Essentiality and functional analysis of type I and type III pantothenate kinases of *Mycobacterium tuberculosis*

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Pantothenate kinase, an essential enzyme in bacteria and eukaryotes, is involved in catalysing the first step of conversion of pantothenate to coenzyme A (CoA). Three isoforms (type I, II and III) of this enzyme have been reported from various organisms, which can be differentiated from each other on the basis of their biochemical and structural characteristics. Though most bacteria carry only one of the isoforms of pantothenate kinases, some of them possess two isoforms. The physiological relevance of the presence of two types of isozymes in a single organism is not clear. *Mycobacterium tuberculosis*, an intracellular pathogen, possesses two isoforms of pantothenate kinases (CoaA and CoaX) belonging to type I and III. In order to determine which pantothenate kinase is essential in mycobacteria, we performed gene inactivation of coaA and coaX of *M. tuberculosis* individually. It was found that coaA could only be inactivated in the presence of an extra copy of the gene, while coaX could be inactivated in the wild-type cells, proving that CoaA is the essential pantothenate kinase in *M. tuberculosis*. Additionally, the coaA gene of *M. tuberculosis* was able to complement a temperature-sensitive coaA mutant of *Escherichia coli* at a non-permissive temperature while coaX could not. The coaX deletion mutant showed no growth defects *in vitro*, in macrophages or in mice. Taken together, our data suggest that CoaX, which is essential in *Bacillus anthracis* and thus had been suggested to be a drug target in this organism, might not be a valid target in *M. tuberculosis*. We have established that the type I isoform, CoaA, is the essential pantothenate kinase in *M. tuberculosis* and thus can be explored as a drug target.

INTRODUCTION

*Mycobacterium tuberculosis*, the causative agent of tuberculosis, infects approximately one-third of the world’s population and causes morbidity and mortality in a large number of people, mostly in underdeveloped countries (Hoft, 2008). Though it is possible to control the disease with a combination of drugs, the long duration of treatment often leads to non-compliance resulting in relapse of active disease and emergence of drug-resistant *M. tuberculosis*. The presence of drug-resistant bacteria in the population poses a serious threat to mankind as a significant number of these bacteria are resistant to multiple drugs, leaving physicians with little choice for prescribing drugs (Chan & Iseman, 2008). Hence there is an urgent need to discover new drugs and vaccines for controlling tuberculosis. As a result of development of good genetic tools and the availability of the complete genome sequence of *M. tuberculosis* (Cole *et al.*, 1998), there have been rapid advances in understanding the molecular mechanisms of pathogenesis of *M. tuberculosis*. A number of new inhibitors of *M. tuberculosis* have also been reported which are undergoing pre-clinical or clinical evaluations, but are yet to be approved for regular use in patients (Rivers & Mancera, 2008). Efforts to design novel vaccines have met with limited success (Hoft, 2008). In order to sustain the efforts towards tuberculosis control there is a need to identify and validate novel *M. tuberculosis* proteins as drug or vaccine candidates, which will help in designing novel therapeutic approaches for controlling this important disease.

Since coenzyme A (CoA) is an essential acyl group carrier co-factor required for growth of various organisms, the enzymes of the CoA biosynthesis pathway have been thought to be good targets for designing inhibitors against bacteria, parasites and fungi (Gerdes *et al.*, 2002; Spry *et al.*, 2008). Pantothenate kinases catalyse the first step in the biosynthesis of CoA. In *Escherichia coli*, pantothenate...
kinase activity has been proposed to be the regulatory step in biosynthesis of CoA (Jackowski & Rock, 1981; Rock et al., 2003), thus making it an attractive target for designing inhibitors. There are three isoforms of pantothenate kinase known in various organisms. The majority of the bacteria possess type I enzyme (e.g. E. coli PanK or EcCoaA) while eukaryotes (e.g. murine enzyme, MmPanK) and a few bacteria (e.g. Staphylococcus aureus SaCoaA) carry a type II enzyme (type II PanK). Although type I and type II isoforms do not possess a high degree of amino acid homology, they both share common regulatory mechanisms and are inhibited by CoA and its thioesters. However, in this aspect, the SaCoaA is an exception, as its activity is not regulated by CoA (Leonardi et al., 2005). The type III isoform discovered recently (CoaX), which lacks any amino acid similarity to type I and type II isoforms has been identified in a number of bacteria including Bacillus subtilis, Helicobacter pylori, Pseudomonas aeruginosa and Bordetella pertussis. Enzymic characterization of CoaX from B. subtilis and H. pylori demonstrated that the type III isoform exhibits distinct biochemical properties such as lack of feedback regulation by CoA and an inability to accept pantothenic acid metabolites (pantothenamides) as substrates (Brand & Strauss, 2005). Though there are no known anti-bacterial which inhibit the CoA biosynthesis pathway, pantothenate analogues and amide derivatives, pantothenol, N-pentylpantothenamide and N-heptylpantothenamide possess antibiotic activity against E. coli, demonstrating the essential nature of this pathway (Snell & Shive, 1945; Choudhry et al., 2003; Zhang et al., 2004). A number of pantothenic acid analogues have been shown to possess anti-malarial activity (Saliba et al., 2005; Spry et al., 2005). In addition, specific inhibitors of another enzyme in the CoA biosynthesis pathway of E. coli, CoaD (PPAT, phosphopantotheine adenyltransferase) have also been reported (Zhao et al., 2003). Based on these observations, it is fair to speculate that inhibition of pantothenate kinase activity in M. tuberculosis will lead to inhibition of cell growth. The coaA gene has been found to be essential by gene inactivation studies in a number of bacteria including E. coli and Salmonella typhimurium (Dunn & Snell 1979; Vallari & Rock, 1987). In S. aureus, which possesses a type II enzyme, pantothenamides were shown to inhibit the growth of the bacteria, thereby demonstrating the essential nature of this enzyme (Choudhry et al., 2003). In B. subtilis, the coaA gene could be inactivated and it was discovered that another gene, designated coaX, could complement the loss of coaA (Yocum & Patterson, 2004). However, double knock-out of coaA and coaX was found to be lethal to the cell, demonstrating that at least one isoform of pantothenate kinase has to be functional. The ability of either of these pantothenate kinases to support the growth of B. subtilis points towards redundancy of this function in this species of bacteria. However, in B. anthracis, the coaX gene has been found to be essential even in the presence of a gene encoding a type II pantothenate kinase, indicating that their functions are not interchangeable in this organism. (Paige et al., 2008). A homologue of CoaX in Bordetella pertussis, BvgS has been shown to be essential for viability (Wood & Friedman, 2000). Thus, from genetic and biochemical studies on various bacteria, it seems that all three types of pantothenate kinases are independently capable of performing the essential function of phosphorylating pantothenate and any one of them could be essential for viability. The reason for the presence of single or multiple isoforms of pantothenate kinases in different bacteria is not clear. The situation in M. tuberculosis is similar to B. subtilis, wherein, in addition to coaA, the coaX gene is also present. In order to identify the essential pantothenate kinase in M. tuberculosis for the purpose of utilizing it as a drug target, we have attempted to inactivate coaA and coaX genes individually. Surprisingly, coaX could be inactivated in M. tuberculosis without any deleterious effects under in vitro or in vivo growth conditions, proving it to be unattractive for drug discovery. CoaA, on the other hand, was found to be essential and hence could be a valid drug target in M. tuberculosis.

