterial expression vector pAZI272, to produce pAZI0259. The sequence of the cloned fragments was confirmed by DNA sequencing. The plasmids were electroporated into *M. tuberculosis* as described by Wards & Collins (1996).

Southern blotting. Genomic DNA from the wild-type *M. tuberculosis* and the *ilvB1* mutant was digested with *NruI* and electrophoresed on a 0.8% agarose gel. A probe was generated by PCR amplification of an internal fragment of the *ilvB1* gene, using primers 356 and 486, corresponding to the upstream and downstream regions of the deletion. The PCR fragment was fluorescein-labelled by random prime labelling (Roche). Transfer, hybridization and washing were performed following the manufacturer's protocol (GE Healthcare). The bound probe was detected by horseradish-peroxidase-conjugated anti-fluorescein antibodies (1:2000 dilution) using chemiluminescence.

Growth and death kinetics *in vitro*. Wild-type and Δ *ilvB1* *M. tuberculosis* cultures were grown in 7H9 in the presence of amino acids, when required, to OD₆₀₀ 1.0. For growth studies, the cultures were washed and resuspended in plain 7H9 broth. These cultures were then divided and grown with increasing concentrations of ILV or different combinations of Ile, Leu and Val. To study the survival kinetics of the Δ *ilvB1* mutant in the absence of ILV, a culture grown in 7H9 containing ILV was washed with plain 7H9 and resuspended in fresh 7H9, with and without amino acids. The cultures were incubated at 37 °C for 4 weeks, and viability of the cultures was monitored at regular intervals by plating various dilutions onto 7H9 plates containing ILV (each 100 μ g ml⁻¹) and 50 μ g pantothenic acid ml⁻¹. C.f.u. were counted after 1 month.

Growth of *M. tuberculosis* H37Rv and the Δ *ilvB1* mutant in macrophages. Intracellular infection and survival kinetics of mycobacterial strains were investigated in gamma-interferon-activated primary cultures of bone-marrow-derived macrophages (BMDM), in a 24-well plate format, as described by Muñoz-Eliás & McKinney (2005). Briefly, bone marrow cells were flushed from mouse femurs and cultured in complete RPMI 1640 medium (Gibco Laboratories) with 20% L929 conditioned medium, for 5 days. The macrophage monolayers were activated by addition of 25 ng ml⁻¹ recombinant IFN- γ (Sigma) for 48 h. Activated and washed

Table 2. Sequence of primers used in this study

Primer	Sequence
355	5'-AATCGCAGCTCGGTACCGTCCTT-3'
356	5'-AGCGACCAACCAAGCCACACTC-3'
357	5'-CGTGTGCATGGCAACGTCGGGT-3'
358	5'-GAGATGTCGGCCTCCTGGAAGG-3'
359	5'-GAGCGGACCAAGAAAGTTGTGCTT-3'
360	5'-CCTGGTTGGTCATCTGGAAGCAA-3'
361	5'-GGCGTGGCCTTCGGTGATG-3'
486	5'-TTCGTGGCGGACCAGCACGT-3'

BMDM monolayers were used for infection. All macrophage infection studies were performed in a custom-made RPMI 1640 medium (Cell Culture Technologies) supplemented with 20 $\mu\text{g ILV ml}^{-1}$. A triplicate set of monolayers was infected with *M. tuberculosis* H37Rv or $\Delta ilvB1$ strains, individually, at an m.o.i. of 1:10. Two hours post-infection, the monolayers were washed with PBS and replaced with RPMI 1640 medium supplemented with 20 $\mu\text{g ILV ml}^{-1}$. A set of BMDM cells infected with the wild-type or the $\Delta ilvB1$ mutant were lysed on days 1, 4, 7 and 11. The lysates were plated onto 7H11 agar plates containing 100 $\mu\text{g ILV ml}^{-1}$ and 50 $\mu\text{g pantothenic acid ml}^{-1}$.

Mouse infection. The Institutional Animal Ethics Committee, registered with the government of India (registration no. CPCSEA 99/5) approved all animal experimental protocols and usage. Six- to eight-week-old BALB/c mice, purchased from the National Institute of Nutrition, Hyderabad, India, were randomly assigned, five per cage, with the restriction that the weight of all cage members was within 1–2 g of each other. The mice were allowed two weeks acclimatization before intake into experiments. For intravenous infection, frozen bacterial samples were thawed, centrifuged, washed in PBS, and diluted appropriately in cold, sterile PBS. The mice were infected with wild-type, mutant or complemented *M. tuberculosis* strains by intravenous inoculation into the tail vein, to obtain approximately 10^5 c.f.u. per animal. Post-infection, the animals were sacrificed at an interval of one week and the bacterial counts present in the organs were determined by plating the lung and spleen homogenates on 7H11 agar supplemented with 100 $\mu\text{g ILV ml}^{-1}$ and 50 $\mu\text{g pantothenic acid ml}^{-1}$.

