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Impairment of D-alanine biosynthesis in *Mycobacterium smegmatis* determines decreased intracellular survival in human macrophages

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Abstract

D-alanine is a structural component of mycobacterial peptidoglycan. The primary route of D-alanine biosynthesis in eubacteria is the enantiomeric conversion from L-alanine, a reaction catalyzed by D-alanine racemase (Alr). *Mycobacterium smegmatis alr* insertion mutants are not dependent on D-alanine for growth and display a metabolic pattern consistent with an alternative pathway for D-alanine biosynthesis. In this study, we demonstrate that the *M. smegmatis alr* insertion mutant TAM23 can synthesize D-alanine at lower levels than the parental strain. The insertional inactivation of the *alr* gene also decreases the intracellular survival of mutant strains within primary human monocyte-derived macrophages. By complementation studies, we confirmed that the impairment of *alr* gene function is responsible for this reduced survival. Inhibition of superoxide anion and nitric oxide formation in macrophages suppresses the differential survival. In contrast, for bacteria grown in broth, both strains had approximately the same susceptibility to hydrogen peroxide, acidified sodium nitrite, low pH and polymyxin B. In contrast, TAM23 exhibited increased resistance to lysozyme. D-alanine supplementation considerably increased TAM23 viability in nutritionally deficient media and within macrophages. These results suggest that nutrient deprivation in phagocytic cells combined with killing mediated by reactive intermediates underlies the decreased survival of *alr* mutants. This knowledge may be valuable in the construction of mycobacterial auxotrophic vaccine candidates.

Abbreviations: DCS, D-cycloserine; **GPL**, glycopeptidolipid; **NM-L-Arg**, *N*-methyl-L-arginine; **OPA**, *o*-phthaldialdehyde; **RNI**, reactive nitrogen intermediates; **ROI**, reactive oxygen intermediates; **SOD**, superoxide dismutase

Three supplementary figures follow the References for this paper; they show growth curves for *M. smegmatis* parental, *alr* mutant, and complemented strains grown in MADC-TW and minimal medium, with or without D-alanine supplementation, and the susceptibility of *M. smegmatis* to hydrogen peroxide and sodium nitrite.

Introduction

Mycobacteria display complex cell walls consisting of glycolipids and proteins linked to the mycolyl-arabinogalactan-peptidoglycan backbone (Brennan & Nikaido, 1995; McNeil & Brennan, 1991). This structure is a major determinant of mycobacterial drug resistance and pathogenesis (Daffé & Draper, 1998; Trias & Benz, 1994), and its biosynthesis is highly conserved among different mycobacterial species (Belanger & Inamine, 2000). Intracellular survival is an essential determinant of mycobacterial pathogenesis. To survive within phagocytic cells, mycobacteria must overcome the bactericidal mechanisms of macrophages, including reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) and lysosomal enzymes (Armstrong & Hart, 1975; Lowrie & Andrew, 1988; Sturgill-Koszycki *et al.*,

1994). Studies on auxotrophic mutants have demonstrated that nutritional status also plays a role in the intracellular survival of mycobacteria within macrophages (De Voss *et al.*, 2000; Hondalus *et al.*, 2000; Jackson *et al.*, 1999).

Mycobacterium smegmatis, a fast-growing species of low pathogenicity, displays a basal but measurable level of resistance to macrophage killing. Studies with this micro-organism have been conducted using a diverse array of macrophages (Jordao *et al.*, 2008), including murine (Anes *et al.*, 2006; Kuehnel *et al.*, 2001) and human (McGarvey *et al.*, 2004; Miller & Shinnick, 2000) cell lines, and tissue-derived macrophages of murine (Kocincova *et al.*, 2009; Singh *et al.*, 2008; Wagner *et al.*, 2005) or human origin (Garg *et al.*, 2006; Lagier *et al.*, 1998). *M. smegmatis* interaction with mouse phagocytic cells results in generation of ROI and RNI, including nitric oxide (Anes *et al.*, 2006). *In vitro* susceptibility of *M. smegmatis* to RNI is well documented (Yu *et al.*, 1999). In addition, expression of *Mycobacterium tuberculosis* genes in *M. smegmatis* alters its resistance to macrophage killing, and this effect has been widely used in the identification of mycobacterial genes involved in intracellular survival of pathogenic mycobacteria (Miller & Shinnick, 2000; Singh *et al.*, 2008). Since *M. smegmatis* has a high-efficiency genetic transfer system and fast growth, it has been used as an affordable model to study mycobacterial physiology, including the effects of metabolic changes on intracellular survival, gene expression and regulation, and this is especially meaningful for the study of highly conserved pathways (Caceres *et al.*, 1997; Danilchanka *et al.*, 2008; Garg *et al.*, 2006; Glover *et al.*, 2007; Lagier *et al.*, 1998; Posey *et al.*, 2006; Provvedi *et al.*, 2008).

D-alanine is a structural component of mycobacterial peptidoglycan (Strominger, 1962). D-alanine racemase catalyses the primary route of D-alanine biosynthesis in mycobacteria (Chacon *et al.*, 2002). D-alanine is also present in the core head group of glycopeptidolipids (GPLs) of many mycobacterial species, with *Mycobacterium xenopi* illustrating the only exception among those species analyzed (Brennan & Nikaido, 1995; Eckstein *et al.*, 2003; Etienne *et al.*, 2005; Riviere & Puzo, 1991). Genetic analysis of the D-alanine branch of peptidoglycan biosynthesis in mycobacteria was first carried out in *M. smegmatis*, which possesses, as does *M. tuberculosis*, only one *alr* gene (Caceres *et al.*, 1997; Cole *et al.*, 1998). We have demonstrated that D-alanine racemase insertion mutants (*alr*) are not dependent on D-alanine for growth (Chacon *et al.*, 2002). These mutants also exhibit increased susceptibility to the bactericidal agent D-cycloserine (DCS), a D-alanine analogue used as a second-line drug in tuberculosis treatment. We hypothesized that disrupting D-alanine biosynthesis would also impair survival in macrophages, as a result of modifications in the cell envelope and/or nutrient deprivation in phagocytic cells. In this study we determine the levels of D-alanine biosynthesis and the survival of *M. smegmatis* parental and *alr* mutant strains under various extracellular conditions and in human monocyte-derived macrophages.

Methods

Bacterial strains, plasmid vectors and growth conditions. Bacterial strains and vectors used in this study have been previously described (Chacon *et al.*, 2002). *Escherichia coli* DH5*a* was grown as described elsewhere (Sambrook *et al.*, 1989). *M. smegmatis* parental strain mc^2155 (Snapper *et al.*, 1990), its derived *alr* mutant TAM23 (Chacon *et al.*, 2002), and their transformants with the integrating vectors pYUB178 (Pascopella *et al.*, 1994), pYUB412 (Pavelka & Jacobs, 1999) and pTAMU3 (Chacon *et al.*, 2002), were grown at 37 °C with shaking (200 r.p.m. with an Innova 4300, New Brunswick Scientific) to OD_{600} ~1.0. Supplements for complete Middlebrook 7H9 (BBL Microbiology Systems) medium were 0.2% (v/v) glycerol (Sigma-Aldrich), 0.05% Tween 80 (Sigma-Aldrich) to prevent clumping, 0.01% cycloheximide (A.G. Scientific) to prevent fungal contamination, and ADC [0.5% bovine fraction V albumin (Amresco), 0.01 M glucose (Acros), and 0.015 M NaCl (MADC-TW)]. Broth minimal medium was prepared as previously described (Chacon *et al.*, 2002). Generation times were determined as described elsewhere (Miller, 1972). c.f.u. were estimated from optical density measurements using appropriate calibration curves. In addition, *M. smegmatis* c.f.u. were determined after incubation at 37 °C on MADC agar (Tween 80 was omitted from all solid media) or appropriately supplemented 7H9 basal medium prepared with 15 g Bacto agar l^{-1} (Difco Laboratories). D-alanine (Sigma-Aldrich) was added at 50 mM as necessary to broth cultures and agar plates.

