Alanine racemase mutants of *Mycobacterium tuberculosis* require D-alanine for growth and are defective for survival in macrophages and mice

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Alanine racemase (Alr) is an essential enzyme in most bacteria; however, some species (e.g. *Listeria monocytogenes*) can utilize D-amino acid transaminase (Dat) to generate D-alanine, which renders Alr non-essential. In addition to the conflicting reports on gene knockout of *alr* in *Mycobacterium smegmatis*, a recent study concluded that depletion of Alr does not affect the growth of *M. smegmatis*. In order to get an unambiguous answer on the essentiality of Alr in *Mycobacterium tuberculosis* and validate it as a drug target *in vitro* and *in vivo*, we have inactivated the *alr* gene of *M. tuberculosis* and found that it was not possible to generate an *alr* knockout in the absence of a complementing gene copy or D-alanine in the growth medium. The growth kinetics of the *alr* mutant revealed that *M. tuberculosis* requires very low amounts of D-alanine (5–10 µg ml⁻¹) for optimum growth. Survival kinetics of the mutant in the absence of D-alanine indicated that depletion of this amino acid results in rapid loss of viability. The *alr* mutant was found to be defective for growth in macrophages. Analysis of phenotype in mice suggested that non-availability of D-alanine in mice leads to clearance of bacteria followed by stabilization of bacterial number in lungs and spleen. Additionally, reversal of D-cycloserine inhibition in the presence of D-alanine in *M. tuberculosis* suggested that Alr is the primary target of D-cycloserine. Thus, Alr of *M. tuberculosis* is a valid drug target and inhibition of Alr alone should result in loss of viability *in vitro* and *in vivo*.

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INTRODUCTION

The presence of drug-resistant bacteria poses a serious challenge to treatment of tuberculosis (TB) as a significant number of bacteria are becoming resistant to currently used drugs. Multidrug resistant and extensively drug resistant strains of *Mycobacterium tuberculosis*, which are resistant to first- and second-line TB drugs, are evolving all over the world (Chan & Iseman, 2008). As TB is the major disease prevalent in immunocompromised HIV patients (Wells *et al.*, 2007), the AIDS epidemic has thrown up additional challenges to treat this ancient scourge of mankind. Though a number of promising *M. tuberculosis* inhibitors have been developed (Ginsberg, 2010), none has been advanced enough to be used clinically. Similarly, efforts to design novel vaccines are under way and a number of candidate vaccines are being tested in clinical trials (Kaufmann *et al.*, 2010). Development of good genetic tools and the availability of genome sequences of *M. tuberculosis* (Cole *et al.*, 1998) are expected to help in

the validation of new *M. tuberculosis* proteins as drug or vaccine candidates.

D-Alanine (D-ala) is a critical component of peptidoglycan and alanine racemase (Alr), the enzyme responsible for racemization of L-ala into D-ala (Fig. 1), is an essential enzyme in most bacteria (Hols *et al.*, 1997; Steen *et al.*, 2005). However, a few bacteria (e.g. *Listeria monocytogenes*) have the capacity to survive in the absence of Alr by virtue of their ability to convert L-ala into D-ala using D-amino acid aminotransferase (Dat). Thus, *L. monocytogenes* mutants lacking Alr are healthy, but double mutants (*alr*, *dat*) are dependent on D-ala for growth (Thompson *et al.*, 1998). Amongst Gram-negative bacteria, Alr has been well characterized in *Escherichia coli* and *Salmonella typhimurium*. Both these organisms carry two isoforms of alanine racemase (Alr and DadX) and it has been demonstrated that inactivation of individual genes does not lead to D-ala dependency. However, inactivation of both the genes together leads to D-ala auxotrophy (Wasserman *et al.*, 1983). Amongst the Gram-positives, Alr has been shown to be essential for the survival of *Bacillus* and *Lactobacillus* species (Hols *et al.*, 1997; Steen *et al.*, 2005). D-Cycloserine (DCS), which is a second-line anti-TB drug, is known to

Abbreviations: BMDM, bone marrow-derived macrophages; DCO, double cross-over; DCS, D-cycloserine; KO, knockout; SCO, single cross-over; TB, tuberculosis.