METHODS

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

M. tuberculosis culture conditions. M. tuberculosis H37Rv (ATCC 27294) cultures were routinely grown in 7H9 or 7H11 medium (Difco) containing ADC enrichment. Hygromycin (50 μg ml⁻¹) or kanamycin (20 μg ml⁻¹) was added to the culture medium when required.

DNA amplification by PCR. The single crossover (SCO) and double crossover (DCO) recombinants in M. tuberculosis were screened by PCR using Taq DNA polymerase. Single isolated bacterial colonies resuspended in 50 μl TE (10 mM Tris, 1 mM EDTA) were boiled for 10 min. An aliquot of the cell lysate (5 μl) was added to 25 μl PCR mixture. Denaturation and extension reactions were performed at 94 and 72 °C, respectively, for 30 s each. The annealing temperature and extension time for each PCR were determined by considering the melting temperature of the primer pair and length of the PCR product, respectively.

RT-PCR. M. tuberculosis cultures were grown in 7H9 medium until the OD600 reached 0.2. The cell suspensions in Trizol (Invitrogen) were subjected to bead beating using 0.1 mm Silica/Zirconia beads (Mini bead beater Biospec Products) at 5000 r.p.m. with 30 s pulses. The samples were extracted with chloroform followed by 2-propanol precipitation at 4 °C overnight. The nucleic acid pellet obtained after centrifugation was resuspended in RNase-free water and treated with DNase I (Ambion) for 30 min at 37 °C. The total RNA was purified using an RNeasy column (Qiagen). Purified RNA (1 μg) was used to synthesize cDNA by a reverse transcriptase (RT) reaction using reverse primers, 0.5 mM dNTP mix and Superscript II (Invitrogen) at 37 °C for 60 min in a 30 μl reaction volume. An RT-minus reaction was set up as a negative control. An aliquot of 1.5 μl of the RT reaction mix was further used to set up the individual PCRs to amplify fragments of coaA and coaX as described above. The sequences of the primers used were as follows: coaA forward primer (TCaARTP) 5'-ACGCTCATGTTGTCGGATGCTT-3', reverse primer (TCaARTR) 5'-GACGGGTGCTTTA-TGGAATGATC-3'; coaX forward primer (472) 5'-AGTGCGGGAT-ACGGAGCGGAT-3', reverse primer (473) 5'-GGCATCGGAG-ACACGTGCAC-3'.
Plasmid constructs. The plasmids used in this study are listed in Table 1. The preparation of constructs used for gene knock-out (KO) of coaA (Rv1092c) and coaX (Rv3600c) was outsourced to Bangalore Geneti (India). The strategy followed for making these constructs was as follows. The recombination substrate for creating a deletion in the coaA gene of M. tuberculosis consisted of 803 bp of the region upstream of coaA, a 739 bp deletion (7–746) in coaA followed by 194 bp of the 3′-end of the gene and 884 bp of the downstream region cloned into a suicide vector, pGOAL19. A 1693 bp DNA fragment generated by overlapping PCR was cloned into the BamHI–HindIII sites of pGOAL19. The coaA gene (Rv3600c) KO construct [consisting of 609 bp of the upstream region, 237 bp of the 5′-end of the gene, 181 bp, 401 bp of the 5′-end of the gene (total gene length, 819 bp) and 433 bp of downstream region] was generated similarly and cloned into the Ncol–BglII sites of pAZI0290 to obtain pAZI0294. The DNA sequences of the cloned fragments were confirmed by sequencing. For generating constructs for complementation in M. tuberculosis, the coaA and coaX genes were amplified from the M. tuberculosis genomic DNA by PCR using high-fidelity Tag DNA polymerase (Phusion, Finnzyme). The primer sequences were as follows: coaA forward (328) 5′-ATAGGTTCCA-TGCGGCTTACGGGACCGC-3′, reverse (329) 5′-AAGTGGATCGTT-TAACAGGTTACGCGACCCG-3′; coaX forward (638) 5′-ATCGCG CTTACGGGATCGGCACGC-3′, reverse (639) 5′-CTAGGGTATACCGGTCGACCGC-3′. The amplified PCR products were cloned into BamHI–HindIII sites of pBAD24 or PvuII–HpalI (coaX) sites of pAZI0288. For expressing CoaX in E. coli, the coaX gene was amplified by PCR from M. tuberculosis genomic DNA and cloned into the Ndel–BamHI sites of pET15b to obtain pAZI9015. The DNA sequences of the cloned coaA and coaX genes were confirmed by sequencing (microsynth, Switzerland). For growth rescue of a temperature-sensitive (ts) mutant of E. coli at a non-permissive temperature, coaA and coaX genes were cloned into the arabinose-inducible expression vector pBAD24. For this, a 1.2 kb DNA fragment encompassing the coaA gene from a T7 expression construct of His:coaA in pET15b (pAZI9004) was cloned into the Ncol–HindIII sites of pBAD24 to obtain pAZI0372. Similarly, the coaX gene from a T7 expression construct pAZI9015 was cloned into Ncol–HindIII sites of pBAD24 to generate pAZI0371.