Analysis of amino acid intracellular pools. Steady-state levels of intracellular amino acid pools were determined as previously described (Feng & Barletta, 2003). *M. smegmatis* strains mc^2155 and TAM23 were grown in 250 ml minimal medium with 21 mM glycerol (carbon source) and 5 mM NH_4Cl (nitrogen source) to exponential phase (OD_{600} 0.5–0.9), treated for 2 h with or without DCS at 75 μg ml^{-1}, harvested, washed and sonicated to prepare cell-free protein-free extracts as previously described (Chacon *et al.*, 2002). Protein was removed from the supernatant by serial passages through YM-10 and YM-3 Centricon concentrators (Millipore Corporation). Determination of amino acids was performed at the Amino Acid Geochronology Laboratory of Northern Arizona University (Flagstaff, AZ) by a reverse-phase HPLC procedure as described elsewhere (Kaufman & Manley, 1998). This procedure was able to detect nine pairs of L- and D-amino acids in the subpicomolar range based on the derivatization reaction of amino acids with *o*-phthaldialdehyde (OPA) and the chiral compound *N*-isobutyryl-L-cysteine. The synthetic amino acid L-*homo*-arginine (L-hArg) was spiked in the samples at 10 μM as an internal standard. The total amount of amino acids in the recovered pools was averaged for each strain/treatment combination and expressed as micromoles total OPA-derivatized product per 1×10^9 cells. Relative abundance percentiles of individual amino acids were calculated as a molar percentage of the total pool for each sample.

Intracellular killing assays. Intracellular killing assays were carried out as described previously, with some modifications (Bermudez & Young, 1988). Prior to infection, *M. smegmatis* cells were grown in MADC-TW with and without D-alanine to OD ~1.0, harvested, and resuspended in Hanks' Balanced Salts Solution (HBSS; Sigma-Aldrich). Cells were passed through a membrane filter (1.2 μm pore-size, Millipore) to obtain a single-cell suspension, and sedimented for 5 min at room temperature. Macrophages were infected with 1.0 ml supernatant appropriately diluted in HBSS. Monocytes (1.0–5.0×10^5 per well) derived from blood of healthy human donors were seeded in tissue-culture plates (Sigma-Aldrich) and incubated for 2 h at 37 °C in 5% CO_2. Procedures for blood collections were performed in compliance with all federal and local regulations. Supernatant fluids with non-adherent cells were removed and the wells were washed twice with 1.0 ml prewarmed HBSS. The adherent

monocytes were maintained in culture for 7 days with 1.5 ml complete RPMI 1640 (Invitrogen) medium supplemented with 2 mM L-glutamine (Sigma-Aldrich) and 10% heat-inactivated fetal bovine serum (Sigma-Aldrich), and D-alanine where indicated; the medium was replaced every 48 h.

Macrophage monolayers were infected at an m.o.i. of 1 or 10 bacilli per cell, as indicated. Monolayers were incubated for 1–2 h at 37 °C in 5% CO_2 followed by three washes with HBSS to remove extracellular micro-organisms. Similar washes were performed at each time point. An m.o.i. of 1 bacillus per cell was used as necessary to maximize the effects of host cell restriction on bacterial growth. For inhibitor treatment assays, cells were split 30 min post-infection and supplemented as indicated with bovine superoxide dismutase (SOD; Sigma-Aldrich), human catalase (Sigma-Aldrich), or the nitric oxide (NO) synthase inhibitor *N*-methyl-L-arginine (NM-L-Arg; Sigma-Aldrich) following previously described procedures (Bermudez & Young, 1989; Bermudez, 1993). Cells were lysed at 1 or 2, 24, 48 (except inhibitor assays) and 72 h with 0.5 ml sterile double-distilled H_2O (ddH_2O) for 10 min and 0.025% SDS (Sigma-Aldrich) in HBSS for another 10 min. SDS was subsequently removed with 0.1% bovine albumin and the lysate was plated on MADC. Three replicate wells were used per experimental group, in at least two independent experiments.

Cell viability was determined by trypan blue exclusion as previously described (Bermudez & Young, 1988). The viability of the cell monolayer post-infection decreased slightly from 98–99% (1 h) to 90–92% (24 h), 84–86% (48 h) and 73–77% (72 h). No significant differences were observed at each time point in the viability of macrophage monolayers subjected to different treatments or infected with various strains. To verify that the addition of SDS did not detrimentally affect the viability of the *M. smegmatis* parental and *alr* mutant strains, ~5.0×10^6 c.f.u. were treated with ddH_2O for 10 min and 0.025% SDS for 10 min, and 0.1% albumin was added. Bacteria were plated to determine c.f.u. There were no significant differences for the titer of either mc^2155 (P = 0.7662) or TAM23 (P = 0.6901) strains before and after treatment, or between mc^2155 and TAM23 (P = 0.9422) in their susceptibility to SDS. Another experiment using 20 μg amikacin ml^{-1} (Sigma-Aldrich) during invasion yielded similar results, demonstrating the effectiveness of the washing procedure for removing extracellular bacteria (data not shown).

Susceptibility assays. Susceptibility assays with *M. smegmatis* cells grown in MADC-TW were carried out at different treatment agent levels (hydrogen peroxide, lysozyme and polymyxin B sulfate) or the kinetics of killing was followed at a standardized level of the agent (sodium nitrite, hydrochloric acid and lysozyme). The effect of hydrogen peroxide (H_2O_2) on the viability of *M. smegmatis* was determined as described elsewhere (Yu *et al.*, 1998). Cell cultures were grown with or without D-alanine, harvested, washed with PBS-Tween 80 (PBS-TW), resuspended in the same volume of PBS-TW, and treated with 0–30 mM H_2O_2 (Sigma-Aldrich) in 5 mM increments. Cells were incubated for 30 min at 37 °C, washed with MADC-TW, and plated on MADC supplemented with D-alanine. To determine the effects of lysozyme, cells were plated on MADC with 0, 500, 750 and 1000 μg ml^{-1} of hen egg-white lysozyme (Sigma-Aldrich). To determine the susceptibility to the model antimicrobial cationic peptide polymyxin B (sulfate salt, Invitrogen), bacteria were plated on MADC with 0, 16, 32, 64 and 96 μg ml^{-1} of this antimicrobial peptide.

Sodium nitrite ($NaNO_2$) susceptibility was determined as previously described (Firmani & Riley, 2002), with modifications. Cell aliquots (0.5 ml) were adjusted to ~1.0×10^6 c.f.u. ml^{-1} and added to MADC-TW at pH 4.0 or 6.5 adjusted with HCl and 0, 2, 4 or 6 mM $NaNO_2$ (Sigma-Aldrich). Samples were collected at 0, 2, 5 and 24 h post-treatment. For susceptibility to HCl alone, 0.5 ml cell aliquots (adjusted to ~1.0×10^8 c.f.u. ml^{-1}) were combined with MADC-TW at pH 4.0 adjusted with HCl. Samples were taken at 0, 7, 12, 24, 48, 72 and 96 h after treatment. To follow the kinetics of lysozyme, cells were grown in MADC-TW with or without 500 μg lysozyme ml^{-1}. Samples were collected at 0, 3, 6, 12 and 24 h post-treatment and washed with MADC-TW. In all cases, c.f.u. were determined on MADC agar.