Fig. 1. Peptidoglycan biosynthesis in bacteria. The functional roles of alanine racemase (Alr) and D-alanine-D-alanine ligase (Ddl) are highlighted in a box. UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-MurNAc, UDP-*N*-acetylmuramic acid; meso-DAP, meso-diaminopimelic acid; PBPs, penicillin-binding proteins; TG, transglycosylase; TP, transpeptidase.

interfere with peptidoglycan synthesis by inhibiting Alr and D-alanine-D-alanine ligase, Ddl (Strych *et al.*, 2001; Bruning *et al.*, 2011). From the published literature, it is not clear whether bacterial killing results from inhibition of both the targets or whether inhibition of either of these can also cause growth inhibition or cell death. The fact that the overexpression of either Alr or Ddl in *Mycobacterium smegmatis* leads to an increase in the MIC of DCS suggests that this drug kills mycobacteria by inhibiting both Alr and Ddl (Cáceres *et al.*, 1997; Feng & Barletta, 2003). The ability of D-alanine-D-alanine to complement a DCS-supersensitive temperature-sensitive Ddl mutant of *Staphylococcus aureus* (Kurokawa *et al.*, 2009) suggested that either Alr or Ddl or both the proteins are targeted by DCS. In a nuclear magnetic resonance-based metabolomics study it was shown that DCS inhibits multiple targets and was suggested that Ddl could be the primary target of DCS in mycobacteria (Halouska *et al.*, 2007). As DCS-mediated inhibition of bacterial growth is reversed in the presence of D-alanine in the medium (Zygmunt, 1963), DCS might kill bacteria by inhibiting Alr alone. Recently reported inhibitors of Alr inhibited the growth of *M. tuberculosis* (Anthony *et al.*, 2011). However, non-reversibility of inhibition in the presence of D-alanine showed that the cellular killing could result from off-target effects.

The genetic approach of proving essentiality of Alr has yielded ambiguous results in *M. smegmatis*. An initial study concluded that Alr activity is not required for the survival of *M. smegmatis* and there could be an alternative pathway for the synthesis of D-alanine in *M. smegmatis* (Chacon *et al.*, 2002). However, in a later study by Milligan *et al.* (2007), it was shown that deletion of the *alr* gene results in D-alanine auxotrophy, thus ruling out the possibility of the existence of an alternative pathway in *M. smegmatis*. Furthermore, in a very recent report (Wei *et al.*, 2011), it was shown that depletion of intracellular Alr by controlled proteolysis did not affect the growth of *M. smegmatis*, which suggested that Alr is not a critical enzyme in this organism. In order to gain a better understanding of the role of Alr in the physiology of *M. tuberculosis* and validate Alr as a drug target, we have inactivated the *alr* gene and studied the mutant phenotype *in vitro*, in macrophages and in mice.

METHODS

Plasmids and strains. The plasmids and the strains used in this study are listed in Table 1.

Statistical analysis. This was performed using GraphPad Prism software.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or relevant feature	Source
<i>E. coli</i> DH5 α	<i>endA1 hsdR17 supE44 recA1 relA1 (lacZYA-argF)</i>	Lab stock
<i>M. tuberculosis</i> H37Rv	Virulent strain of <i>M. tuberculosis</i> (ATCC 27294)	Lab stock
pGOAL19	<i>E. coli ori sacB β-gal, Hyg^r</i>	Parish & Stoker (2000)
pAZI272	Integrating mycobacterial expression vector with <i>hsp70</i> promoter	Awasthy <i>et al.</i> (2009)
pAZI0240	1.9 kb DNA fragment with Δ <i>alr</i> flanked by upstream and downstream sequences cloned into <i>ScaI</i> – <i>SspI</i> sites of pGOAL19	This study
pAZI0256	<i>alr</i> gene of <i>M. tuberculosis</i> cloned into <i>EcoRI</i> – <i>HindIII</i> sites of pAZI0272	This study

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

Gene knockout plasmid constructs. The plasmids used in this study are listed in Table 1. The constructs used for gene knockout (KO) of *alr* were outsourced to Bangalore Genei (India). The recombination substrate for creating a deletion in the *alr* gene of *M. tuberculosis* consisted of 682 bp of the region upstream of *alr*, 147 bp of *alr* followed by a 223 bp deletion in *alr* which rendered the remaining 840 bp *alr* out of frame. This DNA fragment was cloned into *ScaI*-*SspI* sites of a suicide vector, pGOAL19 (Parish & Stoker, 2000). The resulting construct was designated pAZI0240. For making the complementation construct, the *alr* gene (Rv3423c) was amplified from *M. tuberculosis* genomic DNA by PCR using high-fidelity *Taq* DNA polymerase (Phusion, Finnzyme). The amplified PCR product was cloned into *EcoRI*-*HindIII* sites of the integrating mycobacterial expression vector pAZI272 (Awasthy *et al.*, 2009). The sequences of the cloned DNA fragments were confirmed by sequencing (Microsynth).

