JBC RESEARCH ARTICLE

Glutamate decarboxylase confers acid tolerance and enhances survival of mycobacteria within macrophages

Received for publication, November 12, 2024, and in revised form, February 3, 2025 Published, Papers in Press, February 21, 2025,
https://doi.org/10.1016/j.jbc.2025.108338

Rupal Rai[1], **Bijina J. Mathew**[1], **Rashmi Chourasia**[2], **Anirudh K. Singh**[3], **and Shivendra K. Chaurasiya**[1,*]

From the [1]*Molecular Signalling Lab, Department of Biological Sciences and Engineering, Maulana Azad National Institute of Technology Bhopal, Bhopal, Madhya Pradesh, India;* [2]*Department of Basic Science, IES College of Technology, Bhopal, Madhya Pradesh, India;* [3]*School of Sciences, SAM Global University, Raisen, Madhya Pradesh, India*

Reviewed by members of the JBC Editorial Board. Edited by Ursula Jakob

Host-induced metabolic adaptations are crucial for *Mycobacterium tuberculosis* (Mtb) survival and drug resistance. Mtb's persistence in the acidic environments of phagosomes and phagolysosomes suggests its initial metabolic adjustments respond to acidic stress. Glutamate decarboxylase (Gad) enzyme, converts glutamate to GABA while consuming a proton, helping regulate intracellular pH in bacteria. However, the role of Gad in mycobacteria has been unexplored. In this study, we investigated the function of Gad in Mtb and *Mycobacterium smegmatis* (MS), which are encoded by *Rv3432c* (*gadB*) and *MSMEG_1574* (*gadA*), an ortholog of *gadB*, respectively. We observed upregulation of *gad* in both Mtb and MS under acidic stress and during infection within macrophages. Additionally, the expression of genes involved in glutamate metabolism and the GABA shunt, such as glutamine synthetase (*glnA1*), glutamate dehydrogenase (*gdh*), glutamate synthase (*gltD*/*B*), GABA-aminotransferase (*gab-T*), succinic semialdehyde dehydrogenase (*gabD1*/*gabD2*), α-ketoglutarate dehydrogenase (*kdh*), and 2-oxoglutarate dehydrogenase (*sucA*), were responsive to acidic conditions, reflecting a metabolic shift. Similar gene expression patterns were observed during macrophage infection. These findings suggest that Gad plays a role in mycobacterial acid stress response. To further elucidate this, we generated an MS *gadA* knockout strain (MSΔ*gadA*) using allelic exchange. MSΔ*gadA* exhibited reduced survival at pH 3.0, a phenotype rescued by gene complementation. MSΔ*gadA* also showed decreased survival within macrophages. Additionally, *Mycobacterium bovis* BCG, which lacks native Gad expression, demonstrated enhanced intracellular survival when overexpressing Mtb *gadB*. These results suggest that Gad confers acid tolerance and promotes intracellular survival in mycobacteria, highlighting its potential role in host adaptation.

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (Mtb) remains the second leading cause of death from a single infectious agent worldwide https://iris.who.int/bitstream/handle/10665/373828/9789240083851-eng.pdf?sequence=1. The primary challenges hindering the global eradication of this otherwise curable disease include the prolonged treatment regimen, which spans 1 to 6 months leading to non-compliance, and the alarming rise in multidrug-resistant (MDR), extensively drug-resistant (XDR), and totally drug-resistant (TDR) strains of TB https://tbcindia.mohfw.gov.in/wp-content/uploads/2023/05/5646719104TB_AR_2023_04-04-2023_LRP_final.pdf. Thus, the world is facing an urgent need for new approaches and drugs to combat TB, which in turn requires a greater understanding of the biochemical and physiological processes that enable Mtb to survive the stressful environment within macrophages and acquire drug tolerance/resistance (1–3). The interaction between Mtb and its host/environment can result in multiple outcomes. It is of utmost importance to understand each point of interaction with the host. In the pre-antibiotic era, Mtb faced natural selection pressures, primarily the host's immune response, as it became internalized by macrophages. While in the post-antibiotic era, Mtb developed drug resistance, mainly by acquiring mutations in drug targets (4, 5). However, before exposure to therapeutic drugs, the pathogen must first survive from the protective responses of macrophages (6, 7). Mycobacteria exhibit remarkable ability to adapt and manipulate host cells, enabling their survival (8). It is believed that pathogenic Mtb strains have evolved from environmental saprophytic mycobacteria under selection pressure in an ever-changing environment (9). Even one of the most pathogenic clades of Mtb—the Beijing clade—is known to arise due to physiological and molecular changes resulting from selective pressure, leading to increased hypervirulence (5).

As soon as bacilli enter into the host, they are engulfed by macrophages where they get exposed to oxidative (10) and acidic stress (11). These components are integral to the immune defense, with acidification signaling the onset of macrophage activity and coordinating with other immune responses (12). The ability of Mtb to adapt and cope with these stresses is crucial for the establishment of infection and the progression of the disease (13). Mtb faces acidic stress in phagosomes within macrophages stimulated with IFN-γ and/or at the centers of caseating granulomas. Being a part of the host defense, phagosome acidification is an important environmental condition. Once bacilli are engulfed, the phagosome pH drops to 6.0 (14). Several known factors contribute to the

* For correspondence: Shivendra K. Chaurasiya, shivendrachaurasiya@gmail.com.

acidification of phagosomes, including the vacuolar proton-ATPase, the Na^+/H^+ exchanger, and proton-conductive pathways (15, 16). Consequently, when phagosomes fuse with lysosomes, the intracellular pH decreases to approximately 4.5 (17). Mtb is known to inhibit this fusion, thereby hindering phagosome maturation and acidification. Nevertheless, various studies demonstrate that Mtb can survive and replicate within the acidified environments of phagosomes and phagolysosomes, where the pH ranges from 6.5 to 4.5 (18–20). Mtb experiences gradual acidification instead of a sudden and lethal drop in pH within macrophages. It has been proposed that Mtb may develop induced acid tolerance by adjusting its metabolism in response to acidic conditions (21, 22). However, the underlying mechanisms remain poorly characterized and require further investigation in Mtb. While acid tolerance and extreme acid resistance mechanisms are well-documented in various other Gram-positive and Gram-negative bacteria (23–25), these are not yet fully deciphered in Mtb. However, evidence of such adaptive acid tolerance mechanisms in Mtb remains lacking, representing a critical area for future research in Mtb pathogenesis.