MICs were determined by a previously described microdilution method (Chacon *et al.*, 2002). Two compounds were tested: imipenem (compound MK0787, US Pharmacopeia), a β-lactam transpeptidase inhibitor with broad-spectrum anti-microbial activity (Kesado *et al.*, 1980); and vancomycin (Sigma-Aldrich), a glycopeptide that binds to D-alanyl-D-alanine moieties (Perkins & Nieto, 1974). Briefly, cells were grown in MADC-TW to mid-exponential phase (OD_{600} 0.6–1.0), adjusted to ~1.0×10^5 c.f.u. in 0.1 ml, and inoculated into 96-well microplates containing serial twofold dilutions of the corresponding antimicrobial agent. The MIC was defined as the minimal drug concentration that prevented visible bacterial growth at 37 °C after 48 h.

Effects of nutritional supplements on *M. smegmatis* growth. Strains mc^2155 and TAM23 were grown in MADC-TW without and with D-alanine, respectively. Bacteria were washed twice with PBS-TW, resuspended in the original volume of PBS-TW, and plated on 7H9 basal agar without and with supplementation with 0.15% (v/v) glycerol; 50 mM D-alanine; 0.5% (w/v) albumin; 10 mM glucose; albumin, glucose and D-alanine; or glycerol, ADC and D-alanine (MADC+D-alanine).

Statistical and graphical analysis. Statistical analyses were performed using SAS version 9.1.3, 2004 (SAS Institute). Log-transformed c.f.u. were analyzed as a repeated measure design and included the effects for strain and treatment, and their interaction. The repeated measures analyses were carried out using the Mixed procedure. The covariance structure for each analysis was selected based on the Schwarz's Bayesian Criterion. Post-hoc comparisons for the amino acid analysis were carried out using Tukey adjusted P values; for the nutritional supplement analysis, Bonferroni adjusted P values were used. The Cluster procedure was used to determine the average linkage distance (joins clusters with small variances) between strains grouped together. For the H_2O_2, lysozyme and polymyxin B analyses, LD_{50} values were estimated for each strain replicate combination using the Probit procedure. These LD_{50} values were then analyzed as a one-way ANOVA. Contrast and estimate statements were used to determine the statistical significance for line graphs. Bar and line graphs were constructed using DeltaGraph version 5.6.2 (Red Rock Software).

Results

Extracellular growth of *M. smegmatis* strains in broth with and without D-alanine

Extracellular growth of *M. smegmatis* strains was evaluated in broth media. In MADC-TW (with glycerol, L-glutamate and ammonium chloride as carbon and nitrogen sources), mc^2155 started growing immediately with a generation time of 165 ±23 min, reaching a maximum at OD_{600} 3.2 (Supplementary Figure S1a). When D-alanine was added, the mc^2155 generation time was longer (195 ±27 min), reaching an upper limit at OD_{600} 1.7. TAM23 grew with about the same generation time in MADC-TW with (225 ±32 min) and without (210 ±30 min) D-alanine. Both values were greater than those for mc^2155 (Supplementary Figure S1b). However, the yield was higher with D-alanine than in its absence, as OD_{600} values were 2.6 and

1.4, respectively. In addition, growth in the absence of D-alanine was characterized by a lag time of 30 ±4 h that did not occur with D-alanine. Complementation of TAM23 with the integrating vector pTAMU3, which carries the wild-type *alr* gene, restored parental properties. The generation times of TAM23 (pTAMU3) in the presence and absence, of D-alanine were 210 ±30 and 180 ±25 min, respectively. More importantly, this recombinant strain grew in the absence of D-alanine without a lag time (Supplementary Figure S1c), reaching OD_{600} 3.0, similar to the wild-type strain. As expected in the presence of D-alanine, TAM23(pTAMU3) reached a lower OD_{600} value of 2.1. Growth patterns were also determined in nutrient-restricted minimal medium with glycerol (sole carbon source) and ammonium chloride (sole nitrogen source). Similar results were obtained for the parental and complemented strains, but TAM23 grew with longer generation and lag times in the absence of D-alanine (Supplementary Figure S2). A second passage of TAM23 without D-alanine resulted in a growth pattern similar to that of mc^2155, displaying no lag time (Chacon *et al.*, 2002).

Biosynthesis of D-alanine in an *M. smegmatis alr* insertion mutant

The D-alanine-independent phenotype of *M. smegmatis alr* insertion mutants suggests that *M. smegmatis* possesses an alternative pathway of D-alanine biosynthesis (Chacon *et al.*, 2002; Halouska *et al.*, 2007). Alternatively, this micro-organism may be able to synthesize peptidoglycan and other essential components without D-alanine. To distinguish between these two alternative hypotheses, we determined whether the mutant strain could synthesize D-alanine.

Strains mc^2155, mc^2155 treated with the Alr inhibitor DCS, and TAM23 synthesized approximately the same level of total amino acids, as quantified by OPA (Figure 1, inset). This result demonstrates that bacteria do not compensate for the *alr* mutation by making more of all amino acids. However, significant changes in their relative abundance were observed (Figure 1). When compared with untreated mc^2155, mc^2155 with DCS (P = 0.0061) and TAM23 (P = 0.0005) yielded a 14 and 25% decrease in the highly abundant L-glutamate-L-glutamine (L-Glx) pool, respectively. This result is consistent with the decrease in the overall glutamate pool observed in our TAM23 metabolomic analysis (Halouska *et al.*, 2007). Significant increases were observed in the L-alanine (L-Ala) pools for TAM23 (about 4.5-fold, $P < 0.0001$) and mc^2155 with DCS (about 1.7-fold, P = 0.0003) compared to mc^2155. A decreasing trend was detected in the D-alanine (D-Ala) pools upon treatment with DCS or in TAM23. These results further confirm our metabolomic studies, but the current analysis shows that the total increase in the alanine pools is determined by higher levels of the L-enantiomer. Though not significant at the $P < 0.05$ level, similar trends were seen for the total L(D)-aspartate-L(D)-asparagine (L-Aspx/D-Aspx) pools.

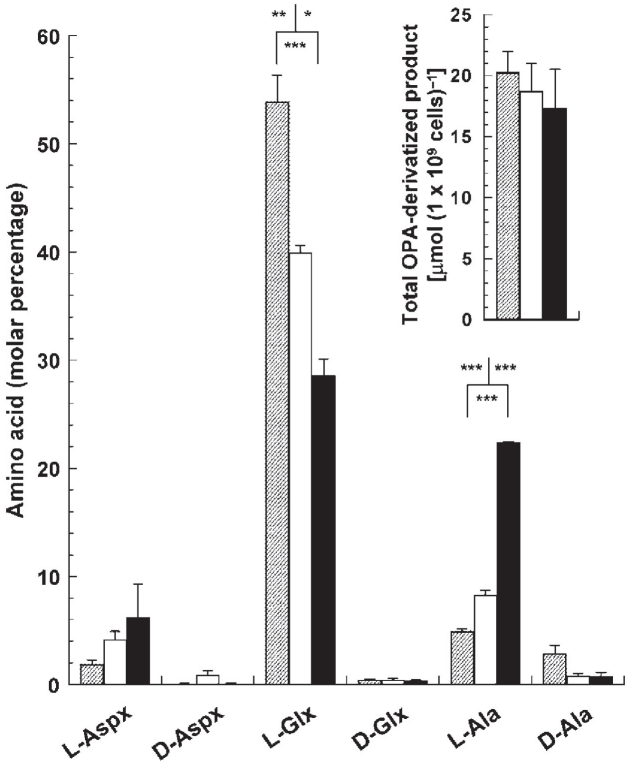

Figure 1. Intracellular *M. smegmatis* steady-state amino acid pools. Standard amino acid nomenclature is used and results are reported as mean ±sem, n = 2–4. Symbols: striped bars, mc^2155 without DCS; white bars, mc^2155 with DCS; black bars, TAM23 without DCS. Inset: the total amino acid pool (total OPA-derivatized product) recovered from 1×10^9 cells. Statistical significance is indicated as follows: *P = 0.01–0.05; **P = 0.001–0.01; ***$P < 0.001$. Pairwise determinations are displayed as follows: the upper left compares mc^2155 without DCS to mc^2155 with DCS, the upper right compares mc^2155 with DCS to TAM23, and the lower middle compares mc^2155 to TAM23. All other comparisons were not significant ($P > 0.05$).