RESULTS

Bioinformatics analysis of AHAS genes in *M. tuberculosis*

In the KEGG and TubercuList genome databases, the following *M. tuberculosis* genes have been suggested to code for large subunits of AHAS: *ilvB1*, *ilvB2*, *ilvG* and *ilvX*. Only one gene, *ilvH*, which was also designated *ilvN* in an earlier study (Choi *et al.*, 2005), has been annotated as the gene coding for the smaller regulatory subunit of AHAS. The genomic organization of the AHAS-coding genes in

M. tuberculosis is shown in Fig. 1. The distribution of genes coding for large subunits of AHAS into different operons probably allows differential transcriptional regulation of these genes under varied environmental conditions. The organization of *ilvB1* and *ilvH* into an operon, *ilvB1HC*, is reminiscent of the transcriptional coupling of *ilvBNC*, in which *ilvB*, *ilvN* and *ilvC* code for large and small subunits of type I AHAS and ketol-acid reductoisomerase, respectively, in a number of Gram-positive bacteria (Supplementary Fig. S1). A number of genes not involved in BCAA synthesis are also present in the operons of *M. tuberculosis*, as shown in Fig. 1. These include a pre-protein translocase subunit (SecA2) and hypothetical proteins (Rv3471c and Rv3510c). The functional significance of these unrelated genes in the *ilv* operons is not understood. In terms of similarity with *E. coli* proteins, IlvB1 of *M. tuberculosis* shows greatest homology to IlvG (type II) and IlvI (type III), followed by IlvB (type I) of *E. coli* (Table 3). Analysis of regions involved in TPP and FAD binding, and active-site residues of IlvBI, also showed maximum similarity with IlvG and IlvI of *E. coli* (Supplementary Fig. S2). The other large subunits of *M. tuberculosis* AHAS (IlvB2, IlvG and IlvX) showed much less homology (approx 21–26 % identity) to the large subunits of *E. coli*. Homology analysis of the smaller regulatory protein (IlvH or IlvN) showed that it had greatest homology to IlvH (smaller subunit of type III) of *E. coli*. Based upon the homology analysis it is tempting to suggest that the AHAS encoded by *ilvB1* and *ilvH* of *M. tuberculosis* is probably a hybrid of the type II and type III isozymes of enterobacteria. A search for genes encoding AHAS in another intracellular mycobacterial pathogen, *Mycobacterium leprae*, showed the presence of the following genes coding for large subunits of AHAS: *ilvB*, *ilvG* and *ilvX* (KEGG database). Since a large number of genes required for *in vitro* growth have been lost in this obligate human pathogen (Cole *et al.*, 2001), these findings suggest that multiple *ilv* genes might play a role in the survival of mycobacteria under *in vivo* conditions.

Fig. 1. Genomic organization of the various *ilv* genes coding for large and small subunits of AHAS in *M. tuberculosis* and *E. coli*.

Table 3. Percentage amino acid sequence identity and similarity between Ilv proteins of *M. tuberculosis* and *E. coli*

Similarity is shown below the diagonal and identity is shown above the diagonal.

				1	2	3	4	5	6	7
1	IlvB	<i>Eco</i>	b3671		40.8	45.6	24.5	38	26	23.6
2	IlvB1	<i>Mtu</i>	Rv3003c	57		43.6	24.9	42.4	24.2	21.6
3	IlvG	<i>Eco</i>	b3767	64.1	58.4		25.2	43.6	25.6	21.8
4	IlvG	<i>Mtu</i>	Rv1820	45	40.3	46		25.3	25.3	21.8
5	IlvI	<i>Eco</i>	b0077	58.7	59.2	63.2	41.1		26.2	21.2
6	IlvB2	<i>Mtu</i>	Rv3470	46.3	41.3	46	42.2	46.5		23.6
7	IlvX	<i>Mtu</i>	Rv3509c	39.5	34	37	39.5	35.4	39.9	