Protein kinase G (PknG) is a recognized virulence factor that enhances mycobacterial survival within macrophages and contributes to antibiotic resistance (26–28). We recently established that PknG regulates acid tolerance in mycobacteria (29). PknG was also shown to regulate glutamate metabolism (30). Glutamate metabolism has also been linked with other stress responses in Mtb (31, 32). The glutamate decarboxylase (Gad)-mediated conversion of glutamate to GABA is a well-known acid resistance mechanism in bacteria (33, 34). While the Mtb genome encodes *gad*, its role in mycobacteria is not well documented. Our previous work demonstrated that Gad is expressed in both Mtb and *Mycobacterium smegmatis* (MS) (35). In this study, we further demonstrated that Gad mediates acid tolerance in mycobacteria leading to enhanced intracellular survival within macrophages.

Results

Gene expression analysis reveals increased gad *expression and modulation of glutamate metabolism-related genes in mycobacteria during acidic stress and macrophage infection*

The gene expression pattern under different conditions provides valuable insights into the function of the gene. To investigate whether Gad is associated with the acidic stress response in mycobacteria, we analyzed the expression of *gad* and other selected genes after exposure to acidic pH and during macrophage infection. These genes were chosen based on their established roles in glutamate metabolism and their potential involvement in acid stress adaptation. The expression patterns of these genes may highlight interconnected pathways contributing to pH homeostasis and intracellular adaptation through Gad-mediated glutamate metabolism. Total RNA was isolated from mycobacterial cultures grown in acidic medium (pH 5.0 ± 0.2) for 4 h, whereas mycobacterial cultures grown in normal medium served as control for the gene expression analysis by qRT-PCR. It was observed that expression of *gad*, GABA-aminotransferase (*gab-T*), glutamate dehydrogenase (*gdh*) and fatty acyl CoA synthetase (*fadD9*) are upregulated, whereas the expression of glutamate synthase (*gltD*), 2-oxoglutarate dehydrogenase (*sucA*) are downregulated following exposure of MS to acidic medium (Fig. 1*A*). No significant change in expression of glutamine synthetase (*glnA*), glutamate synthase (*gltD6458*), succinic semialdehyde dehydrogenase (*gabD2*), isocitratelyase (*aceA*) was observed in MS following exposure to acidic medium (Fig. 1*A*). Upregulation of *gad*, *gaba-at* along with other genes suggests that these changes may be a protective response in the stressed mycobacteria. Mycobacterial pathogens are also exposed to acidic stress during infection in macrophages. To investigate if similar changes are exhibited by mycobacteria during infection of the macrophages, we analyzed the expression of these genes in MS during infection of macrophages. Our results revealed a comparable gene expression profile with upregulation of *gad, gaba-at, glnA, gabD2, fadD9, aceA*, and downregulation of *gdh, gltD, gltD6458, and sucA* compared to control MS strain during macrophage infection (Fig. 1*B*). Taken together, these results indicate a possible role of *gad* in bacterial survival within the acidic environment found in the macrophages.

We next sought to determine whether Mtb also exhibits a similar response during exposure to acidic stress and infection of macrophages. Gene expression analysis of Mtb cells exposed to acidic stress showed similar pattern of gene expression. The expression of *gad*, *gab-T*, *gltD, gltB*, *fadD9* and *icl1* were upregulated whereas expression of *glnA1*, *gdh*, succinic semialdehyde dehydrogenase (*gabD1*) were downregulated in Mtb cells following exposure to acidic medium (Fig. 1*C*). The expression of *gabD2 and* α-keto-glutarate dehydrogenase (*kdh*) showed no changes in Mtb after exposure to acidic medium (Fig. 1*C*). The upregulation of *gad* following acidic stress suggests its role in Mtb's response to acidic environment such as those observed within macrophages. To further confirm *gad* expression in Mtb during infection, gene expression analysis was performed in these bacteria during infection of macrophages, which showed increased expression of *gad*, *gdh*, *gltD*, *gltB*, and *glnA1* (Fig. 1*D*).

Overall, the expression data of the selected genes clearly demonstrate alterations in glutamate metabolism in mycobacteria during acidic stress and infection within host cells.