In all cases, mc^2155 with DCS followed the same trend as TAM23, but TAM23 displayed the most extreme values, as expected from the complete inactivation of the *alr* gene, in contrast to the DCS inhibition of Alr enzyme activity. Importantly, the internal D-Ala pools, although approximately 3.5-fold less than those of mc^2155, were above background levels. Thus, inhibition of the Alr enzyme by DCS or impairment of *alr* gene function has a significant influence on alanine metabolism, leading to a decrease in the D-Ala pool and an accumulation of L-Ala. The L-Ala:D-Ala ratio was 1.7:1 for mc^2155, 10:1 for mc^2155 with DCS, and 28:1 for TAM23.

M. smegmatis alr mutants display impaired survival in human macrophages

Since *alr* insertion mutants displayed poor growth in media without D-alanine, we hypothesized that D-alanine limitation would also lead to decreased intracellular survival in the nutrient-limiting macrophage environment. To max-

imize potential differences, macrophage monolayers were infected with bacilli grown in medium without D-alanine. The uptake levels at 2 h post-infection for both parental and mutant strains were similar (Figure 2). As expected, mc^{2}155 displayed moderate survival in macrophages, while TAM23 survived very poorly ($P < 0.0001$). The complemented strain TAM23 (pTAMU3) displayed similar survival levels at 48 h ($P = 0.3867$) and 72 h ($P = 0.2473$) compared with mc^{2}155. This result confirmed that the inactivation of the *alr* gene is responsible for the reduced intracellular survival of TAM23.

In addition, we tested the effects of selection markers on the survival of *M. smegmatis* in phagocytic cells (Figure 2). Strain mc^{2}155 (pYUB178), which carries the kanamycin-resistance marker used to inactivate the *alr* gene, displayed a survival trend similar to that of the parental strain at 48 h ($P = 0.3475$), indicating that this marker does not affect the intracellular survival of *M. smegmatis*. Strain mc^{2}155 (pYUB412), which carries the hygromycin-resistance complementing vector, displayed an increase in intracellular survival at all time points as compared with TAM23 ($P < 0.0001$). At 24 h post-infection, TAM23 (pYUB412) displayed a similar fivefold reduction in intracellular survival as seen for TAM23; however, at 48 and 72 h, TAM23 (pYUB412) displayed an intermediate level of survival when compared with mc^{2}155 and TAM23. Thus, the hygromycin-resistance marker influences intracellular survival at longer times post-infection. Nonetheless, the effect is different from the complete recovery of TAM23 survival conferred, at every time point post-infection, by the hygromycin-resistance integrating vector pTAMU3 carrying the wild-type *alr* gene. In summary, global analysis of survival data indicated that each strain displayed a unique survival curve ($P < 0.0001$). However, cluster analysis grouped mc^{2}155, TAM23 (pTAMU3) and mc^{2}155 (pYUB178) together with a normalized root mean square distance (NRMSD) of 0.27. This cluster was separated from the TAM23 (pYUB412) (NRMSD = 0.73) and TAM23 (NRMSD = 1.43) clusters. Thus, the inactivation of the *alr* gene has a significant effect on the ability of *M. smegmatis* to survive within phagocytic cells ($P < 0.0001$).

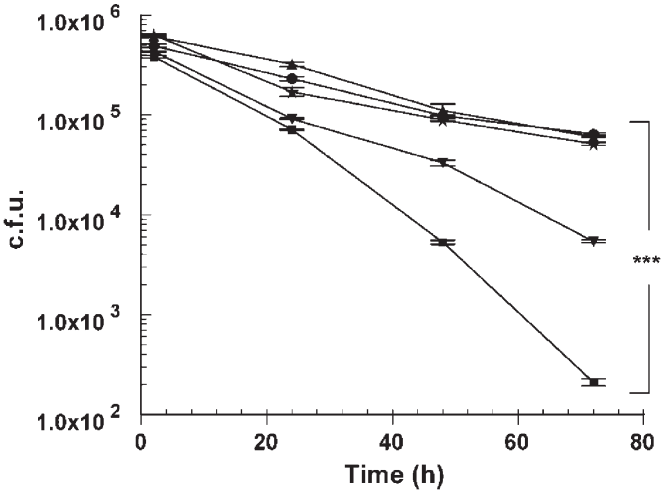

Figure 2. Intracellular survival of *M. smegmatis* in human macrophages. Monolayers were infected at an m.o.i. of 10 bacilli per cell with mc^{2}155 (●), TAM23 (■), TAM23 (pTAMU3) (▲), TAM23 (pYUB412) (▼) or mc^{2}155 (pYUB178) (★) grown in Middlebrook media without D-alanine. Results are reported as mean ±sem, $n = 3$, with statistical significance indicated as ***, $P < 0.001$.

Susceptibility of *M. smegmatis* to bactericidal agents

Experiments were performed to further define the susceptibilities of wild-type and *alr* mutant strains to agents that mediate or enhance intracellular bactericidal action in macrophages. Susceptibilities to reactive intermediates were first assessed on bacteria grown in broth by determining the effects of H_2O_2 and acidified $NaNO_2$. As shown in Supplementary Figure S3(a), no significant differences were observed in the susceptibilities to H_2O_2 ($P = 0.0544$) of mc^{2}155 without D-alanine (LD_{50} 9.73%, v/v), mc^{2}155 with D-alanine (LD_{50} 9.57%, v/v), TAM23 without D-alanine (LD_{50} 13.34%, v/v) and TAM23 with D-alanine (LD_{50} 9.10%, v/v). Likewise, no significant differences were observed between mc^{2}155 and TAM23 treated and not treated with $NaNO_2$ ($P = 0.1198$) over a 24 h period (Supplementary Figure S3b).

Susceptibilities to ROI and RNI within macrophages were also tested using inhibitors (Figure 3). Treatment of monolayers with the H_2O_2-detoxifying enzyme catalase did not result in significant differences for mc^{2}155 ($P = 0.1544$) or TAM23 ($P = 0.6225$) as compared with the corresponding untreated controls, while major differences were observed between strains ($P = 0.0002$). Scavenging of superoxide anion with SOD led to significant differences for TAM23 ($P < 0.0001$) as compared with the untreated control, while a minor effect was observed for mc^{2}155 ($P = 0.1316$ comparing all time points, $P = 0.0158$ at 72 h). However, no significant differences were observed between strains upon SOD treatment ($P = 0.6116$). Inhibition of NO synthase with NM-L-Arg resulted in a significant increase in the survival of mc^{2}155 ($P = 0.0238$) and TAM23 ($P < 0.0001$) as compared with the corresponding untreated controls, with no difference between the strains ($P = 0.9171$), indicating that production of reactive intermediates plays an important role in differential killing.