Gene KO and excision in M. tuberculosis. The recombinant plasmids were electroporated into M. tuberculosis by the procedure described by Wards & Collins (1996). The gene KO was performed by using the two-step method as described by Parish & Stoker (2000). For excisionase activity, pAZI0288 was transformed into the wild-type M. tuberculosis and the coaA mutant strains and plated in the presence of hygromycin. The transformation frequencies were compared, and the excision of the integrated plasmid was monitored by PCR.

Southern blotting. Genomic DNA from the wild-type or mutant M. tuberculosis strains was digested with KpnI (for coaA) or SphI (for coaX) and separated by electrophoresis on 0.8% agarose gels. The probe was generated by amplification of DNA fragments encompassing coaA and coaX genes by PCR, as shown in Figs 1 and 2. The primer sequences were as follows: coaA forward (471) 5′-AGGCGCATGCGCAACGGCTGG-3′, reverse (578) 5′-GTCGCGGGGATGTGGATCC-3′; and coaA forward (422A) 5′-TCAGTTGCACACCGGACCCG-3′, reverse (423) 5′-ATCGTGGCGGATGTAAGTC-3′. The probes were labelled with fluorescein by random prime labelling (Roche). DNA transfer, hybridization and washing were performed by using the protocol provided by the manufacturer (GE Healthcare). The bound DNA probes were detected by horseradish peroxidase (HRP)-conjugated anti-fluorescein antibodies (1:2000 dilution) using chemiluminescence.

Complementation in E. coli DV62. pAZI0371, pAZI0372 and pBAD24 (vector) were transformed into E. coli DV62, plated on LB plates containing ampicillin (50 μg ml⁻¹) and 0.5 mM arabinose (ara) and incubated at 30 or 42 °C for 24 h. Alternatively, colonies from plates incubated at 30 °C were picked up and grown in broth until the OD₆₀₀ reached 0.2, and dilutions were plated on LB plates containing amoxicillin and ara at 30 and 42 °C for 24 h.