Ectopic overexpression of gad *supports acidic tolerance in mycobacteria*

The upregulation of *gad* in mycobacteria in the acidic medium suggests its role in acid tolerance in mycobacteria. To further investigate whether the upregulation of *gad* during acidic stress contributes to the ability of mycobacteria to survive under acidic environment, recombinant MS overexpressing *gadA* or *gadB* were created. For this purpose, *gadB* from Mtb and *gadA* from MS were cloned separately into an integrative vector, pMV361, followed by verification of the

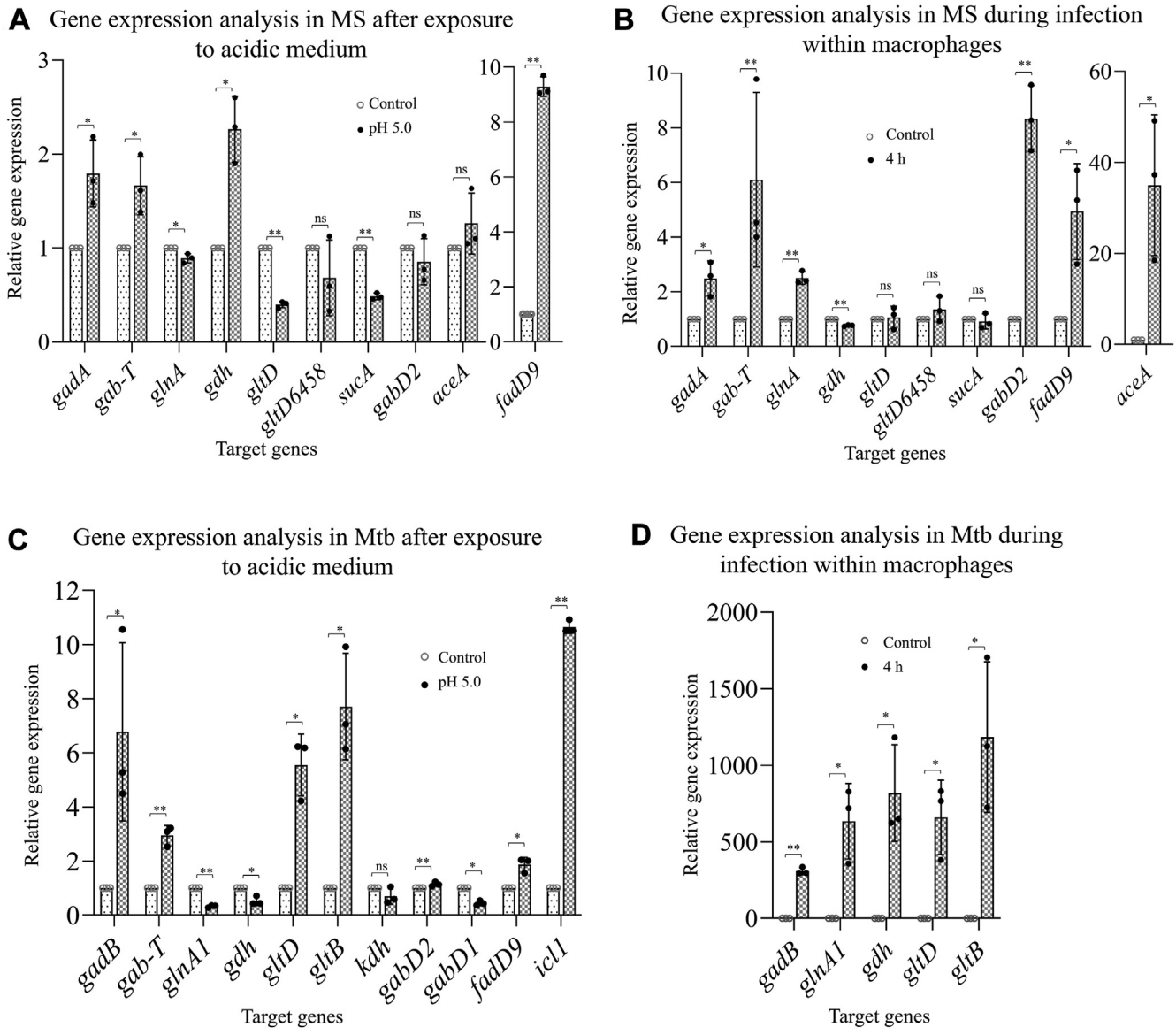

Figure 1. Expression of *gad* and glutamate metabolism related genes in MS and Mtb in acidic conditions and during infection in macrophages. The relative expression of genes was measured using reverse transcriptase qRT-PCR using SYBR *Green* I chemistry. Relative gene expression was determined using the comparative $2^{-\Delta\Delta Ct}$ method. *A*, effect of acid stress, on gene expression in MS cells grown for 4 h in LBGT medium, pH 5.0 ± 0.2. The relative expression of the target genes in acidic medium was compared with MS cells grown in LBGT medium, pH 7.0 ± 0.2. *16SrRNA* was used as the internal reference gene for normalization of the expression data. *B*, expression of the target genes in MS cells during infection in J774.A.1 cells for 4 h at MOI 10:1. A portion of MS cells used for the infection were taken as control, *sigA* was used as the internal reference gene to normalize the expression data. *C*, the relative expression of target genes in Mtb cells grown in MB7H9 medium, pH 5.0 ± 0.2. Cells grown in MB7H9, pH 7.0 ± 0.2 were used as control and *16SrRNA* was used as the internal reference for normalizing the expression data. *D*, the relative expression of target genes in Mtb cells during infection in THP1 cells for 4 h at MOI 10:1. A portion of Mtb cells used for the infection served as control and *16SrRNA* was used as the internal reference to normalize the expression data. Each experiment was done at three independent occasions in triplicates and the data represent the mean ± SD. (*$p < 0.05$, **$p < 0.005$, ns $p > 0.0.5$; one-tailed Student's *t* test using GraphPad Prism software).