Upon phagolysosomal fusion, mycobacteria may be exposed to acid pH, lysosomal enzymes and cationic peptides. Exposure of mc^{2}155 and TAM23 to pH 4.0 (without $NaNO_2$) for 24 h did not result in significant differences (Supplementary Figure S3b). Furthermore, extending the pH treatment to 96 h yielded the same outcome (data not shown). The potential effects of the *alr* mutation on the peptidoglycan structure may result in altered susceptibil-

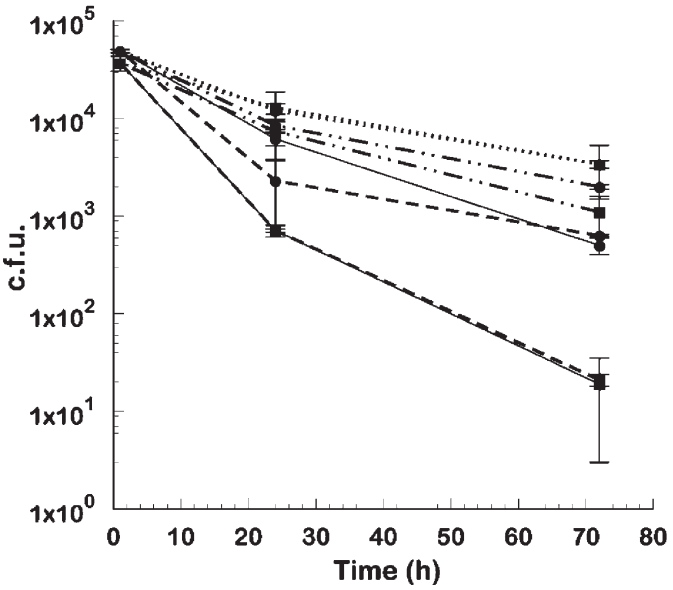

Figure 3. Effect of reactive intermediate inhibitors on the intracellular survival of *M. smegmatis* in human macrophages. Monolayers were infected at an m.o.i. of 1 bacillus per cell with either mc²155 (●) or TAM23 (■) grown in Middlebrook medium without D-alanine. After 30 min, cells were split and either not treated (unbroken lines) or treated with SOD (dashed–dotted lines), catalase (dashed lines) or NM-L-Arg (nearly overlapping; dotted lines). Results are reported as mean ±sem, $n = 2$.

ity to lysozyme and polymyxin B. Treatment with 500 or 750 μg lysozyme ml^{-1} decreased survival of mc²155 (~95%) and TAM23 (~85%) ($P = 0.0344$), with a more pronounced effect (~98% for both strains) at 1000 μg lysozyme ml^{-1} (Figure 4a). Significant differences were observed in the susceptibilities to lysozyme of mc²155 (LD_{50} 47 μg ml^{-1}) and TAM23 (LD_{50} 205 μg ml^{-1}). To confirm these results, the bactericidal action of 500 μg lysozyme ml^{-1} was also followed in MADC-TW 24 h post-treatment (Figure 4b). The survival dropped 500- (TAM23) to 4500-fold (mc²155), indicating that lysozyme has a greater bactericidal action on the parental strain ($P < 0.0001$), although the survival curves follow similar trends. Thus, the changes in TAM23 appear to make its peptidoglycan less susceptible to lysozyme. In contrast, TAM23 was slightly more susceptible to polymyxin B than mc²155 (Figure 4c), although this trend did not reach statistical significance ($P = 0.0660$).

We have reported the hypersusceptibility of TAM23 to DCS with an MIC of 2.56 μg ml^{-1}, as compared with 75 μg ml^{-1} for mc²155, while the susceptibilities to amikacin, ethambutol and ribafutin remain at parental levels (Chacon *et al.*, 2002). In this study, the MICs against two additional drugs related to peptidoglycan structure were determined. Mutant TAM23 was significantly more susceptible to imipenem (MIC 0.625 versus 20 μg ml^{-1}) and vancomycin (MIC 1.56 versus 12.5 μg ml^{-1}). These results are consistent with peptidoglycan alterations as a result of the downshift in the D-alanine pool.

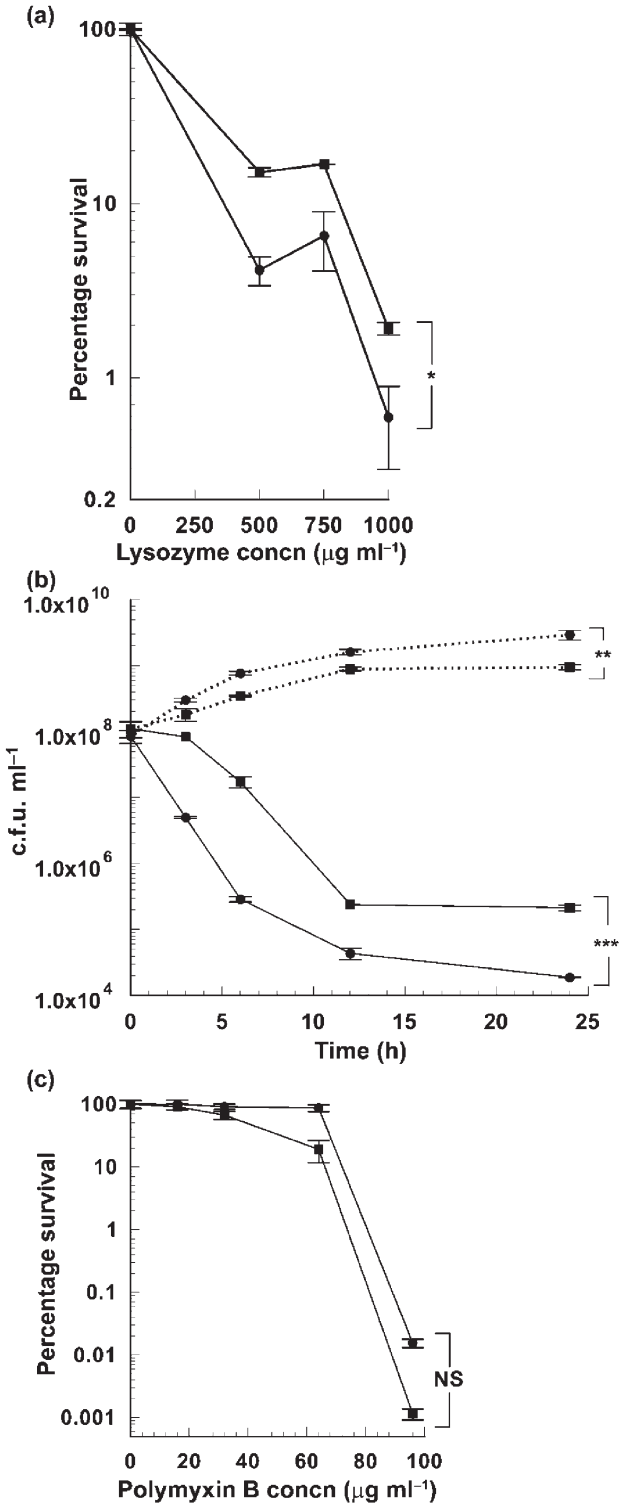

Figure 4. Susceptibility of *M. smegmatis* to lysozyme and polymyxin B. For lysozyme assays, cells were plated at various concentrations (a) or treated with 500 μg ml^{-1} for different time periods (b). For polymyxin B assays (c), cells were plated at various concentrations. Symbols and line patterns are: ●, mc²155; ■, TAM23; ——, mc²155 and TAM23 with lysozyme; ·····, mc²155 and TAM23 without lysozyme. Results are reported as mean ±sem, $n = 3$, with statistical significance displayed as NS, not significant ($P > 0.05$); *, $P = 0.01$–0.05; **, $P = 0.001$–0.01; ***, $P < 0.001$.