IlvB1 is essential in the absence of BCAA

To decipher the roles of the multiple genes coding for large subunits of AHAS, we inactivated the *ilvB1* gene of *M. tuberculosis* H37Rv by homologous recombination, following a two-step method described by Parish & Stoker (2000). To ensure that no active IlvB protein was made, the portion of the *ilvB* gene downstream of the introduced deletion was rendered out of frame, which meant that the construct could only code for an 85 aa (out of 618 residues) truncated polypeptide. Since the mutation in the *ilvB1* gene was an unmarked one, free from polar effects associated with antibiotic markers, the expression of the downstream genes of the operon (*ilvN* and *ilvC*) should not have been affected. Out of 16 colonies of SCO recombinants screened, 3 colonies had the plasmid integrated in the proper locus, as confirmed by PCR using primers 355 and 359 (Supplementary Fig. S3a). One of the SCO recombinants (Supplementary Fig. S3b, lane 4) was grown in the absence of hygromycin for 3 weeks and subsequently plated either on 7H9 plates containing 2% sucrose or on 7H9 plates containing 2% sucrose and supplemented with ILV (each 100 µg ml⁻¹) and pantothenic acid (50 µg ml⁻¹). The colonies growing in the presence of sucrose were screened for loss of the plasmid by

PCR amplification of the *hyg^r* and *sacB* genes; 80 % of the colonies growing on sucrose plates were found to have lost the plasmid. No *ilvB1* mutant colonies were obtained in the absence of BCAA in the medium, indicating that the *ilvB1* gene is essential in the absence of BCAA and that the genes coding for putative large subunits of AHAS (*ilvB2*, *ilvG* and *ilvX*) were unable to complement the loss of *ilvB1* under the conditions employed. Screening of colonies from ILV-containing plates, by PCR using gene-specific primers, showed that 94 % of the colonies had retained the wild-type copy of the gene, while the remaining 6 % were found to harbour a deletion in the *ilvB1* gene (data not shown). The absence of the wild-type copy in one of the DCO mutants (no. 19) was further confirmed by PCR (Fig. 2b), using primers corresponding to the regions indicated in Fig. 2(a). Finally, Southern blot analysis, using a probe corresponding to the internal regions of the *ilvB1* gene, showed the presence of an expected smaller fragment in the Δ *ilvB1*/19 mutant (Fig. 2c).

In order to understand the expression profile of IlvB1 under active-growth and stationary-phase conditions, the *M. tuberculosis* cell lysates were subjected to Western analysis using antibodies raised against the IlvB1 protein. However, because multiple protein bands were detected,

Fig. 2. Confirmation of *ilvB1* gene inactivation in *M. tuberculosis* by PCR and Southern blotting. (a) Positions of primers used for PCR amplification to differentiate between wild-type and Δ *ilvB1* DCO recombinants. (b) PCR amplification of one of the DCO recombinants (no. 19) using the primer combinations indicated. Lanes: 1, *ilvB1* knockout (Δ *ilvB1*/19); 2, wild-type; 3, SCO recombinant; M, molecular size marker. (c) Confirmation of *ilvB1* gene deletion in DCO no. 19 (Δ *ilvB1*/19) by Southern blotting of *NruI* digested genomic DNA. Lanes: 1, Δ *ilvB1*/19 mutant; 2, wild-type *M. tuberculosis*.

probably due to antibody cross-reactivity with the putative large subunits of AHAS (IlvB1, IlvB2, IlvG and IlvX), the results were inconclusive (data not shown). Further studies are required to understand the changes in expression of IlvB1 and other AHAS subunits under varied physiological conditions.

***In vitro* growth characteristics of *M. tuberculosis* Δ ilvB1/19**

Since 2-ketoisovalerate is a common intermediate for synthesis of BCAA and pantothenate, we analysed the nutritional requirements of *M. tuberculosis* Δ ilvB1/19 for ILV and pantothenate. No growth was seen in the absence of ILV (Fig. 3a). Addition of pantothenic acid stimulated growth, but the mutant cultures were found to grow in its absence also (data not shown). To ascertain whether the mutant was auxotrophic for all three amino acids, cultures were grown in the presence of IL, IV or LV. The Δ ilvB1/19 mutant was found to be auxotrophic for all three BCAA, as no growth of this mutant was observed in the absence of any one of these amino acids from the medium (Fig. 3b). The growth kinetics following addition of increasing concentrations of the amino acids indicated that the mutant strain required a minimum of $50 \mu\text{g ml}^{-1}$ each amino acid for growth. Growth was poor at $20 \mu\text{g ml}^{-1}$ and no growth was observed below $20 \mu\text{g ml}^{-1}$ (Fig. 3a). Upon complementation with a copy of the *ilvB1* gene in an integrating vector, the wild-type phenotype could be restored and the cultures could grow in the absence of the three amino acids, confirming that the inactivation of the *ilvB1* gene leads to auxotrophic requirement for BCAA (data not shown). Since inactivation of individual *ilv* genes in enterobacteria leads to BCAA auxotrophy only when the bacteria are grown on glucose or a C_2 carbon source (Dailey *et al.*, 1987), we investigated whether *M. tuberculosis* Δ ilvB1 was prototrophic when grown in the presence of a C_2 carbon source. For this purpose, the mutant culture was diluted 1:100 and inoculated into Dubos medium containing palmitate as a source of C_2 units. No growth could be seen in the absence of ILV, indicating the requirement of these amino acids for growth in a C_2 carbon source (data not shown).