DNA amplification by PCR. The single cross-over (SCO) and double cross-over (DCO) recombinants in *M. tuberculosis* were screened by colony PCR as described previously (Awasthy *et al.*, 2009). 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 was decided by considering the melting temperature of the primer pair and length of the PCR product, respectively. The sequences of primers used for PCR amplification were as follows: 366, 5'-TTTGGTCTTCAGCTAGATCA-3'; 367, 5'-AAAGCCAAACGACACGACAG-3'; 368, 5'-GCAACAGTTGCGAGTTGG-3'.

Southern blotting. Genomic DNA from the wild-type and *alr* KOs of *M. tuberculosis* were digested with *XmaI* and *XhoI* and separated by electrophoresis on 0.8 % agarose gels. A DNA fragment amplified by PCR encompassing the upstream region and 5' end of *alr*, which was labelled with fluorescein by random prime labelling (Roche), was used as a probe. DNA transfer, hybridization and washings were performed according to the protocol provided by the manufacturer (GE, Amersham). The bound probes were detected by horseradish peroxidase-conjugated anti-fluorescein antibodies (1:2000 dilution) using chemiluminescence.

MIC reversal with D-alala or D-alala-D-alala. The MIC of DCS on *M. tuberculosis* was determined by using the Alamar blue method using microtitre plates (Franzblau *et al.*, 1998). In order to look for reversal of the DCS effect, the MIC experiment was set up with cultures containing increasing concentrations of D-alala (0, 2, 5, 10, 20, 50 and 100 µg ml⁻¹) or D-alala-D-alala (up to 1 mg ml⁻¹). The plates were incubated for 6 days at 37 °C. Alamar blue dye was added to the culture at the end of day 6 and the plates were further incubated for 24 h. The change in dye colour was noted at the end of day 7.

Growth and survival kinetics of *M. tuberculosis alr* KO in vitro. For growth studies in liquid culture, the *alr* mutant culture was grown in the presence of 50 µg D-alala ml⁻¹, diluted in fresh 7H9 medium containing increasing concentrations of D-alala or D-alala-D-alala and OD₆₀₀ was measured. For growth kinetics on agar plates, the mutant culture was grown up to OD₆₀₀ 0.8 in the presence of 50 µg D-alala ml⁻¹, washed and resuspended in plain 7H9 medium. Appropriate culture dilutions were plated on plates containing 0–20 µg D-alala ml⁻¹ to obtain isolated colonies. The plates were incubated at 37 °C and colonies were counted after 3 and 8 weeks of incubation. To study the survival kinetics of the *alr* mutant in the absence of D-alala, the mutant

culture grown in the presence of 10 µg D-alala ml⁻¹ was washed free of extracellular D-alala and resuspended in plain 7H9. The culture was incubated on a roller for 7 days and aliquots were monitored for viability by plating various dilutions of the culture on 7H9 containing 10 µg D-alala ml⁻¹. The colonies were counted after incubation for 1 month at 37 °C.

Growth of *M. tuberculosis* H37Rv and *alr* KO mutant in macrophages. Intracellular infection and survival kinetics of mycobacterial strains were investigated in IFN-γ-activated primary cultures of bone marrow-derived macrophages (BMDM) in a 24-well plate format. 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⁻¹ (Sigma) for 48 h. Activated and washed BMDM monolayers were used for infection. The monolayers were infected with *M. tuberculosis* H37Rv and *alr* KO mutant 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 and the monolayers were incubated for 7 days. On days 0, 3 and 7 the monolayers were lysed and the lysates were plated on 7H9 + 10 µg D-alala ml⁻¹. Before lysing, the viability of the cells was assessed microscopically by observing the adherence and intactness of the monolayers. Plate wells with more than 90 % intact cells were processed further.