Table 1. *M. smegmatis* cell viability on Middlebrook 7H9 basal agar with and without supplementation

Supplementation	Mean log(c.f.u.)*		*P* value†
	mc²155	TAM23	
None	3.67	0.60	<0.0001
Glycerol (0.15 %, v/v)	4.91	4.29	<0.0001
D-Alanine (50 mM)	4.92	3.74	<0.0001
Albumin (0.5 %, w/v)	4.80	4.57	0.2435
Glucose (10 mM)	4.96	4.79	0.6492
Albumin + glucose + D-alanine	5.77	5.71	0.9999
Glycerol + ADC + D-alanine (MADC + D-alanine)	5.78	5.76	1.0000

*Strains mc²155 (without 50 mM D-alanine) and TAM23 (with 50 mM D-alanine) were grown at 37 °C in MADC-TW to OD_{600} ~1.0, washed twice in PBS-TW, and plated on solid media (SEM = 0.056, n = 3).

† Bonferroni adjustment (significant at $P < 0.007$).

Effect of nutritional supplements on *M. smegmatis* growth

Macrophages restrict nutrients to phagocytosed bacteria, an effect that has been termed a nutriprive mechanism (Appelberg, 2006). This restriction may include essential ions (Wagner *et al.*, 2005), purines, pyrimidines, amino acids and vitamins. To test the effects of nutritional supplementation, parental and mutant cells were plated on various solid media. As shown in Table 1, significant differences were found between mc²155 and TAM23 on 7H9 basal medium and 7H9 supplemented with glycerol or D-alanine. No significant differences were observed for the remaining supplementations. Comparing the growth performance of each strain individually in the various media, both strains performed best and equally well on basal agar with albumin, glucose and D-alanine; and MADC plus D-alanine (P = 1.00). Intermediate growth was observed for both strains with the addition of glycerol, D-alanine, albumin or glucose to the basal medium. Basal agar yielded the poorest growth for both strains with a more prominent effect on TAM23. These observations confirmed that *alr* insertional mutants grow better with D-alanine, but are not dependent on this nutrient.

To test for the effect of D-alanine in macrophages, parental and mutant strains were grown in MADC with or without D-alanine prior to the infection of monolayers cultured without D-alanine. The uptake of mc²155 and TAM23 by monolayers of adherent human macrophages 1 h post-infection was similar, regardless of D-alanine supplementation (Figure 5a). As expected, mc²155 grown in broth with or without D-alanine displayed similar survival in macrophages (P = 0.309), but TAM23 survived better when grown in broth with D-alanine (P < 0.0001). As described above (Figure 2), mc²155 survived better than TAM23 (P < 0.0001, for all pairwise comparisons). A similar effect was obtained when TAM23 was grown without D-alanine and macrophage monolayers were incubated with or without D-alanine during the course of the infection (Figure 5b). In the absence of D-alanine, TAM23 was less able to survive as compared with conditions in which monolayers were supplemented with D-alanine (P = 0.0166). In summary, the difference in the viability of both strains in basal media reflected the intracellular survival data well.

Discussion

The goal of this study was to analyze the physiological consequences of the insertional inactivation of the *alr* gene in *M. smegmatis* in the context of both extracellular and intracellular survival. We have shown that *alr* insertion mutants are not dependent on D-alanine for growth, and display changes in colony morphology, metabolomic profiling and hypersusceptibility to DCS (Chacon *et al.*, 2002; Halouska *et al.*, 2007). We hypothesized that *alr* mutants synthesize D-alanine by an alternative pathway. In contrast, another study indicates that *M. smegmatis alr* deletion mutants are auxotrophic for D-alanine (Milligan *et al.*, 2007). However, there are significant differences in the experimental design that led to mutant isolation and characterization in the two studies, including a two-step procedure and shorter follow-up incubation in the study of the *alr* deletion mutants. Furthermore, metabolomic analysis of the TAM23 *alr* insertion mutant indicates metabolic changes more readily compatible with the activation of an alternative pathway of D-alanine biosynthesis (Halouska *et al.*, 2007). Thus, the differences between these two studies may reflect changes in the experimental conditions.

In this study, the analysis of the amino acid pools indicates that the TAM23 *alr* mutant exhibits lower, but above background, levels of D-alanine and L-glutamate and accumulation of L-alanine, as compared with the parental strain. These results are consistent with our hypothesis and previous studies (Chacon *et al.*, 2002; Halouska *et al.*, 2007). As expected, the effect of *alr* insertional inactivation on these amino acid pools is more pronounced than the effect of DCS, an Alr inhibitor. Thus, insertional inactivation of the *alr* gene in TAM23 results in markedly reduced availability of D-alanine (indicated by a low D-alanine pool in the mutant), possibly leading to decreased incorporation of this amino acid into peptidoglycan and glycolipids. Structural analyses will be conducted to test this hypothesis.

The insertional inactivation of the *M. smegmatis alr* gene resulted in 80% reduction in the intracellular survival of the mutant strain in monocyte-derived human macrophages at 24 h post-infection (Figure 2). This effect is considerably more pronounced than the 20–50% reduction observed for other *M. smegmatis* mutants under similar conditions (Lagier *et al.*, 1998). The conservation of basic

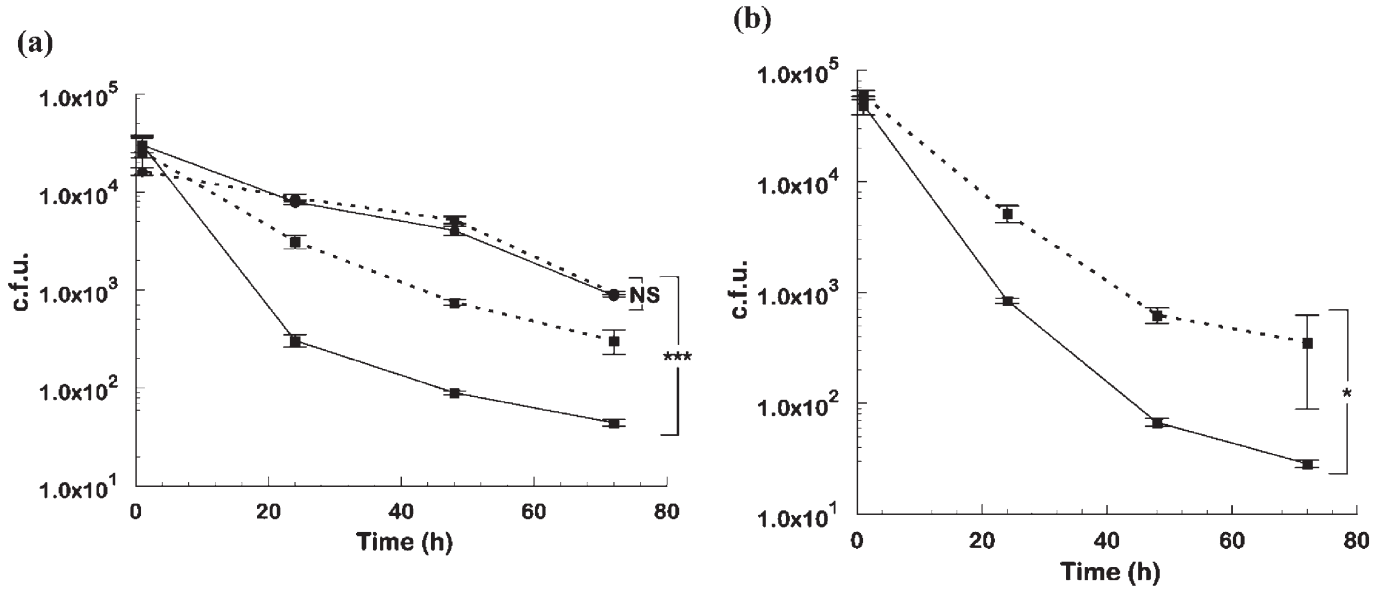

Figure 5. Effect of D-alanine on the intracellular survival of *M. smegmatis* in human macrophages. Monolayers were infected at an m.o.i. of 1 bacillus per cell with either mc^{2}155 (●) or TAM23 (■) grown in Middlebrook media. (a) Bacilli were grown in broth with (dashed lines) or without (unbroken lines) D-alanine. (b) Bacilli were grown in broth without D-alanine and macrophage monolayers were incubated with (dashed lines) or without (unbroken lines) D-alanine. Results are reported as mean ±sem, $n = 3$, with statistical significance displayed as NS, not significant ($P > 0.05$); *, $P = 0.01$–0.05; ***, $P < 0.001$.