***In vitro* survival kinetics of *M. tuberculosis* Δ ilvB1/19**

In order to determine whether ILV depletion leads to cessation of growth (static effect) or loss of viability (cidal effect) in *M. tuberculosis* *in vitro*, the viability of the mutant culture, maintained at different concentrations of ILV, was measured over a period of time. For this purpose, *M. tuberculosis* Δ ilvB1/19 grown with ILV was washed and resuspended in 7H9 containing 0, 20 or $100 \mu\text{g ml}^{-1}$ ILV. Samples were taken at various time intervals and plated on 7H9 containing ILV ($100 \mu\text{g ml}^{-1}$) and pantothenate ($50 \mu\text{g ml}^{-1}$). As shown in Fig. 3(c), there was a continuous drop in the number of c.f.u. of *M. tuberculosis*

Fig. 3. *In vitro* growth and survival kinetics of the Δ ilvB1/19 mutant of *M. tuberculosis* grown in 7H9 broth at 37 °C. (a) Growth of the mutant in the presence of increasing concentrations of ILV (0–100 $\mu\text{g ml}^{-1}$). (b) Growth of the mutant in the presence of different combinations of ILV (100 $\mu\text{g ml}^{-1}$ of each amino acid). A slight drop in OD₆₀₀ at the last time point in (a) and (b) is due to clumping of the culture. The experiments were repeated three times and results from a representative experiment are shown (mean $\pm$ SEM; error bars in (a) are not shown where smaller than symbols). (c) Survival/death kinetics of the *ilvB1* mutant in the presence and absence of ILV. The concentrations of amino acids used ($\mu\text{g ml}^{-1}$) are indicated. Pan50, pantothenate (50 $\mu\text{g ml}^{-1}$).

$\Delta ilvB1/19$ grown in the absence of ILV. The culture maintained in $20 \mu\text{g ILV ml}^{-1}$ also showed loss of viability, but the rate of death was slower than that of cultures with no ILV. The *M. tuberculosis* $\Delta ilvB1/19$ culture grown in the presence of $100 \mu\text{g ILV ml}^{-1}$ showed an increase in the number of c.f.u. (Fig. 3c). This clearly proves that depletion of the intracellular pool of BCAA results in death of *M. tuberculosis*.

Growth of *M. tuberculosis* $\Delta ilvB1/19$ in macrophages

In order to assess the contribution of *ilvB1* to survival in macrophages, an *M. tuberculosis* $\Delta ilvB1/19$ culture, washed free of amino acids, was used to infect BMDM at an m.o.i. of 10. Since the macrophages did not grow well in the complete absence of ILV, the survival of the mutant bacteria inside macrophages in the absence of BCAA could not be studied. Instead, the macrophages were maintained in the presence of $20 \mu\text{g ILV ml}^{-1}$, which is close to the physiological concentration of BCAA *in vivo* in humans (Filho *et al.*, 1997). At this concentration of ILV, the macrophages proliferated well and were found to be healthy. The infected macrophages were lysed at regular intervals (1, 4, 7 and 11 days post-infection) and plated on 7H9 medium containing ILV ($100 \mu\text{g ml}^{-1}$) and pantothenate ($50 \mu\text{g ml}^{-1}$). The number of c.f.u. obtained from the *ilvB1/19* mutant was similar to the bacterial counts obtained from the wild-type *M. tuberculosis* H37Rv on all days of sampling (Fig. 4). The mutant cultures harvested from the macrophages did not grow in the absence of ILV, showing that they had not reverted to

Fig. 4. Growth of the $\Delta ilvB1/19$ mutant in BMDM. The macrophages were infected with the wild-type and the mutant *M. tuberculosis* in triplicate. The infected macrophages were lysed at the indicated time intervals and plated on 7H10 in the presence of ILV ($100 \mu\text{g ml}^{-1}$) and pantothenate ($50 \mu\text{g ml}^{-1}$). The experiment was repeated twice and results from one experiment are shown (mean $\pm$ SEM).

prototrophy (data not shown). Since a suboptimal concentration of ILV ($20 \mu\text{g ml}^{-1}$) does not support the growth of the *M. tuberculosis* $\Delta ilvB1/19$ mutant culture *in vitro*, the macrophage survival kinetics suggested that intracellular survival of $\Delta ilvB1/19$ in the presence of a suboptimal concentration of ILV was due either to an accumulation of ILV inside macrophages by active uptake, or to other genes encoding the large subunit of AHAS being active in the intracellular environment.