recombinant clones by restriction enzyme digestion (Fig. S1). The pMV361 is a shuttle vector used for mycobacterial studies enabling efficient expression of genes of interest under the control of a constitutive *hsp*60 promoter. The recombinant plasmids were subsequently electroporated into MS to yield kanR MS::*gadA* and MS::*gadB* strains. The presence of recombinant pMV361:*gadA* and pMV361:*gadB* integrated in the respective strains, MS::*gadA* and MS::*gadB*, was confirmed by colony PCR using specific primer pairs and genomic DNA as template. The results showed the presence of PCR amplicons with the DNA from only the recombinant strains whereas the empty vector control strain did not yield any PCR amplification product (Fig. 2*A*). Ectopic overexpression of *gad* in the recombinant strains was then confirmed at the mRNA transcript level by qRT-PCR as well as at the protein level by the Gad assay. As presented in Figure 2*B*, both MS::*gadA* and MS::*gadB* exhibited higher activity in Gad assay compared to the MS::EV. Appearance of blue color in Gad assay is an indicator of positive Gad activity. In case of recombinant MS strains Gad reagent turned blue within 30 min of incubation,

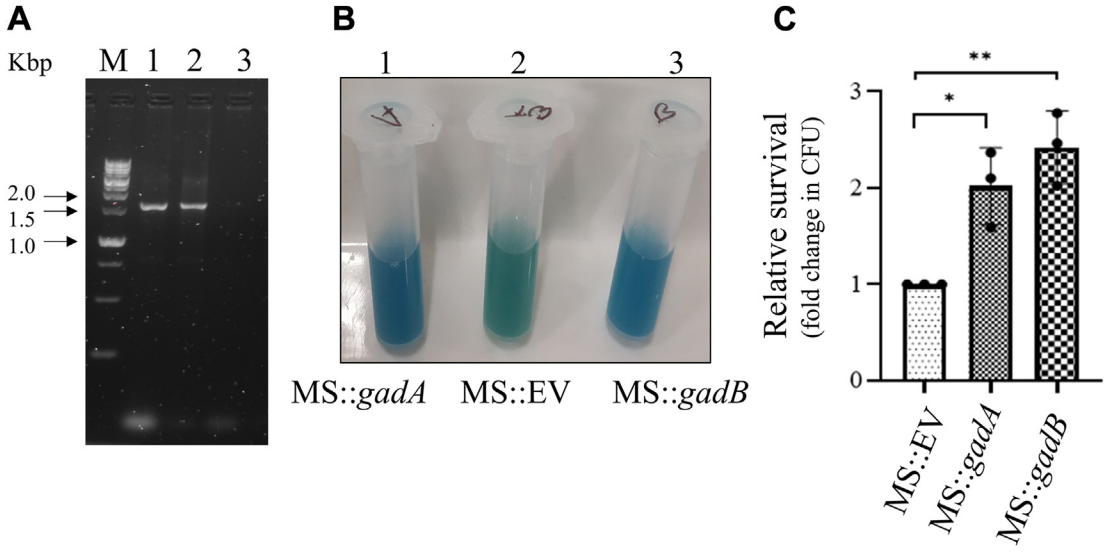

Figure 2. Ectopic overexpression of *gad* supports acid tolerance in mycobacteria. *A*, confirmation of the integration of *gadA* and *gadB* in the genome of MS by colony PCR. M- DNA ladder 1. MS::*gadA* (1566 bp); 2. MS::*gadB* (1569 bp); 3. MS:EV. *B*, detection of Gad activity in MS recombinants overexpressing Gad. Mycobacterial cultures at OD_{600}-0.6 to 0.8 were harvested by centrifugation, washed with PBS and resuspended in Gad reagent. The Gad overexpressing MS strain produced *blue* colour within 30 min of incubation. 1. MS::*gadA*; 2. MS::EV; 3. MS::*gadB*. *C*, ectopic overexpression of *gad* in MS confers higher acid resistance at a lethal pH 3.0. MS recombinants were cultured at OD_{600}-0.6 to 0.8 and were harvested by centrifugation, washed with PBS and resuspended in pH 3.0 ± 0.2 and further incubated for 4 h at 37 °C, 200 rpm. The values represent relative survival of MS::*gadA* and MS::*gadB* compared with MS::EV separately (*p –0.0229, **p –0.0470; two-tailed Student's t test using GraphPad Prism software). The data represent the mean ± SD from three independent experiments, each performed in triplicates.

compared to 4 h for MS::EV, thus confirming overexpression of Gad in the recombinant strains (Fig. 2*B*).

Next, we examined the role of *gad* in acid tolerance. All three strains, namely, MS::EV, MS::*gadA*, and MS::*gadB* were subjected to acid survival assay. Both MS::*gadA* and MS::*gadB* showed significantly higher survival at the pH 3.0 when compared with MS:EV (p = 0.0229, p = 0.0470). By CFU estimation, it was found that the survival of Gad-overexpressing strains is almost twofold higher in comparison to the MS::EV (Fig. 2*C*). These results thus indicate that Gad confers acid resistance in mycobacteria.

Deletion of gad *reduces survival of MS in acidic medium*

To further confirm the role of *gad* in acid tolerance in mycobacteria, a *gad* deletion mutant of MS (MSΔ*gadA*) was constructed using the allelic exchange method. The schematic of allelic exchange cassette for *gadA* deletion is shown in Figure 3*A*. The deletion of *gad* in the knockout strain was confirmed by Southern blotting (Fig. 3*B*) and Sanger sequencing. MSΔ*gadA* was complemented with either *gadA* gene (designated as MSΔ*gadA*::*gadA*) or *gadB* (MSΔ*gadA*::*gadB*), which were cloned downstream of the *hsp60* promoter in the pMV361 vector at an ectopic locus in the MSΔ*gadA*