metabolic pathways in mycobacteria plus the effect of *alr* mutation on extracellular and intracellular survival of *M. smegmatis* would predict a considerable degree of attenuation for *alr* mutant strains of pathogenic mycobacterial species, provided viable mutants could be obtained.

Pathogenic mycobacteria have greater ability to evade bactericidal mechanisms such as phagosome acidification, while *M. smegmatis* fails to prevent this process (Sturgill-Koszycki *et al.*, 1994). However, intracellular bacilli of both *M. smegmatis* and pathogenic mycobacterial species are exposed to killing by reactive intermediates (Anes *et al.*, 2006; Jordao *et al.*, 2008; Nathan & Shiloh, 2000; Shiloh & Nathan, 2000), though controversial results have been obtained (Ehrt *et al.*, 1997). In our case, extracellular treatment with H_2O_2, $NaNO_2$ and acidic pH had a bactericidal action on *M. smegmatis*, but did not reveal any major survival differences between the strains. H_2O_2 scavenging by catalase treatment of infected monolayers had no effect on the viability of any of the strains as compared with untreated controls. However, scavenging of superoxide anion by SOD or inhibition of NO synthase by the specific inhibitor NM-L-Arg resulted in decreased bactericidal activities in macrophage monolayers. This effect was more pronounced in the *alr* mutant. This suggests that in our experimental conditions, reactive intermediates have an important role in *M. smegmatis* killing by human macrophages, as indicated in Figure 3. Since RNI inhibition may not affect superoxide anion production by the phagocyte oxidase pathway, it is possible that SOD scavenging of superoxide only limits its conversion to peroxynitrite, a highly bactericidal product formed from superoxide and NO. In contrast, other studies have failed to detect reactive intermediates and conclude that these molecules do not have a major role in *M. smegmatis* killing by human monocytes (Jordao *et al.*, 2008). However, these molecules are difficult to detect, given their short life and low concentrations in the macrophages (Forman & Torres, 2001; Valko *et al.*, 2007).

The results presented herein suggest that the use of inhibitors is a more reliable indicator of the involvement of reactive intermediates than the failure to detect relatively low levels of these molecules by the Griess reaction. This situation is particularly critical in our study, since the human monocytes were not activated with γ-interferon. Nonetheless, infection with *M. smegmatis* (Ehrt *et al.*, 1997), maturation in culture (Martin & Edwards, 1993), and specific experimental conditions may sufficiently increase reactive intermediate levels, especially for RNI, to have bactericidal action on this micro-organism, as suggested by our inhibition studies. Activation of the arginase pathway reduces arginine availability for the NO synthase reaction in murine macrophages (Martin *et al.*, 2006). This leads to an increase in intracellular levels of L-glutamate. A similar effect might be possible upon NO synthase inhibition by NM-L-Arg. Since L-glutamate seems to be involved in the alternative pathway of D-alanine biosynthesis in *M. smegmatis alr* mutants, this effect could increase the survival of the mutant strain to wild-type levels in the absence of D-alanine (Figure 5). The differential microbicidal effect on TAM23 in untreated macrophages may also reflect the increased permeability of the mutant strain to reactive intermediates. In either case, preloading of TAM23 cells with D-alanine or its addition to monolayers had a partially com-

pensatory effect (Figure 5). Thus, the effects of nutrient deprivation in phagocytic cells combined with killing mediated by reactive intermediates determined the differential survival of the strains. The decreased pool of D-alanine might also result in a further reduction in the GPL content of TAM23, affecting both colony morphology and susceptibility to macrophage killing (Kocincova *et al.*, 2009).

Additional bactericidal mechanisms operate in lysosomal compartments. In this context, we observed a slight decrease rather than an increased susceptibility to lysozyme, an enzyme that hydrolyses the longitudinal peptidoglycan strands. This effect may be due to subtle changes in the peptidoglycan layer. However, a weakened cell wall structure was suggested by an increased susceptibility to DCS (Chacon *et al.*, 2002), imipenem, polymyxin B and vancomycin. These findings are consistent with peptidoglycan alterations as a result of the downshift in the D-alanine pool.

Auxotrophic mutations decrease the intracellular survival of pathogenic micro-organisms, including mycobacteria (Appelberg, 2006; Crawford *et al.*, 1996; Guleria *et al.*, 1996; Mintz *et al.*, 1988). This effect may be related to the reduced availability of nutrients in the phagosome, in contrast to the relatively rich cytoplasmic compartment. In the case of mycobacteria, the vacuole is deficient in purines, amino acids, vitamins and other nutrients (Brown *et al.*, 2005; Hondalus *et al.*, 2000; Jackson *et al.*, 1999; Pavelka *et al.*, 2003). Our studies suggest a combined effect of nutrient deficiency and reactive intermediate molecules on the intracellular survival of mycobacteria inside nutrient-deficient vacuoles. Inhibition of ROI and/or RNI may also result in metabolic changes in the phagosome that could alter the attenuation of auxotrophic mutant strains. Mutants absolutely dependent on D-alanine for growth would likely autolyse *in vivo*, providing potentially safer auxotrophic vaccines. If the *alr* gene is essential in *M. tuberculosis*, as suggested by global genomic analysis (Sassetti *et al.*, 2003), it may still be possible to engineer viable mutants by introducing the *M. smegmatis* alternative pathway genes under the control of a regulated promoter. Thus, these studies are significant for the development of novel live attenuated vaccines to control mycobacterial diseases.