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics</th>
<th>Source/Ref</th>
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<tbody>
<tr>
<td>E. coli DH5α</td>
<td>endA1 hsdR17 supE44 recA1 lacZΔM15 argF</td>
<td>Lab, stock</td>
</tr>
<tr>
<td>E. coli DV62</td>
<td>panD2 gyrA21 spoT1 metB zif-2009::Tn10 coaA15(ts)</td>
<td>CGSC</td>
</tr>
<tr>
<td>M. tuberculosis H37Rv (ATCC 27294)</td>
<td>Virulent strain of M. tuberculosis</td>
<td>Lab stock</td>
</tr>
<tr>
<td>pAZI272</td>
<td>Integrating mycobacterial expression vector</td>
<td>Awasthy et al. (2009)</td>
</tr>
<tr>
<td>pAZI0221</td>
<td>1.9 kb fragment carrying ΔcoaA with upstream and downstream sequences cloned into BamHI–HindIII sites of pUC18</td>
<td>This study</td>
</tr>
<tr>
<td>pAZI0222</td>
<td>hyg' cassette cloned into NruI site of pAZI0221</td>
<td>This study</td>
</tr>
<tr>
<td>pAZI0228</td>
<td>BamHI–HindIII fragment from pAZI0221 cloned into similar sites of pGOAL19</td>
<td>This study</td>
</tr>
<tr>
<td>pAZI0290</td>
<td>pGOAL19 with a deletion of lacZ gene</td>
<td>This study</td>
</tr>
<tr>
<td>pAZI0294</td>
<td>ΔcoaX with upstream and downstream sequences cloned into BglII–Ncol sites of pAZI0290</td>
<td>This study</td>
</tr>
<tr>
<td>pAZI0234</td>
<td>coaA of M. tuberculosis cloned into BamHI–HindIII sites of pAZI0272</td>
<td>This study</td>
</tr>
<tr>
<td>pAZI0297</td>
<td>coaX of M. tuberculosis cloned into PvuII–HpalI sites of pAZI0272</td>
<td>This study</td>
</tr>
<tr>
<td>pAZI9004</td>
<td>coaA of M. tuberculosis cloned into Ndel–BamHI sites of pET15b</td>
<td>This study</td>
</tr>
<tr>
<td>pAZI9015</td>
<td>coaX of M. tuberculosis cloned into Ndel–BamHI sites of pET15b</td>
<td>This study</td>
</tr>
<tr>
<td>pBAD24</td>
<td>Arabinose inducible E. coli expression vector</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pAZI0371</td>
<td>HisCoaX coding DNA fragment cloned into Ncol–HindIII sites of pBAD24</td>
<td>This study</td>
</tr>
<tr>
<td>pAZI0372</td>
<td>HisCoaX coding DNA fragment cloned into Ncol–HindIII sites of pBAD24</td>
<td>This study</td>
</tr>
<tr>
<td>pL28</td>
<td>Mycobacterial plasmid carrying a gene coding for L5 phage excisionase</td>
<td>Parish et al. (2001)</td>
</tr>
<tr>
<td>pAZI0288</td>
<td>Gm' marker of pL28 replaced by hyg' gene</td>
<td>This study</td>
</tr>
</tbody>
</table>

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Western blotting. The cytosolic fractions of cell lysates were separated by 12% SDS-PAGE and transferred onto a nitrocellulose membrane. BSA (3%) in PBS containing 0.1% Tween-20 (PBST) was used for blocking and the blot was incubated in the presence of rabbit anti-CoaX antibodies diluted 1:200,000 for 1 h. After washing with PBST, HRP-conjugated anti-rabbit antibodies (diluted 1:20,000) were added to the blot and the incubation was continued for 1 h. The blot was washed with PBST and the induced proteins were detected by chemiluminescence using ECL Western blotting kit (GE Healthcare) by using the method provided by the manufacturer.

Purification of *M. tuberculosis* CoaA and CoaX. *E. coli* BL21 (DE3) transformed with pAZI9015 was grown in LB to OD<sub>600</sub> 0.5, and was induced with 1 mM IPTG for 3 h. The cell pellet obtained from 2 l IPTG-induced cultures was resuspended in 50 ml buffer A (50 mM Tris/HCl, pH 7.5, 300 mM NaCl, 1 mM DTT, 10% glycerol, 1 mM PMSF and 100 μg lysozyme ml<sup>-1</sup>). The resuspended cells were kept on ice for 30 min and were lysed by sonication (Branson sonifier-450) at 4°C. The cell lysate was centrifuged at 100,000 g for 1 h. The supernatant was recovered and loaded on a Ni-NTA column, pre-equilibrated in buffer A. The flow-through was collected and the column was washed with 50–100 ml wash buffer (buffer A containing 10 mM imidazole) and dialysed against gel filtration buffer (50 mM Tris/HCl, pH 7.5, 500 mM NaCl, 2 mM DTT, 10% glycerol and 1 mM EDTA) overnight at 4°C. The dialysed protein sample (30 ml) was concentrated to 2 ml and loaded on a pre-equilibrated gel filtration column (Superdex pg 200) at a flow rate of 1.0 ml min<sup>-1</sup>. Fractions of 5 ml were collected, and fractions E14–18 were pooled and concentrated to 1 ml using a Millipore concentrator (MWCO-10 kDa). The protein sample was stored at −70°C in 0.25 ml aliquots. For CoaA expression, *E. coli* BL21 star (DE3) transformed with pAZI9004 was grown in LB medium at 25°C overnight in the absence of the inducer, as the optimum amount of protein was expressed under these conditions. CoaA was purified by using the procedure described above, except that 100 mM NaCl was used in the gel filtration buffer.

Biochemical assay. Pantothenate kinase activity was measured by detecting adenosine diphosphate formation using pyruvate kinase and lactate dehydrogenase as described previously (Yang et al., 2008). The activity was assayed in a 100 μl reaction mixture containing 50 mM PIPES NaOH, pH 7.0, 25 mM KCl, 20 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 2 mM DTT, 0.002% Brij-35, 0.5 mM PEP, 0.24 mM NADH, 10 units PK/LDH ml<sup>−1</sup> (Sigma), 0.12 mM ATP and 0.3 mM L-pantothenate. The reaction was started by the addition of CoaA (20–100 nM) or CoaX (100 nM–3.2 μM) to the reaction mix. The reaction was monitored by observing the change in A<sub>340</sub> for 60 min at 25°C in a Spectramax spectrophotometer. The velocity of the reaction was calculated from the linear range of the reaction by estimating the amount of NADH converted per second. For this purpose, the molar absorption coefficient of NADH was taken as 6220 M<sup>−1</sup> cm<sup>−1</sup>, and the path length considered for a 100 μl volume was 0.275 cm. The catalytic constant (k<sub>cat</sub>) was determined by using the formula k<sub>cat</sub> = V<sub>max</sub>/E<sub>i</sub>, where V<sub>max</sub> is the maximum enzyme velocity, and E<sub>i</sub> is the total enzyme concentration.