Mouse infection. The animal usage and experimental protocols were approved by the Institutional Animal Ethics Committee, registered with the government of India (registration no. CPCSEA 99/5). To infect BALB/c mice by the intravenous route, the frozen bacterial samples were thawed, centrifuged, washed in PBS and diluted appropriately in cold, sterile PBS. The animals were infected with the wild-type or mutant *M. tuberculosis* strains to obtain 10⁵ c.f.u. per animal. After infection, the animals were sacrificed at an interval of 1 week and the bacterial counts present in the organs were determined by plating the lung and spleen homogenates on 7H11 agar supplemented with D-alala (10 µg ml⁻¹).

RESULTS

alr gene of *M. tuberculosis* can only be inactivated in the presence of an extra copy of *alr* or D-alala in the growth medium

To determine whether *Alr* is essential for the survival of *M. tuberculosis*, we decided to inactivate the *alr* gene by a two-step homologous recombination procedure involving an SCO followed by a DCO recombination event as described previously (Awasthy *et al.*, 2009; Parish & Stoker, 2000). In order to avoid any polar effects on downstream genes, inactivation of *alr* was attempted by introducing a markerless deletion as described in Methods. The gene KO was performed under the following three conditions: 1) in the presence of single copy of the *alr* gene (wild-type *M. tuberculosis*), 2) in the presence of two copies of *alr* (merodiploid) and 3) in the presence of D-alala (100 µg ml⁻¹), the reaction product of *Alr*, in the growth medium. The ability to obtain a gene KO only in the presence of an additional copy of the gene in the merodiploid strain or D-alala in the medium was taken as evidence of essentiality of the gene. The third condition (D-alala in the medium) was

expected to provide an opportunity to study the strain's phenotype in the absence of D-ala *in vitro* and in macrophages and mice.

After electroporation of pAZI0401 into *M. tuberculosis*, an SCO recombinant (*hyg*⁺, *sacB*⁺; *alr*/SCO) was identified by PCR to detect *hyg* and *sacB* genes (data not shown) and by primers corresponding to the *alr* locus as shown in Fig. 2(b). *alr*/SCO was further used for obtaining DCO recombinants as described previously (Awasthy *et al.*, 2009). Briefly, *alr*/SCO was grown in the absence of hygromycin for 3 weeks and subsequently plated on 7H9 plates containing 2 % sucrose. The colonies growing in the presence of sucrose were screened for the loss of the plasmid by PCR amplification of *hyg* and *sacB* genes (data not shown). In the wild-type *M. tuberculosis* background, out of 177 colonies screened from sucrose plates, 11 had not undergone recombination and the remaining 166 were found to have lost the plasmid (*hyg*[−], *sacB*[−]; 94 % recombination efficiency); however, all had retained the wild-type copy of the gene indicating that inactivation of the *alr* gene leads to loss of viability. Following a similar procedure, we screened for the presence of KO colonies

amongst DCO recombinants obtained from a merodiploid strain (*alr*/SCO with the *alr* gene on the *att* site). It was found that 10 of 66 DCOs (*hyg*[−], *sacB*[−]) were carrying the deleted copy of the gene, proving that when two copies of *alr* are present in the organism, it is possible to inactivate one of them. Next, we attempted inactivation of *alr* in the presence of D-ala (100 µg ml^{−1}). PCR screening of 88 colonies from sucrose plates for the loss of plasmid showed that 92 % had lost the plasmid (*hyg*[−], *sacB*[−]) by a DCO event, which showed that the recombination frequency of the cultures grown in the absence and the presence of D-ala was similar. After screening colonies by gene-specific primers, it was found that 13 % of the DCO colonies had a deleted copy of the gene in the chromosome, while the remaining had the wild-type copy of *alr*. This showed that it was possible to obtain a KO of *alr* provided the cells had access to D-ala. The inactivation of the *alr* gene in one of the DCO recombinants was confirmed by PCR (Fig. 2a, b) and by Southern blotting (Fig. 2c). The observation that *alr* can only be inactivated in the presence of either an extra copy of *alr* or D-ala in the growth medium suggested that *alr* is an essential gene in *M. tuberculosis*.