M. tuberculosis $\Delta ilvB1/19$ is attenuated for growth in mice

In order to study the effect of *ilvB1* inactivation on replication and survival of *M. tuberculosis* in mice, the mutant strain was used to infect mice intravenously and the growth of bacteria was monitored by quantification of bacterial loads in the lungs and spleen at regular intervals, for 4 weeks. All cultures were plated on 7H9 containing $100 \mu\text{g ILV ml}^{-1}$. As shown in Fig. 5a, the initial bacterial loads of the three strains in the lungs and spleens varied slightly, with the mutant strain showing higher c.f.u. in both organs. Small differences in the initial bacterial loads in the organs of mice have been observed previously, and this is not expected to influence the outcome of infection (Ewann *et al.*, 2002; Sun *et al.*, 2004). In contrast to the wild-type bacteria, which increased in numbers in the lungs of infected animals over a period of 4 weeks (from 1.3×10^4 to 2.6×10^5 c.f.u.), the *ilvB1* mutant was found to be attenuated for growth in the lungs, since despite a higher bacterial load at the beginning of infection, it showed a slow reduction in c.f.u. over this period (from 1.8×10^4 to 4.6×10^2 c.f.u., Fig. 5a). The *M. tuberculosis* mutant strain complemented with a copy of the *ilvB* gene behaved in a similar manner to the wild-type strain, and showed an increase in c.f.u. in the lungs of infected animals (from 1.0×10^3 to 1.6×10^5 c.f.u.). A similar picture was seen in the spleen, where the wild-type and the complemented strain showed an increase in the number of bacteria recovered, whereas the mutant strain showed a small decline in the number of c.f.u. recovered over a period of 4 weeks (Fig. 5b). Even though the mutant was attenuated in virulence compared to the wild-type *M. tuberculosis*, it persisted in the organs of the infected mice and was not eliminated completely in four weeks. The bacteria obtained from organs of animals infected with *M. tuberculosis* $\Delta ilvB1/19$ were dependent on ILV for growth *in vitro*, ruling out the possibility of reversion to prototrophy under *in vivo* conditions.

DISCUSSION

Although described as *IlvBN* in earlier reports (Zohar *et al.*, 2003; Choi *et al.*, 2005), which suggested similarity to type I AHAS of *E. coli*, our bioinformatics analysis predicts the major AHAS of *M. tuberculosis* to be a hybrid of the type II and type III *E. coli* AHAS. This view is supported by earlier

Fig. 5. Growth kinetics of wild-type *M. tuberculosis* H37Rv, the $\Delta ilvB1/19$ mutant and the complemented mutant *in vivo* in mice. (a) Lungs, (b) spleen. BALB/c mice were injected with the mycobacterial cultures and bacterial growth was monitored by quantification of the c.f.u. obtained by plating organ homogenates on 7H11 plates supplemented with ILV ($100 \mu\text{g ml}^{-1}$) and pantothenate ($50 \mu\text{g ml}^{-1}$). The data represent the mean $\pm$ SEM c.f.u. of wild-type, $\Delta ilvB1/19$ mutant and complemented *M. tuberculosis* strains, obtained from the lungs or spleens of five animals at each time point.

studies on AHAS of the Gram-positive bacterium *B. stearothermophilus*, in which ALS (encoded by *ilvBN*) was proposed to be similar to AHAS III of *E. coli* (Porat *et al.*, 2004). Biochemical characterization of IlvBN from *M. tuberculosis* and *M. avium* showed that the recombinant enzyme was sensitive to valine, and that the AHAS activity could be inhibited by sulfonylureas (Zohar *et al.*, 2003; Choi *et al.*, 2005), thus making it a functional equivalent of the type II enzyme of enterobacteria. Although Gram-positive bacteria possess a single species of AHAS, mostly classified as IlvBN type (Keilhauer *et al.*, 1993; Porat *et al.*, 2004), the presence of multiple genes coding for the

catalytic subunits of AHAS in *M. tuberculosis* points towards the possible existence of multiple AHAS isozymes in this organism.