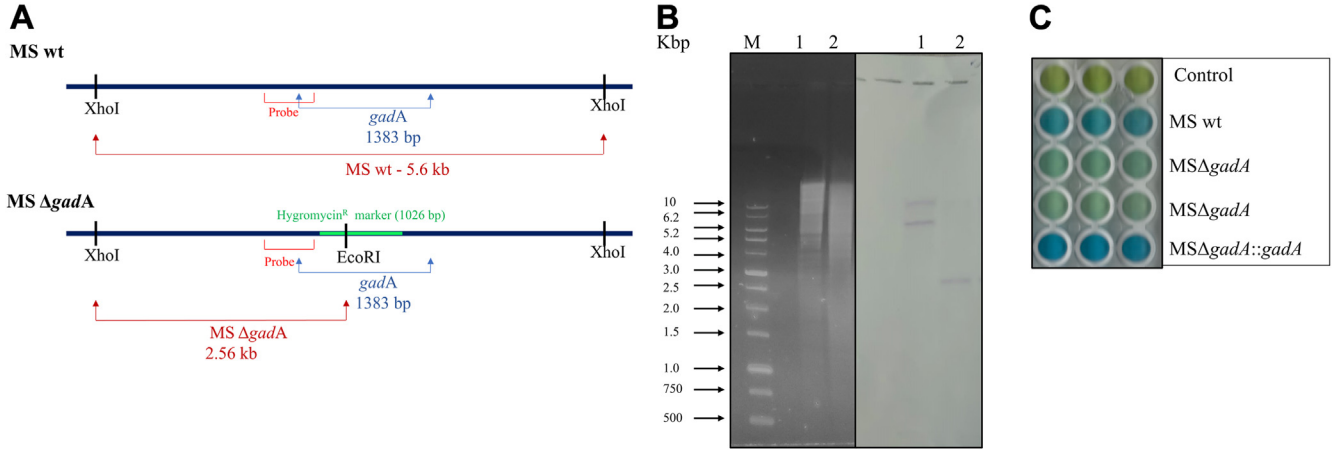

Figure 3. Generation of MS *gadA* deletion mutant. *A*, a schematic of allelic exchange cassette for the *gadA* deletion in MS. *B*, southern Hybridization. DNA of MS wt and MSΔ*gad*A were digested with EcoRI and XhoI and transferred to a Nylon membrane and probed with a digoxygenin labelled probe complementary to *gad*A and its upstream sequence 1. MS wt DNA, fragment of 5664 bp confirms the wt; 2. MSΔ*gadA* DNA, fragment of 2569 bp confirms the deletion mutant. *C*, Gad assay to confirm the loss of Gad activity in MSΔ*gadA* strain and restoration by complementation. Mycobacterial cultures at OD_{600}-0.6 to 0.8 were harvested by centrifugation, washed with PBS and resuspended in Gad reagent. Cultures producing *blue* colour within 4 h were considered positive.

genome. To confirm the loss of Gad activity in the mutant and restoration in the MSΔ*gadA*::*gadA* and MSΔ*gadA*::*gadB* complemented strains, these strains were subjected to the Gad assay. As expected, the *Gad* assay confirmed the loss of Gad activity in MSΔ*gadA*, which was restored by *gad* gene complementation in both MSΔ*gadA*::*gadA* and MSΔ*gadA*::*gadB* (*Fig. 3C*). These observations confirmed the loss of the *gad* gene and Gad activity in MSΔ*gadA*. To test the contribution of Gad in supporting acid resistance in mycobacteria, deletion mutant as well as the complemented strains were subjected to acid survival assay. The mycobacterial cultures were grown until the OD_{600} reached 0.6 to 0.8, and then subcultured after thorough washing in the medium of either pH 7.0 ± 0.2 or pH 3.0 ± 0.2 for 4 h. The viability of cultures was then determined by CFU plating on LBGT agar plates. CFUs were enumerated after 3 days of incubation, and relative survival of the mycobacterial strains was determined. Our results show lower survival of MSΔ*gadA* at pH 3.0 ± 0.2 when compared to the wild-type MS (MS wt). A significant decrease in the survival of MSΔ*gadA* compared to MS wt at pH 3.0 (p = 0.0226), and the restoration of MSΔ*gadA* viability by complementation with *gad* gene from both MS and Mtb origin (p = 0.0057, p = 0.028, respectively) together establish the role of *gad* in acid tolerance in mycobacteria (Fig. 4*A*). Indeed, the complemented strains showed even more survival compared to MS wt strain which may be due to the fact that complemented strain carries the gene under a strong constitutively expressing promoter. These observations are in accordance with our previous observations when we compared MS wt with *gad* overexpressing MS strains for their ability to survive at acidic pH 3.0 (Fig. 2*C*). Previous studies suggest that pH 3.0 is lethal for mycobacteria. To further analyze whether *gad* also supports mycobacterial growth at a sublethal pH, mycobacterial cultures were exposed to pH 5.0 ± 0.2 for 6 h. We observed significantly reduced survival in MSΔ*gadA* cultures treated with pH 5.0 when compared to MS wt and complemented strain (p = 0.02, NS p = 0.3750, respectively) (Fig. 4*B*). These observations confirm the role of *gad* in supporting mycobacterial adaptation and fitness to cope up with acidic environment.