Growth of *M. tuberculosis* H37Rv and coaX KO mutant in macrophages. Intracellular infection and survival kinetics of mycobacterial strains were investigated in gamma-interferon (IFN-γ)-activated bone-marrow-derived macrophages (BMDM) in a 24-well plate format as described by Munoz-Elías & 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 recombinant IFN-γ ml<sup>−1</sup> (Sigma) for 48 h. Activated and washed BMDM monolayers were used for infection. All macrophage infection studies were performed in RPMI 1640 medium (Cell Culture Technologies). Monolayers were infected with *M. tuberculosis* H37Rv and coaX KO mutant strains individually at an m.o.i. of 1:10. Two hours post-infection, the monolayer was washed with PBS and replaced with RPMI 1640 medium. A set of BMDM cells infected with wild-type and coaX KO mutant were lysed on day 0, 3, 7 and 10. The lysates were plated onto 7H11 agar plates.

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 to cages at five mice per cage, with the restriction that all cage members were within a 1–2 g weight of each other. They were allowed to acclimatize for 2 weeks before intake into experiments. For intravenous infection, frozen bacterial samples were thawed, centrifuged and washed in PBS,
and bacteria were diluted appropriately in cold, sterile PBS. The mice were infected with the wild-type, the KO mutant or the complemented M. tuberculosis strain by intravenous inoculation into the tail vein to obtain 10⁵ c.f.u. per animal. The animals were sacrificed at intervals of 1 week post-infection, and the bacterial counts present in the organs were determined by plating the lung and spleen homogenates on 7H11 agar.

RESULTS

Mycobacteria carry both type I and type III pantothenate kinases

Type III is the most common type of pantothenate kinase found in bacteria followed by type I and II (Yang et al., 2006). Several bacteria harbour combinations of type I and III or type II and III enzymes. Mycobacteria, including M. tuberculosis, M. leprae, M. bovis, M. avium and M. smegmatis, show the presence of genes encoding type I and type III pantothenate kinase in their genomes. Genomic organization of coaA in M. tuberculosis revealed that it is present as a single gene, whereas coaX forms an operon with genes involved in pantothenate biosynthesis (Figs 1a and 2a). The presence of coaX in the pantothenate biosynthesis operon suggests that it plays a role in this pathway.

CoaA (Rv1092c) is essential for the survival of M. tuberculosis

Based upon the existing knowledge of in vitro gene essentiality of pantothenate kinases in various bacteria.
(Dunn & Snell 1979; Vallari & Rock, 1987; Wood & Friedman, 2000; Liberati et al., 2006; Paige et al., 2008), it can be speculated that either of the two kinase coding genes (coaA or coaX) could potentially be capable of providing the essential enzymic function for the survival of M. tuberculosis. In order to prove whether either of them could keep the cells alive in the absence of other isoforms (as in B. subtilis) or only one is essential even in the presence of the other isoform (as in B. anthracis), we attempted to inactivate the coaA and coaX genes of M. tuberculosis individually. This was achieved by homologous recombination using a deleted copy of either of the genes using the procedure described by Parish & Stoker (2000). The ability to obtain a gene KO only in the presence of an additional copy of the gene in the merodiploid strain and not in the wild-type M. tuberculosis H37Rv was taken as evidence of the essentiality of the gene. The recombination substrate for coaA gene KO on pAZI0228 consisted of a mutant coaA gene with a 739 bp markerless deletion (7–746) with 810 bp upstream and 1078 bp downstream flanking the deleted region. Additionally, a construct with a hyg' insertion in the deleted region of coaA (pAZI0222) was also tried for allele exchange. As described in Methods, the construct was designed in such a way that it would not express any intact CoaA protein. The single cross-over (SCO) recombinants obtained by transformation of M. tuberculosis with pAZI0228 were confirmed by PCR using primers indicated in Fig. 1(b). One of the recombinants showing integration in the proper locus (Mtb/coaA/SCO3) was further taken up for replacement of the wild-type coaA gene by the deleted copy of the gene by a double cross-over (DCO) recombination event. Mtb/coaA/SCO3 was grown in 7H9 broth in the absence of hygromycin for 3 weeks and subsequently plated on 7H9 plates containing 2% sucrose. The loss of plasmid integrated in the chromosome was monitored by amplification of hyg' and the sacB genes by PCR. Of 200 colonies screened (150 from hyg' insertion and 50 from deletion construct), 140 had lost the plasmid, indicating that 70% of the colonies had undergone recombination by a DCO event. The putative DCO recombinants (showing absence of sacB and hyg' genes) were further screened for the presence of the mutated copy of the gene in the chromosome by PCR using primers shown in Fig. 1(b). None of the colonies showed the presence of a mutant copy of coaA in the genome. Despite a high frequency of DCO recombination events, the inability to generate a KO mutant indicated that the coaA gene is likely to be essential for the survival of M. tuberculosis. In order to confirm this experimentally, the coaA gene KO was attempted in a merodiploid background. For this purpose, pAZI0234, which carries the M. tuberculosis coaA gene under the control of hsp60 promoter in the mycobacterial integrating vector, pAZI0272, was electroporated into Mtb/coaA/SCO3 strain as described above and the transformed cells were plated on 7H9 agar containing hygromycin and kanamycin. The integration of pAZI0234 into the chromosome of the Mtb/coaA/SCO3 was confirmed by amplification of the kan' gene (data not shown) and the strain was designated Mtb/coaA/SCO3/mcro. As described above for Mtb/coaA/SCO3, Mtb/coaA/SCO3/mcro culture was grown up and plated on 7H9 plates for DCO recombinants, and the loss of the integrated plasmid was monitored by PCR. The frequency of DCO recombination in Mtb/coaA/SCO3 and Mtb/coaA/SCO3/mcro was in a similar range. The colonies that had lost the plasmid were further screened for the presence of a deleted copy of coaA by PCR by using gene-specific primers (Fig. 1c). In contrast with Mtb/SCO3, DCO recombination in Mtb/SCO3/mcro resulted in replacement of the wild-type coaA gene by a deleted copy in 20% of the colonies (18 of 88). The presence of a deleted copy of the coaA gene in the chromosome of one such DCO recombinant (Mtb/coaA/ KO) was finally confirmed by Southern blotting using a 340 bp PCR product as a probe. As shown in Fig. 1(d), mto/coaA/KO showed the presence of a smaller DNA fragment corresponding to the deleted coaA in addition to the full-length wild-type coaA gene present in the att site.