The inability of the *ilvB1* mutant to grow in the absence of ILV in the medium showed that none of the other AHAS catalytic subunits could compensate for the loss of IlvB1. This indicates either that these proteins are not expressed optimally under *in vitro* conditions, or that they exhibit insufficient activity to synthesize the required amounts of BCAA. The physiological role of the multiple large subunits of *M. tuberculosis* AHAS under other physiological states remains unclear. Since most of the genes present in the bacterial genome are active under the correct physiological condition (Tamburini & Mastromei, 2000), it will be interesting to see under what growth conditions *ilvB2*, *ilvG* and *ilvI* are expressed. The lack of a growth defect shown by the *ilvB1* mutant in macrophages in the presence of a suboptimal concentration of ILV could be due either to uptake of BCAA from the *in vivo* environment or to the mutants having the ability to synthesize some BCAA using an alternative mechanism, or to a combination of both. Although the $\Delta ilvB1$ mutant is attenuated for replication in mice, it manages to persist in the organs of infected mice. The growth defects shown by amino acid auxotrophs of *M. tuberculosis* in animals suggest that sufficient amounts of amino acids are not available to *M. tuberculosis* in mice (Hondalus *et al.*, 2000; Smith *et al.*, 2001; Sambandamurthy & Jacobs, 2005). This raises the possibility that the other AHAS enzymes, IlvB2, IlvG and IlvX, which do not seem to have any role in BCAA synthesis *in vitro*, might be able to provide some activity *in vivo*, thus enabling the bacteria to synthesize suboptimal levels of BCAA, allowing survival, but not providing enough BCAA for growth. Further studies on expression profiling, gene inactivation and *in vitro* biochemical characterization of IlvB2, IlvG and IlvX will help to resolve these issues. Earlier studies on gene inactivation in enterobacteria have demonstrated that the multiplicity of AHAS has evolved to help the organisms in adapting to growth on C_2 or C_6 carbon sources, in the absence of external supplies of amino acids (Barak *et al.*, 1987; Dailey *et al.*, 1987). Since *M. tuberculosis* is known to utilize C_2 as the preferred carbon source for intracellular survival and replication (Muñoz-Elias & McKinney, 2006), it can be speculated that some AHAS isozymes might be functional under similar alternative physiological conditions. In the absence of genes coding for multiple regulatory subunits (small subunit), it can be postulated either that these catalytic subunits do not require the regulatory subunits for activity, or that all of them can associate with the lone regulatory subunit, IlvH. Evidence for both of these possibilities emerges from the earlier studies on the activity shown by the isolated catalytic AHAS subunit of *B. stearothermophilus* and subsequent increase in activity upon reconstitution with an *E. coli* AHAS III regulatory subunit (Porat *et al.*, 2004). The retention of genes coding for multiple catalytic subunits of AHAS in *M. leprae*, an organism that has lost a

large number of genes not required for growth *in vitro* (Cole *et al.*, 2001), also indicates that multiple AHAS might help in survival *in vivo*.

Although the rapid loss of viability observed with the *ilvB1* mutant in a medium lacking ILV suggested that inhibition of IlvB1 alone would lead to killing of *M. tuberculosis* under *in vitro* growth conditions, persistence of the *ilvB1* mutant in mice indicates that IlvB1 inhibition alone would not lead to rapid killing of *M. tuberculosis* *in vivo*. The *in vivo* and *ex vivo* growth inhibition of *M. tuberculosis* by sulfometuron methyl and its derivatives, shown in earlier studies (Grandoni *et al.*, 1998; Sohn *et al.*, 2008), may possibly have resulted from inhibition of all of the *in vivo*-active isozymes of AHAS. *In vitro* inhibition studies with other AHAS isozymes of *M. tuberculosis* will provide the necessary evidence for this contention.

Studies of a number of BCAA auxotrophic mutants of intracellular pathogens, such as *S. typhimurium*, *B. pseudomallei* and *Listeria monocytogenes*, have shown that due to limited availability of these amino acids *in vivo*, the mutant bacteria are attenuated in virulence, and thus have been proposed as vaccine candidates (Hoiseth & Stocker, 1981; Alexander *et al.*, 1993; Atkins *et al.*, 2002). This has been found to be true for *M. tuberculosis* as well, where it has been observed that mutants requiring purines, leucine, pantothenate or arginine were defective for growth in macrophages and mice, and these have proven to be good vaccine candidates, as shown by their efficacy in animals, which has been comparable to the current BCG vaccine (Jackson *et al.*, 1999; Hondalus *et al.*, 2000; Smith *et al.*, 2001; Sambandamurthy & Jacobs, 2005). As the *ilvB1* mutant is sufficiently attenuated and can persist in mice for long periods, there is a possibility of using this mutant as a vaccine candidate.