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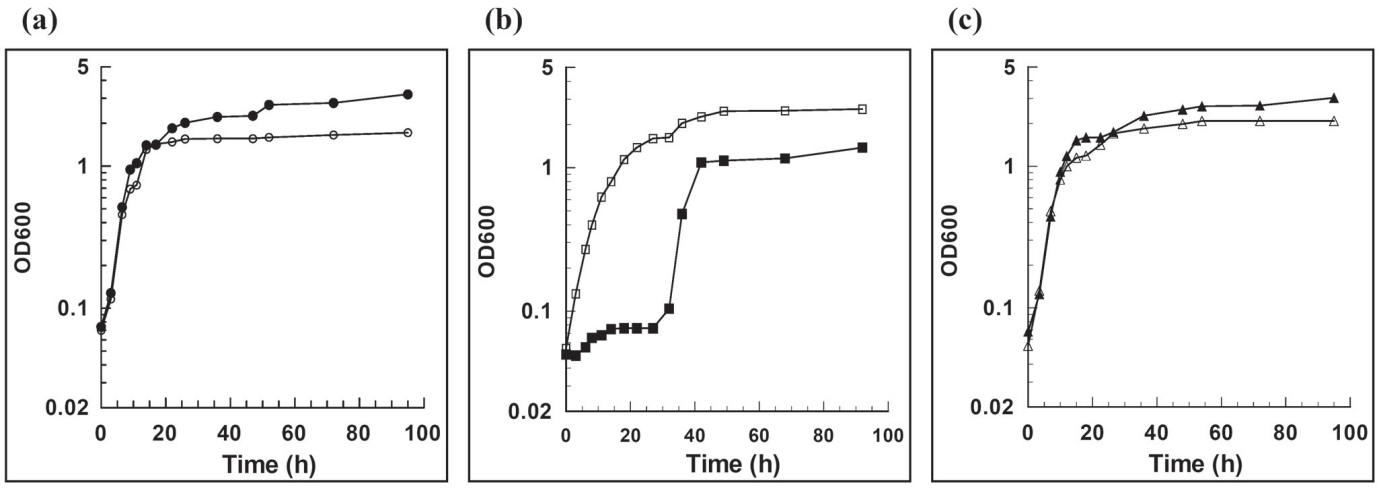
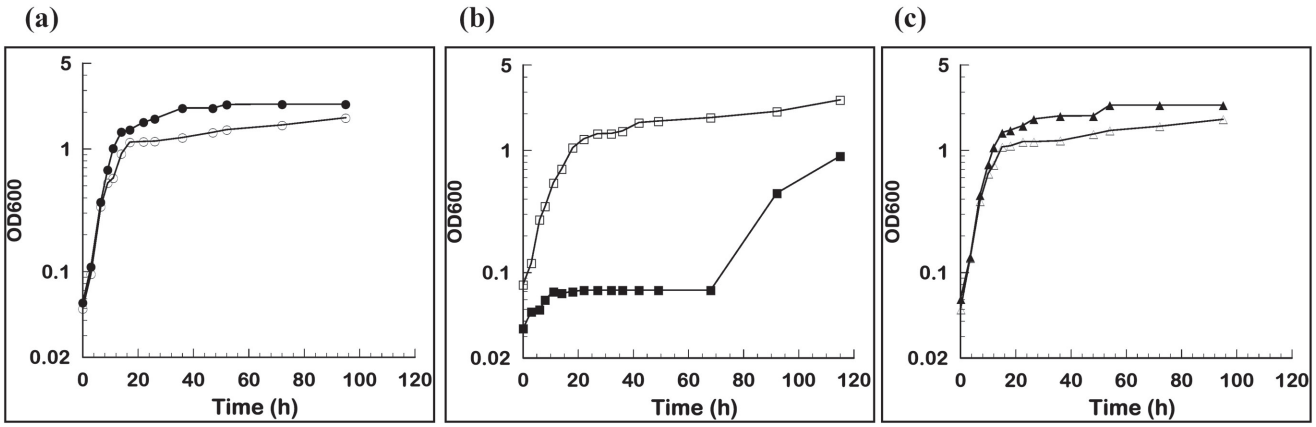
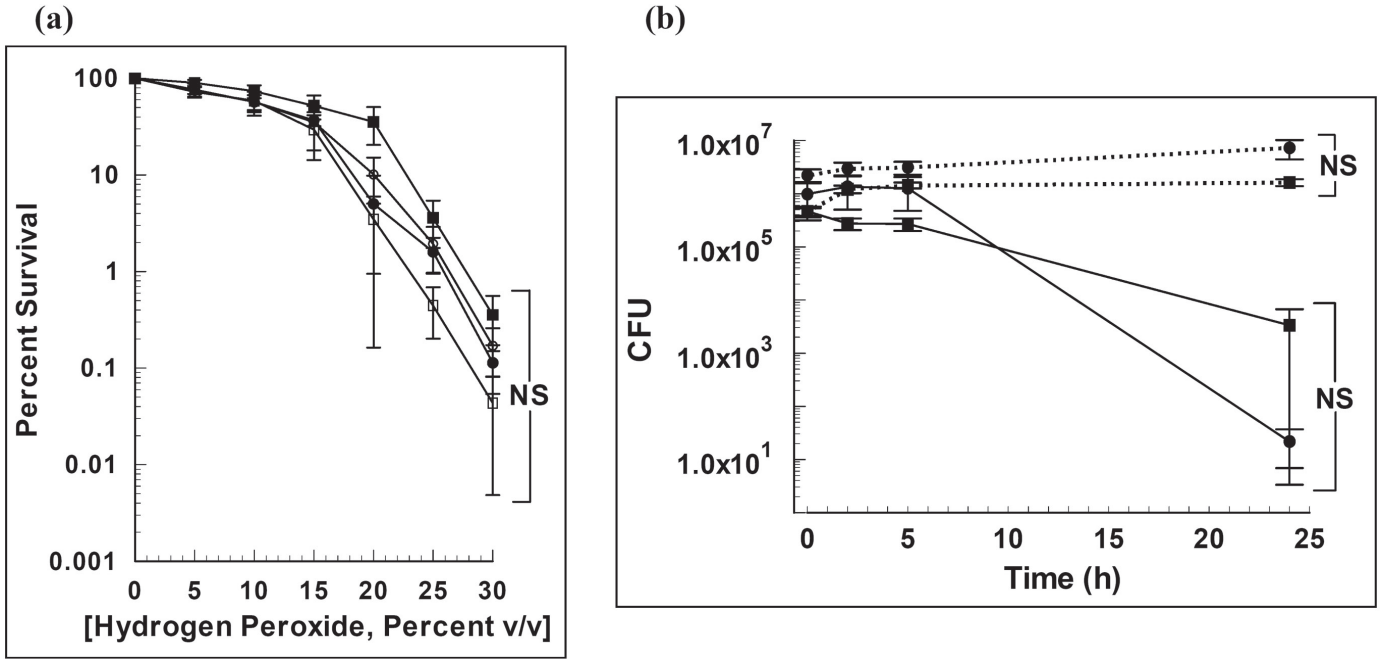
Supplementary Figures



Supplementary Figure S1. Growth curves of *M. smegmatis* parental, *alr* mutant and complemented strains in Middlebrook medium. Strains mc^{2}155 (a), TAM23 (b), and TAM23 (pTAMU3) (c) were grown in MADC-TW with D-alanine. Cells were harvested, washed twice with PBS-TW, and inoculated 1:50 into MADC-TW. Growth (OD_{600}) was recorded for 95 h. Symbols indicate growth with (open symbols) or without (filled symbols) D-alanine supplementation.



Supplementary Figure S2. Growth curves of *M. smegmatis* parental, *alr* mutant, and complemented strains in minimal medium. Strains mc^{2}155 (a), TAM23 (b), and TAM23 (pTAMU3) (c) were grown in MADC-TW with D-alanine. Cells were harvested, washed twice with PBS-TW, and inoculated 1:50 into minimal medium. Growth (OD_{600}) was recorded for 95 h for all strains except TAM23 which was grown for 115 h. Symbols indicate growth with (open symbols) or without (filled symbols) D-alanine supplementation.



Supplementary Figure S3. Susceptibility of *M. smegmatis* to reactive-intermediate-generating compounds. For H_2O_2 assays (a), cells were treated with increasing concentrations for 30 min and plated. For $NaNO_2$ assays (b), cells were treated at pH 4.0 with or without 2 mM $NaNO_2$ as these conditions yielded significant overnight killing. Symbols and line patterns are ●, mc^{2}155 without D-alanine; ■, TAM23 without D-alanine; ○, mc^{2}155 with D-alanine; □, TAM23 with D-alanine; unbroken lines, mc^{2}155 and TAM23 with $NaNO_2$; and dashed lines, mc$^2_{155}$ and TAM23 without $NaNO_2$. Results are reported as means ±SEM, n = 3, with statistical significance displayed as NS, not significant ($P > 0.05$).