Gad promotes intracellular survival of mycobacteria within macrophages

During infection, Mtb is known to survive within macrophages. As soon as the pathogen enters macrophages, the intracellular environment becomes acidic, which acts as a primary defense against the pathogen. Mtb is known to survive under the acidic environment within phagosomes and phagolysosomes. Considering our earlier observations, we next sought to determine whether Gad activity can promote intracellular survival of mycobacteria within macrophages. For this purpose, we infected THP-1 and J774.A.1 cells with recombinant MS strains. We observed significantly lower survival of MSΔ*gadA* in both THP-1 as well as J774.A.1 cells when compared to MS wt and *gadA* complemented

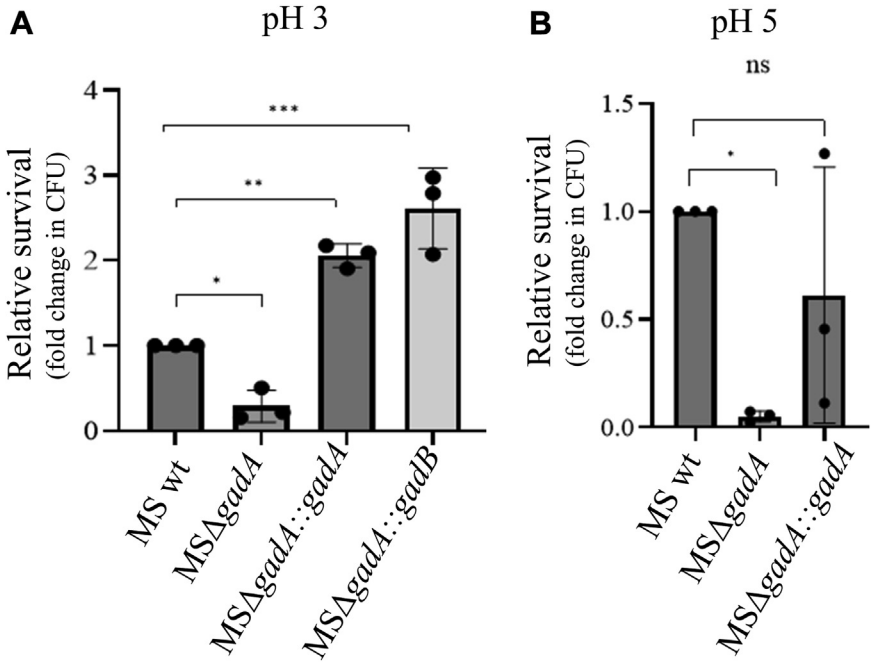

Figure 4. Deletion of *gadA* reduces survival of MS in acidic medium. *A*, *gadA* in MS confers higher acid resistance at a lethal pH 3.0. MS recombinants were cultured at OD_{600}-0.6 to 0.8 and were harvested by centrifugation, washed with PBS and resuspended in pH 3.0 ± 0.2 and further incubated for 4 h at 37 °C, 200 rpm. The values represent relative survival of each strain compared with MS wt (*p –0.0226, **p –0.0057, ***p –0.028; two-tailed Student's t test using GraphPad Prism software). Error bars represent standard deviations from three independent experiments performed in triplicates. *B*, *gadA* in MS supports survival at a sub lethal pH 5.0. MS recombinants were cultured at OD_{600}-0.6 to 0.8 and were harvested by centrifugation, washed with PBS and resuspended in pH 5.0 ± 0.2 and further incubated for 6 h at 37 °C, 200 rpm. The values represent the relative survival of each strain compared with MS wt (*p –0.02, ns p –0.3750; two-tailed Student's t test using GraphPad Prism software). The data represent the mean ± SD from three independent experiments performed in triplicates.

counterparts. The survival of MSΔ*gadA* was almost twofold less in J774.A.1 cells when compared to MS wt (p = 0.0417), and this reduced survival was restored by *gadA* complementation (Fig. 5*A*). Similar pattern of survival was also observed in THP1 cells (p = 0.0045 respectively) (Fig. 5*B*). These findings suggest that *gad* may promote intracellular survival of mycobacteria within macrophages. While MS is a useful model for studying mycobacteria, it does not naturally infect the host and survive within macrophages for long. Hence, we further confirmed role of *gad* in intracellular survival of BCG, which is an attenuated strain derived from the Mtb-complex bacterium *Mycobacterium bovis*, as a complementary model to support the findings obtained from the MS strain. Importantly, BCG does not express Gad and tested negative Gad activity in the Gad assay. This allowed us to ectopically express the *gad* gene and directly compare the intracellular survival of recombinant