Fig. 2. Gene KO of *alr* in *M. tuberculosis* by introducing a marker-less deletion by homologous recombination. (a) The position of primers used for PCR amplification of gene fragments from wild-type and Δalr *M. tuberculosis* strains. The expected sizes of the PCR products of wild-type, *alr*/SCO and the Δalr mutant PCR products are, respectively, 401, 401 and 178, and 178 bp for primers 367 + 368, and 1177, 954 and 954 bp for primers 366 + 368. (b) PCR profiles of gene fragments obtained from wild-type, *alr*/SCO and Δalr mutants. Lanes: 1, wild-type; 2, Δalr ; 3, *alr*/SCO; M, molecular size marker consisting of 100 bp ladder (left) or 1 kb ladder (right). (c) Southern blotting of *Xma*I-digested genomic DNA from wild-type and the Δalr mutant following the procedure described in Methods. Lanes: 1, wild-type H37Rv; 2, Δalr . The position of the probe used in Southern blotting is shown by a heavy line. The faint bands appearing in the Southern blot result from binding of the probe to the undigested genomic DNA.

M. tuberculosis requires a very small amount of D-ala for growth

As seen in Fig. 3(a) there was no visible growth in liquid cultures of the Δalr mutant lacking D-ala, while cultures containing 5 $\mu\text{g D-ala ml}^{-1}$ showed poor growth. Addition of more than 10 $\mu\text{g D-ala ml}^{-1}$ to the medium did not make any difference to the growth rates, showing that 10 $\mu\text{g ml}^{-1}$ is sufficient for optimum growth of the mutant strain. Interestingly, D-ala-D-ala, the end product of the pathway, was also able to support the growth of the Δalr mutant. However, it required a much higher concentration of D-ala-D-ala (500 $\mu\text{g ml}^{-1}$) to support optimum growth (Fig. 3b), which could be because of poor uptake of the dipeptide by the cells. This is the first report of D-ala-D-ala supporting the growth of an auxotrophic mutant of a mycobacterial species.

As seen in Fig. 3(c), the agar plates containing 10 and 20 $\mu\text{g D-ala ml}^{-1}$ showed similar c.f.u. at 3 and 8 weeks of

incubation. In plates supplemented with 5 $\mu\text{g D-ala ml}^{-1}$, the number of colonies after 3 weeks was significantly less ($P=0.0004$) than on plates containing 10 or 20 $\mu\text{g D-ala ml}^{-1}$, but by 8 weeks, the bacterial counts on all three concentrations of D-ala were found to be very similar. Under similar conditions, in plates containing 2 $\mu\text{g D-ala ml}^{-1}$ a significant increase in c.f.u. was seen after 8 weeks (from 10^4 to 10^6 c.f.u. ml^{-1} , $P=0.0007$). A further increase in c.f.u. was not seen by incubating the plates beyond 8 weeks. The data indicate that 5–10 $\mu\text{g D-ala ml}^{-1}$ is the optimum amount of D-ala required for growth on agar. Interestingly, on plates without D-ala, no colonies could be seen at 3 weeks, but there was a significant number of small colonies by 8 weeks (Fig. 3c) The colonies picked up from the plate without D-ala and replated onto increasing concentrations of D-ala did not show any colonies in the absence of D-ala (data not shown), indicating that the growth on the plate with no D-ala in the earlier experiment was because of carry over of the D-ala pool present inside

Fig. 3. Growth and survival kinetics of the Δalr mutant *in vitro*. (a) Growth of wild-type *M. tuberculosis* and the Δalr mutant in liquid culture in the presence of the indicated concentrations of D-ala. (b) Growth of wild-type *M. tuberculosis* and the Δalr mutant in liquid culture in the presence of the indicated concentrations of D-ala-D-ala. (c) Growth of the Δalr mutant in agar medium in the presence of the indicated concentrations of D-ala. The platings were done in triplicate and the average c.f.u. obtained after 3 and 8 weeks of incubation is shown. The error bars indicate SD of c.f.u. obtained in triplicate. *, Very small numbers of colonies were obtained in plates lacking D-ala when the starting culture was grown in the presence of 50 $\mu\text{g D-ala ml}^{-1}$, whereas no colonies were obtained when the starting culture was grown in 20 $\mu\text{g D-ala ml}^{-1}$. (d) Survival of wild-type *M. tuberculosis*, Δalr mutant and Δalr -complemented strains in the presence or absence of D-ala. LOQ, limit of quantification.