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REFERENCES

- Alexander, J. E., Andrew, P. W., Jones, D. & Roberts, I. S. (1993). Characterization of an aromatic amino acid-dependent *Listeria monocytogenes* mutant: attenuation, persistence, and ability to produce protective immunity in mice. *Infect Immun* **61**, 2245–2248.
- Atkins, T., Prior, R. G., Mack, K., Russell, P., Nelson, M., Oyston, P. C., Dougan, G. & Tittball, R. W. (2002). A mutant of *Burkholderia pseudomallei*, auxotrophic in the branched chain amino acid biosynthetic pathway, is attenuated and protective in a murine model of melioidosis. *Infect Immun* **70**, 5290–5294.

- Barak, Z., Chipman, D. M. & Gollop, N. (1987). Physiological implications of the specificity of acetohydroxy acid synthase isozymes of enteric bacteria. *J Bacteriol* **169**, 3750–3756.
- Boigegrain, R. A., Liautard, J. P. & Köhler, S. (2005). Targeting of the virulence factor acetohydroxyacid synthase by sulfonylureas results in inhibition of intramacrophagic multiplication of *Brucella suis*. *Antimicrob Agents Chemother* **49**, 3922–3925.
- Chan, E. D. & Iseman, M. D. (2008). Multidrug-resistant and extensively drug-resistant tuberculosis: a review. *Curr Opin Infect Dis* **21**, 587–595.
- Choi, K. J., Yu, Y. G., Hahn, H. G., Choi, J. D. & Yoon, M. Y. (2005). Characterization of acetohydroxyacid synthase from *Mycobacterium tuberculosis* and the identification of its new inhibitor from the screening of a chemical library. *FEBS Lett* **579**, 4903–4910.
- Cole, S. T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., Gordon, S. V., Eiglmeier, K., Gas, S. & other authors (1998). Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**, 537–544.
- Cole, S. T., Eiglmeier, K., Parkhill, J., James, K. D., Thomson, N. R., Wheeler, P. R., Honoré, N., Garnier, T., Churcher, C. & other authors (2001). Massive gene decay in the leprosy bacillus. *Nature* **409**, 1007–1011.
- Dailey, F. E., Cronan, J. E., Jr & Maloy, S. R. (1987). Acetohydroxy acid synthase I is required for isoleucine and valine biosynthesis by *Salmonella typhimurium* LT2 during growth on acetate or long-chain fatty acids. *J Bacteriol* **169**, 917–919.
- Ewann, F., Jackson, M., Pethe, K., Cooper, A., Mielcarek, N., Ensergueix, D., Gicquel, B., Loch, C. & Supply, P. (2002). Transient requirement of the PrrA-PrrB two-component system for early intracellular multiplication of *Mycobacterium tuberculosis*. *Infect Immun* **70**, 2256–2263.
- Filho, J. C., Bergström, J., Stehle, P. & Fürst, P. (1997). Simultaneous measurements of free amino acid patterns of plasma, muscle and erythrocytes in healthy human subjects. *Clin Nutr* **16**, 299–305.
- Grandoni, J. A., Marta, P. T. & Schloss, J. V. (1998). Inhibitors of branched-chain amino acid biosynthesis as potential antituberculosis agents. *J Antimicrob Chemother* **42**, 475–482.
- Guardiola, J., De Felice, M. & Iaccarino, M. (1974). Mutant of *Escherichia coli* K-12 missing acetolactate synthase activity. *J Bacteriol* **120**, 536–538.
- Hoft, D. F. (2008). Tuberculosis vaccine development: goals, immunological design, and evaluation. *Lancet* **372**, 164–175.
- Hoiseth, S. K. & Stocker, B. A. (1981). Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. *Nature* **291**, 238–239.
- Hondalus, M. K., Bardarov, S., Russell, R., Chan, J., Jacobs, W. R. & Bloom, B. R. (2000). Attenuation of and protection induced by a leucine auxotroph of *Mycobacterium tuberculosis*. *Infect Immun* **68**, 2888–2898.
- Jackson, M., Phalen, S. W., Lagranderie, M., Ensergueix, D., Chavarot, P., Marchal, G., McMurray, D. N., Gicquel, B. & Guilhot, C. (1999). Persistence and protective efficacy of a *Mycobacterium tuberculosis* auxotroph vaccine. *Infect Immun* **67**, 2867–2873.
- Keilhauer, C., Eggeling, L. & Sahm, H. (1993). Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. *J Bacteriol* **175**, 5595–5603.
- LaRossa, R. A. & Schloss, J. V. (1984). The sulfonylurea herbicide sulfometuron methyl is an extremely potent and selective inhibitor of acetolactate synthase in *Salmonella typhimurium*. *J Biol Chem* **259**, 8753–8757.
- LaRossa, R. A. & Smulski, D. R. (1984). *ilvB*-encoded acetolactate synthase is resistant to the herbicide sulfometuron methyl. *J Bacteriol* **160**, 391–394.