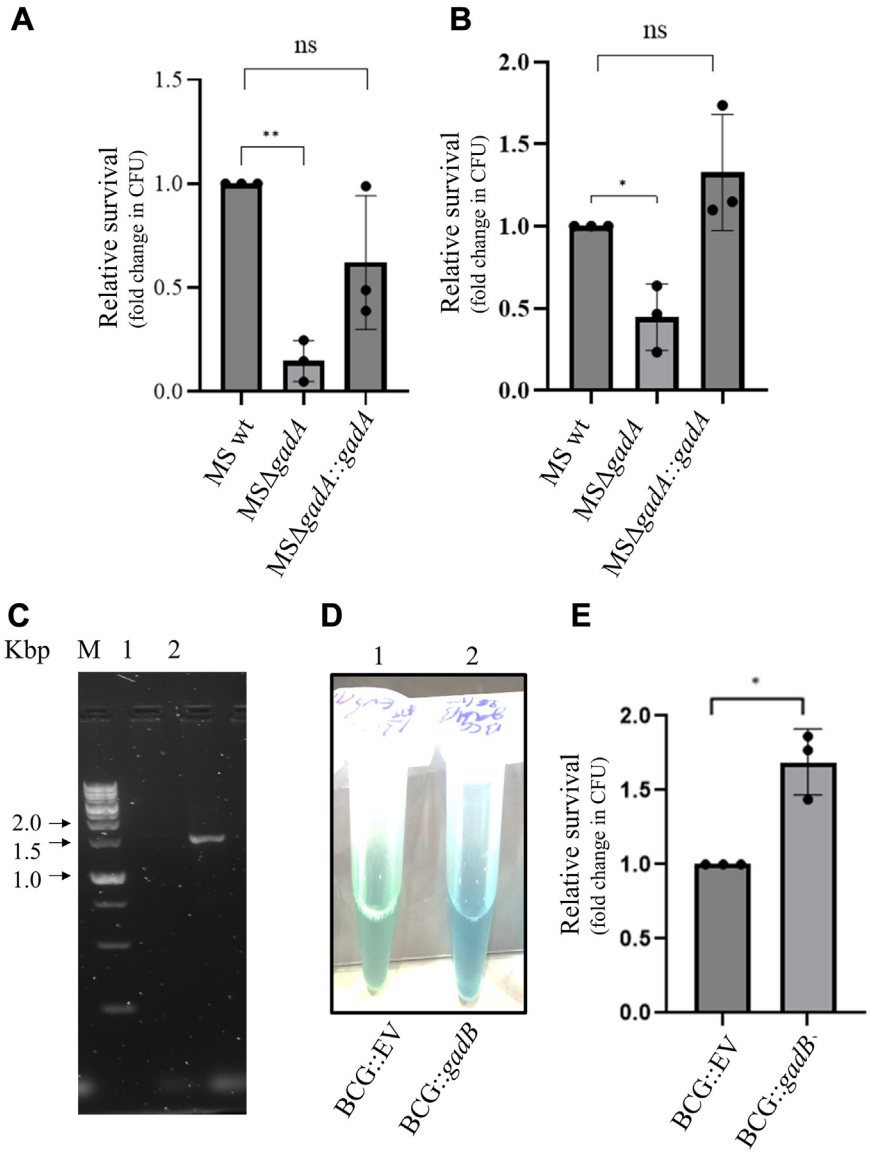

Figure 5. Gad promotes intracellular survival of mycobacteria within macrophages. *A*, intracellular survival of recombinant MS in THP-1 cells. Mycobacterial cultures were grown in normal LBGT medium, pelleted and resuspended complete RPMI, single cell suspension was prepared and infected in THP-1 cells with MOI 10:1. The values represent relative survival of MSΔ*gadA* when compared to MS wt after 24 h of infection (**p* –0.0045, two-tailed Student's *t* test using GraphPad Prism software). The data represent the mean ± SD from three independent experiments performed in triplicates. *B*, intracellular survival of recombinant MS in J774.A.1 cells. Mycobacterial cultures were grown in normal LBGT medium, pelleted and resuspended in complete DMEM, single cell suspension was prepared and infected in differentiated J774.A.1 cells with MOI 10:1. The values represent relative survival of MSΔ*gadA* when compared to MS wt after 24 h of infection (**p* –0.0417, two-tailed Student's *t* test using GraphPad Prism software). Data represent the mean ± SD from three independent experiments performed in triplicates. *C*, cloning of *gadB* in BCG. M – DNA ladder; 1. BCG::EV; 2. BCG::*gadB*, DNA fragment of size 1569 bp confirms the integration of gene. *D*, detection of Gad activity in Gad overexpressing BCG strain. Mycobacterial cultures at OD_{600}-0.6 to 0.8 were harvested by centrifugation, washed with PBS and resuspended in Gad reagent. Cultures producing blue colour within 4 h were considered positive. 1. BCG::EV; 2. BCG::*gadB*. *E*, intracellular survival of BCG overexpressing *gad* in J774.A.1 cells. Mycobacterial cultures were grown in 7H9 medium, pelleted and resuspended complete DMEM, single cell suspension was prepared and infected in J774.A.1 cells. The values represent relative survival of BCG::*gadB* when compared to BCG::EV after 48 h of infection (**p* –0.0331, two-tailed Student's *t* test using GraphPad Prism software). The data represent the mean ± SD from three independent experiments performed in triplicates.

BCG to wild-type BCG. Gad expressing BCG was constructed by electroporating pMV361:*gadB* in BCG (BCG::*gadB*) and the integration of the gene was confirmed by PCR (Fig. 5*C*). Gad activity in BCG::*gadB* as determined by the Gad assay further confirmed the expression of *gad* in the recombinant BCG strain (Fig. 5*D*). The BCG::*gadB* showed positive Gad activity while empty vector transfected BCG did not show Gad activity (Fig. 5*D*). Notably, expression of Gad increased the intracellular survival of BCG in J774.A.1 cells by 1.8 fold (p = 0.0331) at 48 h post infection (Fig. 5*E*). These observations corroborate our findings and further suggest that Gad promotes the intracellular survival of mycobacteria within macrophages.

Discussion

Mtb has the ability to survive and multiply inside host macrophages for a long period of time. Although drugs are available to treat TB infection with considerable success, but the currently used long treatment regime, high drug toxicity and emergence of drug resistance have alarmed the need for new and better drugs (13). In order to discover new drugs and to reduce incidences of drug resistance, new drug targets and mechanisms conferring drug resistance need to be discovered. Metabolic pathways in bacterial pathogens have been implicated in adaptation inside host cells and drug resistance (36). Pathogens alter their metabolic pathways to survive host defense and to counter the effects of antibiotics (36, 37). The exposure of *Escherichia coli* (*E. coli)* to low pH promotes intrinsic resistance to antibiotics (38). Gad, a major component of the acid resistance system in bacteria, has been associated with increased antibiotic tolerance in *E. coli* (39). We earlier demonstrated that Gad is expressed in Mtb as well as in MS (35). The expression of Gad in Mtb was detected at both acidic as well as at normal pH (35). In this study, we aimed to investigate the expression of *gad* both in Mtb and MS, and its effect on bacterial survival under acid stress and during macrophage infection. Gene expression analysis revealed that *gad* was upregulated in Mtb at acidic pH as well as within macrophages. Similarly, the expression of *gad* in MS was also upregulated at acidic pH as well within macrophages. Gene expression analysis suggests the role of Gad in mycobacterial acid resistance as well as its role in altered metabolic tricarboxylic acid (TCA) cycle within mycobacteria.