In order to gain more confidence into the essentiality of CoaA in M. tuberculosis, the coaA genes present at the att site in H37Rv and Mtb/coaA/KO were subjected to excision as described in Methods. The H37Rv cultures could be transformed with excisionase expressing pAZI0288 at high frequency (5 × 10^8 c.f.u. per electroporation), whereas only six colonies were obtained in the case of Mtb/coaA/KO. These six colonies were found to have retained the integrated plasmid at the att site (data not shown). Both H37Rv and Mtb/coaA/KO strains could be transformed with a control plasmid with equal frequency. This clearly demonstrated that at least one copy of the intact gene has to be present inside the cell for the colonies to grow in vitro. This clearly proved that coaA is essential for survival of M. tuberculosis, and the coaX gene present in this organism is unable to complement the loss of coaA, suggesting non-redundancy of their functions under the conditions tested.

coaX (Rv3600c) gene is not essential for the survival of M. tuberculosis

CoaX of M. tuberculosis has been predicted to be a pantothenate kinase based upon its high degree of amino acid homology to the biochemically characterized type III isomerase of pantothenate kinase of H. pylori (Brand & Strauss, 2005). The coaX gene has been shown to be essential for survival of B. anthracis, P. aeruginosa and Bordetella pertussis (Wood & Friedman, 2000; Liberati et al., 2006; Paige et al., 2008). In order to assess whether CoaX is essential for the survival of M. tuberculosis, we decided to inactivate the coaX gene by homologous recombination using the strategy applied for coaA KO described above. The suicide plasmid pAZI0294 carried a mutant copy of coaX, which could only code for 79 N-terminal amino acids (out of 272) as a result of a deletion of 181 bp after bp 237, which renders the downstream sequence out of frame. The SCO recombinants obtained
by transformation of pAZI0294 into *M. tuberculosis* were confirmed by PCR (Fig. 2c) and one of them (Mtb/coaX/Sco27) was grown in 7H9 and plated on 2% sucrose to obtain DCOs. Of the 130 sucrose-resistant resistant colonies selected for analysis, more than 90% were found to have lost the plasmid, as determined by the absence of *sacB* and *hyg* genes in PCR analysis of DCO colonies (data not shown). Analysis of these colonies by gene-specific PCRs using primers 472 and 473 showed that 4 of 130 had only the mutant copy of the gene while the remainder had retained the wild-type copy of the gene. Further confirmation of the absence of wild-type *coaX* gene copy in one of the mutants (Mtb/coaX/KO) was done by PCR using primers shown in Fig. 2(c). The absence of wild-type *coaX* gene in Mtb/coaX/KO was also confirmed by Southern blot analysis using a PCR fragment corresponding to bp 85–486 of the *coaX* gene as a probe. As shown in Fig. 2(d), the size of the *SphI–XhoI* fragment was found to be smaller than the fragment in the wild-type *M. tuberculosis* H37Rv. Thus it was clearly demonstrated that in contrast with *coaA*, the *coaX* gene could be deleted in the wild-type *M. tuberculosis*, proving that it is non-essential for survival. Mtb/coaX/KO was further studied for its phenotype under *in vitro*, *ex vivo* and *in vivo* growth conditions.

Is it possible that *coaX* happens to be non-essential in *M. tuberculosis* because it is not expressed? In order to answer this question, RT-PCR analysis of mRNA isolated from actively growing *M. tuberculosis* cells was performed. Amplification of both *coaA* and *coaX* cDNAs gave rise to DNA fragments of the expected sizes (292 bp and 401 bp, respectively; Fig. 2e). No DNA fragments were generated in RT-minus or template minus samples (Fig. 2e, lanes 3, 4, 8 and 9) confirming the amplification of specific transcripts. This shows that both *coaA* and the *coaX* genes are transcribed in *M. tuberculosis*, and the non-essential nature of *coaX* cannot be ascribed to the lack of expression.