Fig. 4. Intracellular survival kinetics of wild-type *M. tuberculosis* H37Rv (hatched) and the Δalr mutant (white) in BMDM. The c.f.u. obtained on days 0, 3 and 7 are shown. The experiment was performed in triplicate and repeated twice. The mean $\pm$ SD c.f.u. values of triplicate experiments obtained in one experiment are shown.

the cells which had been grown in $50 \mu\text{g D-ala ml}^{-1}$. This set of experiments suggested that *M. tuberculosis* requires very small amounts of D-ala for growth. The amount of D-ala required for growing various *alr* mutants of Gram-positive bacteria ranges from 100 to $300 \mu\text{g ml}^{-1}$ (Thompson *et al.*, 1998; Palumbo *et al.*, 2004; Milligan *et al.*, 2007). This suggests that, compared with other bacteria, *M. tuberculosis* requires very small amounts of D-ala for growth.

Depletion of intracellular D-ala pool leads to cell death of *M. tuberculosis*

Since our data showed that *M. tuberculosis* requires small amounts of D-ala for growth, does this mean that depletion of the intracellular D-ala pool will only lead to cessation of cell growth or can it result in loss of viability? To resolve this, the Δalr mutant was incubated without D-ala for 7 days and the viability was determined by measuring c.f.u. on 7H9 containing $10 \mu\text{g D-ala ml}^{-1}$. As shown in Fig. 3(d), in the Δalr mutant, there was a continuous drop in

c.f.u. over a period of 7 days. As expected, the Δalr culture supplemented with D-ala or complemented with *alr*, and the wild-type *M. tuberculosis* strains showed an increase in c.f.u. in the first week, after which the c.f.u. remained constant. Thus, it is clear that depletion of the D-ala pool in *M. tuberculosis* leads to death of the cells.

An *Alr* mutant of *M. tuberculosis* is defective for survival in macrophages

In order to assess the role of D-ala in survival of *M. tuberculosis* inside macrophages, the survival of the Δalr mutant was studied in BMDM cells made immune-competent by activation with gamma-interferon. As seen in Fig. 4, in the wild-type there was small increase in c.f.u. on day 3 followed by no change in c.f.u. till day 7, whereas the Δalr mutant showed 70 and 89% reduction in c.f.u. by day 3 and day 7, respectively. The reduction in the c.f.u. observed with the Δalr mutant was statistically significant ($P=0.0008$). Thus, D-ala is required for intracellular survival of *M. tuberculosis*. This finding is consistent with the results of Chacon *et al.* (2009) where an *M. smegmatis* mutant was used. As there was no net increase in the number of wild-type bacteria inside the macrophages during this time period, the presence of D-ala is probably required to maintain the integrity of the cell wall.

An *Alr* mutant of *M. tuberculosis* is defective for survival in mice

The D-ala-requiring mutant of *M. tuberculosis* is expected to face absolute shortage of D-ala inside the animals since the eukaryotic host tissues lack D-amino acids. As seen in Fig. 5, both the wild-type and mutant *M. tuberculosis* were able to establish infection in mice, though the c.f.u. of Δalr seen in lungs and spleen of mice was ten times less than that of wild-type bacteria. Since the c.f.u. of the mutant and the wild-type H37Rv inoculated into the mice was the same, the lesser number of c.f.u. obtained on day 1 from lungs and spleen of mice infected with the Δalr mutant indicates that the absence of D-ala probably interferes with establishing infection. The growth profile of the wild-type bacteria showed that after a small drop in the number of c.f.u., there was a gradual increase in the number of

Fig. 5. Survival kinetics of wild-type *M. tuberculosis* H37Rv (■) and the Δalr mutant (▲) in mice. C.f.u. obtained from lungs and spleen of mice at time intervals ranging from 0 to 9 weeks. The mean $\pm$ SD c.f.u. values of triplicates obtained from three mice for each time point is shown. LOQ, limit of quantification.