- Martin, C. (2006).** Tuberculosis vaccines: past, present and future. *Curr Opin Pulm Med* **12**, 186–191.
- Muñoz-Elías, E. J. & McKinney, J. D. (2005).** *Mycobacterium tuberculosis* isocitrate lyases 1 and 2 are jointly required for *in vivo* growth and virulence. *Nat Med* **11**, 638–644.
- Muñoz-Elías, E. J. & McKinney, J. D. (2006).** Carbon metabolism of intracellular bacteria. *Cell Microbiol* **8**, 10–22.
- Parish, T. & Stoker, N. G. (2000).** Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis* *tlyA plcABC* mutant by gene replacement. *Microbiology* **146**, 1969–1975.
- Pascopeella, L., Collins, F. M., Martin, J. M., Lee, M. H., Hatfull, G. F., Stover, C. K., Bloom, B. R. & Jacobs, W. R., Jr (1994).** Use of *in vivo* complementation in *Mycobacterium tuberculosis* to identify a genomic fragment associated with virulence. *Infect Immun* **62**, 1313–1319.
- Porat, I., Vinogradov, M., Vyazmensky, M., Lu, C. D., Chipman, D. M., Abdelal, A. T. & Barak, Z. (2004).** Cloning and characterization of acetohydroxyacid synthase from *Bacillus stearothermophilus*. *J Bacteriol* **186**, 570–574.
- Rivers, E. C. & Mancera, R. L. (2008).** New anti-tuberculosis drugs in clinical trials with novel mechanisms of action. *Drug Discov Today* **13**, 1090–1098.
- Sambandamurthy, V. K. & Jacobs, W. R., Jr (2005).** Live attenuated mutants of *Mycobacterium tuberculosis* as candidate vaccines against tuberculosis. *Microbes Infect* **7**, 955–961.
- Shaw, K. J., Berg, C. M. & Sobol, T. J. (1980).** *Salmonella typhimurium* mutants defective in acetohydroxy acid synthases I and II. *J Bacteriol* **141**, 1258–1263.
- Smith, D. A., Parish, T., Stoker, N. G. & Bancroft, G. J. (2001).** Characterization of auxotrophic mutants of *Mycobacterium tuberculosis* and their potential as vaccine candidates. *Infect Immun* **69**, 1142–1150.
- Sohn, H., Lee, K. S., Ko, Y. K., Ryu, J. W., Woo, J. C., Koo, D. W., Shin, S. J., Ahn, S. J., Shin, A. R. & other authors (2008).** *In vitro* and *ex vivo* activity of new derivatives of acetohydroxyacid synthase inhibitors against *Mycobacterium tuberculosis* and non-tuberculous mycobacteria. *Int J Antimicrob Agents* **31**, 567–571.
- Stover, C. K., de la Cruz, V. F., Fuerst, T. R., Burlein, J. E., Benson, L. A., Bennett, L. T., Bansal, G. P., Young, J. F., Lee, M. H. & other authors (1991).** New use of BCG for recombinant vaccines. *Nature* **351**, 456–460.
- Sun, R., Converse, P. J., Ko, C., Tyagi, S., Morrison, N. E. & Bishai, W. R. (2004).** *Mycobacterium tuberculosis* ECF sigma factor *sigC* is required for lethality in mice and for the conditional expression of a defined gene set. *Mol Microbiol* **52**, 25–38.
- Tamburini, E. & Mastromei, G. (2000).** Do bacterial cryptic genes really exist? *Res Microbiol* **151**, 179–182.
- Wards, B. J. & Collins, D. M. (1996).** Electroporation at elevated temperatures substantially improves transformation efficiency of slow-growing mycobacteria. *FEMS Microbiol Lett* **145**, 101–105.
- Zohar, Y., Einav, M., Chipman, D. M. & Barak, Z. (2003).** Acetohydroxyacid synthase from *Mycobacterium avium* and its inhibition by sulfonylureas and imidazolinones. *Biochim Biophys Acta* **1649**, 97–105.

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