**CoaX is not required for survival in macrophages and mice**

Mtb/coaX/KO did not show any significant growth defect in 7H9 medium (data not shown). Next, we asked whether CoaX could be critical for survival under *ex vivo* and/or *in vivo* conditions. In order to answer this question, we first compared the growth and survival of the wild-type and the *coaX* mutant in BMDMs. The infected macrophages were lysed and plated to estimate the bacterial load on days 0, 3, 7 and 10. There was no difference in the growth characteristics of wild-type and Mtb/coaX/KO (Fig. 3a), as in both cases the bacterial number remained unchanged between days 0 and 10. In order to understand the contribution of CoaX towards survival *in vivo*, growth kinetics of the Mtb/coaX/KO mutant were compared with the wild-type *M. tuberculosis*. The c.f.u. values obtained from lung and spleen homogenates of mice infected with wild-type, mutant or the complemented strains were similar (Fig. 3b and c). Statistical analysis (Student’s *t*-test) of the c.f.u. values obtained from lungs of wild-type and the mutant on day 56 showed that these values were significantly different (*P*≤0.007), though the difference in the c.f.u. values of similar samples from spleens was not significant (*P > 0.5*). The course of infection of the complemented strain in the lungs and the spleens was similar to that of the wild-type strain, though the starting conditions.
The c.f.u. of the complemented strain was slightly less than the wild-type. Small variations in initial bacterial loads were seen earlier and are not expected to influence the course of infection (Sun et al., 2004). Overall, the absence of coaX does not lead to any kind of change in virulence in M. tuberculosis. Thus, it was quite puzzling to see that CoaX, which is non-essential for survival in vitro, does not contribute towards survival of M. tuberculosis under ex vivo or in vivo conditions.

CoA of M. tuberculosis complements a CoaA ts mutant of E. coli while CoaX does not

In B. subtilis, both CoaX and CoaA were shown to be functional by their ability to keep the cells alive in the absence of the other isoforms, indicating that both type I and type III pantothenate kinases are active in B. subtilis. Additionally, both coaA and coaX could rescue the growth of a CoaA ts mutant of E. coli at non-permissive temperature (Yocum & Patterson, 2004). Since we found that CoaX was non-essential for survival in vitro or in vivo and could not complement the loss of CoaA in M. tuberculosis, we wanted to see whether this protein could function as a pantothenate kinase in E. coli. For this purpose we tested the ability of M. tuberculosis CoaA and CoaX to complement a ts mutation in CoaA of E. coli at non-permissive temperature. The individual plasmids coding for M. tuberculosis CoaX and CoaA under the control of an arabinose-inducible promoter (pAZI0371 and pAZI0372) were transformed into E. coli DV62 at 30 °C and plated at 30 and 42 °C in the presence of arabinose. It was found that E. coli DV62 transformed with any of the plasmids was able to grow at 30 °C whereas only M. tuberculosis CoaX was able to support the growth of E. coli DV62 at 42 °C (Fig. 4a). The ability of M. tuberculosis CoaA to complement a ts CoaA of E. coli is consistent with the earlier findings of Kumar et al. (2007). In order to rule out the possibility that the lack of protein expression or lack of soluble protein is responsible for the inability of the coaX gene to complement the ts phenotype in E. coli, we analysed the protein expression in E. coli DV62 pAZI0371 by Western blotting using anti-CoaX antibodies. As shown in Fig. 4(b), sufficient amounts of CoaX protein were present in the cytosolic fractions of cell lysates. This set of experiments proved that CoaX of M. tuberculosis does not function as a pantothenate kinase in E. coli.

CoaX of M. tuberculosis lacks pantothenate kinase activity in vitro

In order to determine why CoaX is unable to function in M. tuberculosis or in E. coli, we analysed the recombinant CoaX for pantothenate kinase activity. CoaX purified from E. coli was assayed for enzymic activity by the PK-LDH coupled assay described in Methods. No enzymic activity could be observed under standard assay conditions using 300 nM of the enzyme. On the other hand, CoaA showed the expected enzymic activity under these assay conditions (Fig. 5b). Analysis of CoaA kinetic parameters revealed that its catalytic constant (kcat) value (3.17 s⁻¹) was in a similar range to those reported for type III pantothenate kinases of H. pylori and B. subtilis and the type II kinase of S. aureus (Brand & Strauss, 2005). Since it had been observed previously that type III pantothenate kinase had higher Km values for ATP (Brand & Strauss, 2005), the assays were repeated with increasing amounts of ATP (up to 12.5 mM) and pantothenate (0–300 μM). However, no activity was observed under these conditions either. Increasing the concentration of CoaX (from 100 nM to 3.2 μM), MgCl₂ (62.5 mM), NADH (0.75 mM) or inclusion of potassium glutamate and ammonium chloride did not make any difference (data not shown). This suggested...
that CoaX of *M. tuberculosis* lacked any observable pantothenate kinase activity *in vitro*, and this is also consistent with the observation that CoaX of *M. tuberculosis* is unable to function as a pantothenate kinase in *E. coli*. Interestingly, in an earlier study, the type II pantothenate kinase of *B. anthracis* was found to have no pantothenate kinase activity and was unable to rescue the growth of the ts CoaA mutant of *E. coli* at a non-permissive temperature (Nicely et al., 2007).