bacteria in lungs while the c.f.u. counts in spleen did not change till day 63 (Fig. 5). On the other hand, the Δalr mutant showed a more than 2- $\log_{10}$ reduction in the bacterial counts obtained from lungs during the first week (Fig. 5a). Thereafter, no further drop in c.f.u. was observed. Overall, the wild-type strain showed a 1.2- $\log_{10}$ c.f.u. increase by day 63, whereas the mutant strain showed a reduction of 2.1- $\log_{10}$ c.f.u. during this period. Though at a slower rate than that in lungs, a continuous drop in c.f.u. was seen in samples obtained from the spleen till day 21 (Fig. 5b). Bacterial numbers in the wild-type strain did not increase from day 1 to day 63, whereas the mutant strain showed a 1.0- $\log_{10}$ reduction in c.f.u. by day 63. After a steady decline in c.f.u. to day 21, there was curiously an increase in c.f.u. by day 28, which is unexplainable at this time. There was no significant change in c.f.u. between days 28 and 63. In order to have better confidence in the data, we performed statistical analysis by normalizing the c.f.u. of the wild-type and the mutant. This was done by increasing the c.f.u. of the mutant in lungs and spleen at all time points by the c.f.u. difference seen between the wild-type and the mutant at the beginning of the infection (i.e. 1.18- $\log_{10}$ in lungs and 1.21- $\log_{10}$ in spleen). Even after the normalization, the reduction in c.f.u. of the mutant in the lungs and the spleen was highly significant. The *P*-values of the Δalr lung c.f.u. on days 14, 21, 28 and 63 were 0.0003, 0.001, 0.002 and 0.004, while the corresponding values for the spleen samples were 0.0002, 0.0004, 0.02 and 0.004, respectively. The significant loss of viability of the Δalr mutant seen in lungs and to a lesser extent in the spleen, demonstrated that *alr* is essential for growth and survival of *M. tuberculosis* in mice.

D-Ala or D-alal-D-alal can reverse the inhibition of *M. tuberculosis* by DCS

Overexpression of Alr and Ddl individually has been shown to result in an increase in the MIC of DCS on *M. smegmatis* (Cáceres *et al.*, 1997; Feng & Barletta, 2003), indicating dual target inhibition by DCS. Reversal of growth inhibition by supplementing with the product

of the target enzyme is one way of proving the mechanism of action of an inhibitor. We hypothesized that if killing of *M. tuberculosis* by DCS was because of inhibition of both Alr and Ddl or if Alr was non-essential, then addition of D-alal will have either a partial effect or no effect on DCS-mediated inhibition of *M. tuberculosis*. As seen in Table 2, the addition of D-alal was able to reverse the DCS inhibition as the MIC changed from 8 $\mu\text{g ml}^{-1}$ in the absence of D-alal to 128 $\mu\text{g ml}^{-1}$ in the presence of 100 $\mu\text{g D-alal ml}^{-1}$. Addition of L-alal (up to 1000 $\mu\text{g ml}^{-1}$) to the growth medium did not have any significant effect on DCS inhibition. D-Alal-D-alal showed modest reversal of DCS inhibition at a very high concentration of the dipeptide (500 $\mu\text{g ml}^{-1}$). This could be because of poor uptake of D-alal-D-alal by the *M. tuberculosis* cells, as observed in growth kinetics of the Δalr strain. The MIC of two other drugs, INH and rifampicin, remained unchanged under these conditions, showing the specificity of MIC reversal by D-alal or D-alal-D-alal (data not shown). To see whether depletion of D-alal will make the cells super-sensitive to DCS, the MIC of this inhibitor on the Δalr strain was tested in cultures grown in the presence of concentrations of D-alal that are suboptimal for growth. It was found that the MIC of DCS was 1, 4 and 32 $\mu\text{g ml}^{-1}$ on cultures grown in the presence of 3, 4 and 10 $\mu\text{g D-alal ml}^{-1}$. This showed that depletion of D-alal makes the *M. tuberculosis* cells super-sensitive to DCS. The modulation of DCS MIC seen in the wild-type and *alr* mutant of *M. tuberculosis* grown in the presence of varying concentrations of D-alal indicated that Alr-driven synthesis of D-alal is essential for the survival of *M. tuberculosis* and also suggests that Alr could be the primary target of DCS in this organism.

DISCUSSION

In target-based drug discovery the compound leads are optimized on a single target and thus, it is crucial to confirm that a particular target (e.g. Alr) is essential and inhibition of the target alone will lead to suppression of mycobacterial growth. The essentiality of a target can be

Table 2. MIC of DCS on wild-type *M. tuberculosis* in the presence of L-alal, D-alal or D-Alal-D-alal

The MIC experiment was done in duplicate and repeated twice. Values obtained from one of the experiments are shown here. Values shown are in $\mu\text{g ml}^{-1}$.

L-Alal		D-Alal		D-Alal-D-alal	
Concentration	DCS MIC	Concentration	DCS MIC	Concentration	DCS MIC
0	4	0	8	0	4
50	8	2	8	50	4
100	4	5	16	100	4
150	8	10	32	150	4
200	8	20	64	200	4
500	8	50	128	500	8
1000	8	100	128	1000	16

determined by either genetic means or the action of a specific inhibitor. DCS inhibits both Alr and Ddl proteins *in vitro* (Bruning *et al.*, 2011), which makes it difficult to decipher whether inhibition of either of these proteins individually will lead to growth inhibition. Studies based on overexpression of Alr or Ddl (Cáceres *et al.*, 1997; Feng & Barletta, 2003), reversal of DCS inhibition in the presence of D-alanine in various bacteria (Wijsman, 1972) and a metabolomics approach in *M. smegmatis* (Halouska *et al.*, 2007) suggest that killing of bacteria by DCS could be mediated through inhibition of either Alr or Ddl.

We have shown that either D-alanine or D-alanine-D-alanine can reverse the effect of DCS inhibition in *M. tuberculosis*. Complete reversal of DCS-driven inhibition by D-alanine alone suggests that, as proposed previously (Halouska *et al.*, 2007), Ddl is not the primary target of DCS in mycobacteria and inhibition of Alr contributes significantly towards killing by DCS. If DCS was killing mycobacteria by inhibiting Ddl alone, then D-alanine alone would not be able to reverse the DCS effect as inhibition of Ddl would deprive the cell of D-alanine-D-alanine which would lead to inhibition of cell growth.

The dependency of the *alr* mutant on D-alanine, coupled with the absence of D-amino acid transaminase (Dat), confirms that Alr activity is essential for the survival of *M. tuberculosis*. In this respect, the mycobacterial mutants behave like many known *alr* mutants of bacteria such as *E. coli*, *S. typhimurium*, *Lactococcus lactis* and *Lactobacillus plantarum* (Wasserman *et al.*, 1983; Hols *et al.*, 1997; Steen *et al.*, 2005). Depletion of intracellular D-alanine pools in *alr* mutants of *E. coli*, *Bacillus subtilis* and *Lactobacillus plantarum* has been shown to result in death of the cells (Wijsman, 1972; Heaton *et al.*, 1988; Palumbo *et al.*, 2004).

The amount of D-alanine required for the optimum growth of *M. tuberculosis* (5 µg ml⁻¹) is the lowest amongst the bacteria (Thompson *et al.*, 1998; Palumbo *et al.*, 2004; Milligan *et al.*, 2007) and is almost ten times less than the amount of other amino acids required for optimum growth of *M. tuberculosis* (Awasthy *et al.*, 2009). The exact reason for this low requirement of D-alanine is not clear, though it can be speculated that it might reflect the poor rate of peptidoglycan biosynthesis because of the very slow growth rate of *M. tuberculosis*.

The low requirement of D-alanine is also reflected in the poor catalytic activity of recombinant mycobacterial Alr *in vitro*, compared with Alr from other bacterial species (Strych *et al.*, 2001). In spite of the low need for D-alanine in *M. tuberculosis*, depletion of the intracellular pool not only leads to growth stasis but also causes cell death, indicating the possibility of obtaining bactericidal inhibitors with this target. This finding is consistent with the observations made with another cell wall target, InhA, whose depletion or inhibition leads to killing of bacteria (Vilchèze *et al.*, 2000). However, as suggested by target depletion experiments (Wei *et al.*, 2011), only a potent inhibitor of Alr is expected to inhibit mycobacterial growth. Availability of the crystal structure of *M. tuberculosis* Alr offers great

opportunity for designing novel inhibitors of this essential enzyme (LeMagueres *et al.*, 2005). Looking at the phenotype of the *alr* mutant in mice, in which the depletion of the bacterial D-alanine pool leads to a rapid loss of viability initially, followed by persistence of the remaining bacteria for several days, we are prompted to predict that the prospective Alr inhibitors, like other cell wall inhibitors (e.g. INH), might show early bactericidal activity, but could be poor in sterilizing activity (Mitchison, 2000).

Bacterial mutants requiring D-alanine for growth have been found to be attenuated for virulence in animal studies (Thompson *et al.*, 1998). An *L. monocytogenes alr dat* double mutant has been used as a vaccine candidate and shown significant protection against the wild-type strain of *L. monocytogenes*. Though the persisting nature of the *alr* mutant in mice does not bode well for obtaining inhibitors with sterilizing activity, the same feature can be utilized in designing a vaccine against TB. The attenuated nature of the *alr* mutant coupled with its ability to survive for long periods of time inside mice suggests that it could be a good vaccine candidate. However, more studies are required to prove this point.

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