**DISCUSSION**

The availability of the genome sequence of *M. tuberculosis* has thrown up enormous possibilities of finding novel drug targets. Amongst these targets, some would be essential for survival under most growth conditions (e.g. RNA polymerase), while others could only be required under certain physiological conditions (e.g. virulence factors). Inhibition of targets, which are required only for growth *in vitro*, is not expected to lead to growth inhibition *in vivo* and thus the inhibitors will have to be tested *in vivo* only. Also, the inhibition of targets that are not essential *in vivo* is not expected to result in elimination of the organism from the host. Therefore, the knowledge of *in vitro* and *in vivo* essentiality of the targets is crucial in designing compound progression strategies in drug discovery.

In organisms that possess a single isoform of pantothenate kinase, the gene encoding the lone form of pantothenate kinase is expected to be essential. This has been proven in a number of bacteria such as *E. coli*, *P. aeruginosa* and *Bordetella pertussis* (Dunn & Snell 1979; Vallari & Rock, 1987; Wood & Friedman, 2000). Since all three forms of pantothenate kinase have been shown to be functionally active, it can be speculated that in organisms possessing multiple forms of pantothenate kinases, any one of the three isoforms should be sufficient for *in vitro* survival of the bacteria. This has been found to be true in *B. subtilis*, wherein it has been demonstrated that the presence of either CoaA or CoaX permits the cell to be viable (Yocum & Patterson, 2004). However, in a related bacterium, *B. anthracis*, which possesses type II and III isoforms of pantothenate kinase (Yang et al., 2006), the coaX gene was found to be essential even in the presence of type II isoform of pantothenate kinase (Paige et al., 2008), which led the authors to propose CoaX as a drug target for developing inhibitors (Paige et al., 2008). Whether the type II pantothenate kinase of *B. anthracis* is also essential or not is not clear at this moment.

Though *in vitro* studies with recombinant *M. tuberculosis* CoA have indicated that pantothenol can act as a substrate for this enzyme, thereby blocking the subsequent steps of CoA biosynthesis (Kumar et al., 2007), the *in vivo* effect of these analogues has not been studied. Our gene inactivation studies demonstrate that under *in vitro* growth conditions, CoA is the functional pantothenate kinase in *M. tuberculosis*, and CoaX is not able to complement the loss of CoaA. This finding is in contrast with the situation that exists in *B. subtilis*. Interestingly, *M. leprae*, which has lost most of the non-essential genes from its genome (Cole et al., 2001), has retained both coaA and coaX, suggesting that both the isoforms might play important roles under varied physiological conditions. Since CoaX of *M. tuberculosis* is not essential *in vitro*, we had hypothesized that it might be required for survival in macrophages or *in vivo* in animals. However, the absence of any growth defect in macrophages or in mice rules out the possibility of CoaX playing a major role in survival in this animal model. The exact reason for this result is not clear at this moment. However, it is possible that these enzymes might have evolved to confer a survival advantage in human hosts and hence might not be crucial for survival in other species. Testing of the phenotype of the coaX mutant in other animal species might help to answer this question. Differences in phenotypes of *M. tuberculosis* mutants in various animal species have been reported previously (Converse et al., 2009).

The inability of CoaX to complement a CoaA ts mutant of *E. coli* and the lack of any demonstrable biochemical activity *in vitro* raises the question of whether CoaX possesses pantothenate kinase activity or not. It might also be possible that it requires an environment for activity that is not present *in vitro* or in heterologous systems. Comparison of the amino acid sequence of *M. tuberculosis* CoaX with CoaX of *H. pylori* and *B. anthracis*, in which pantothenate kinase activity has been demonstrated (Yang et al., 2006), show that the substrate-binding residues and other characteristic motifs are conserved amongst the enzymes from these species. Thus, lack of functional and biochemical activity in CoaX of *M. tuberculosis* cannot be explained on the basis of the amino acid sequence of the protein. Also, the existence of an entirely different enzymic activity in CoaX of *M. tuberculosis* cannot be ruled out. The presence of a seemingly inactive pantothenate kinase in two intracellular pathogens, *B. anthracis* (Nicely et al., 2007) and *M. tuberculosis*, both carrying two isoforms of this enzyme, is intriguing and lacks any valid explanation at this moment. Further biochemical and structural studies will be required to find an answer to this complex question.

The presence of multiple isoforms of enzymes is quite common in *M. tuberculosis*, which is perhaps reflected in the bigger genome size of this organism compared with other pathogenic bacteria (Cole et al., 1998). Glutamine synthases, DNA ligases, ketoacyl synthases, thymidylate synthase and methionine aminopeptidases are some of the examples of enzymes that exist as multiple isoforms in *M. tuberculosis*. The redundancy of putative drug targets in *M. tuberculosis* calls for stringent target validation efforts before commencement of target-based drug discovery programs to identify potential inhibitors. In summary, we have established that CoaA is a valid drug target in *M. tuberculosis*, whereas inhibition of CoaX will not result in growth inhibition *in vitro* or *in vivo*. This, along with the
recently solved crystal structure of *M. tuberculosis* CoaA protein (Das et al., 2006), will be of great value in identification and characterization of CoaA inhibitors.

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