Altered levels of metabolites of the TCA cycle have been shown to confer antibiotic resistance in Mtb (40). Mtb was proposed to lack Kdh activity limiting the conversion of α-ketoglutarate to succinyl CoA (41). Instead, *kdh* codes for α -ketoglutarate decarboxylase which converts α-ketoglutarate into succinic semialdehyde (SSA) (41). Tian *et al.* proposed that α-ketoglutarate decarboxylase bifurcates the TCA cycle by converting α-ketoglutarate into SSA (41). Consistent with this finding, we observed downregulation of *kdh*/*sucA* in Mtb and MS at both acidic conditions and within macrophages. We also observed the upregulation of *gab-T* in Mtb and MS during acidic stress and during infection within macrophages. *gabD1/gabD2* was found to be upregulated during infection of MS within macrophages. The increased expression of these genes suggests a coordinated metabolic response where Gad-mediated glutamate decarboxylation may be coupled with the TCA cycle *via* the GABA shunt. The increased expression of *gab-T* and simultaneous decrease in *kdh* expression suggests that, under acidic conditions, Mtb may metabolize α-ketoglutarate through Gab-T. Gab-T converts GABA and α-ketoglutarate into SSA and glutamate. This process may provide an alternate pathway for the TCA cycle which also regenerates glutamate for further decarboxylation by Gad. During acidic stress, Mtb metabolizes glutamate to GABA *via* Gad, utilizing a proton, which may be beneficial for the cell under acidic conditions by removing excess protons at low pH. It has been inferred that GABA can neutralize protons and simultaneously contribute to the NAD^+/NADH balance, playing a role in Mtb's early adaptation to both acidic and oxidative stresses (42). Findings such as high levels of α-ketoglutarate also suggest the presence of an alternate pathway, *i.e.*, the GABA shunt, which has been identified as one of the metabolic pathways for lactate and pyruvate metabolism in Mtb in studies combining microbial physiology analysis with transposon-directed insertion site sequencing (43).

Mtb is known to alter or integrate its metabolic pathways depending on nutrient availability and environmental stress (44). Changes in the expression of glutamate metabolism genes i,e *gdh*, *glnA*/*glnA1* and *gltD*/*gltB* in MS and Mtb during acidic stress and during infection suggest similar metabolic adaptation in mycobacteria to the acidic environment encountered within macrophages. Mycobacteria also face nutritional scarcity, and in order to survive, further changes in metabolic pathways are reported (45, 46). Mtb cannot utilize lactate or pyruvate under oxygen-limiting conditions. Transcriptomic and proteomic analyses reveal that Mtb utilizes the glyoxylate shunt and methyl citrate cycle to metabolize lactate and pyruvate (43). Mtb acquires glutamate from host macrophages, and high levels of glutamate was found in the cell lysates of Mtb (41) which may serve as both a carbon and a nitrogen sources for its intracellular survival within the host (47, 48). Glutamate can be used as both a carbon and nitrogen source *via* GDH which converts glutamate to ammonium, thus providing protection against acidic stress by buffering with ammonia. This also enables carbon assimilation by degrading glutamate to 2-oxoglutarate (49). The depletion of intracellular glutamate in the Δ*gltBD* double mutant and its accumulation in Δ*gdh* mutant suggest that glutamate homeostasis is critical under acidic stress conditions in BCG. Gdh was reported to be essential for optimal growth in BCG and also contributes to nitrosative stress resistance and intracellular survival in macrophages (49, 50). GltB/D facilitates the conversion of glutamine to glutamate, ensuring a stable intracellular glutamate pool. Its role in GABA synthesis becomes critical under propionate stress induced by the methylcitrate cycle (MCC) in BCG (32). The growth defects caused by propionate toxicity were alleviated by monosodium glutamate supplementation, highlighting the metabolic link between MCC and glutamate pathways. The conservation of glutamate pool may be utilized either by Gdh associated anaplerosis of TCA cycle intermediates or by Gad for proton neutralization (32). These

mechanisms likely sustain TCA activity and pH balance under acidic or nutrient-limited conditions. GlnA1 one of the four glutamine synthetase present in Mtb, is the only enzyme detected, isolated, and assayed in growing mycobacteria (51). In MS, *glnA1* expression and glutamine synthetase activity are tightly regulated by nitrogen availability, downregulated under excess ammonium and upregulated under nitrogen starvation (52). In Mtb, *glnA1* has been associated with virulence and intracellular survival. Mutants lacking *glnA1* were attenuated in THP-1 macrophages and failed to infect guinea pigs (53). Moreover, *glnA1* was shown to be essential for *in vitro* growth and involved cell wall remodeling through poly-L-glutamate synthesis (51, 54). Proteomic studies also indicate upregulation of *glnA1* during acidic stress which are consistent with our observations of increased *glnA1* expression in acidic conditions, suggesting its role in pH adaptation and intracellular persistence (55).We observed the upregulation of *fadD9* and *icl1* in Mtb and MS during acidic stress. Deletion of *icl1/2* impairs growth at acidic pH but not at neutral pH, highlighting the importance of anaplerotic metabolism during acidic stress (56). While *icl1/2* does not directly channel GABA into the TCA cycle, its upregulation, along with *gabT*, supports the hypothesis that alternate metabolic pathways are active under these conditions to sustain TCA cycle function. Consistent with our observations, proteomic studies by Choudhary *et al.* have also demonstrated the upregulation of FadD9 during acidic stress in Mtb (55). FadD9 may channel fatty acid-derived acetyl-CoA into the TCA cycle, providing carbon input and supporting energy metabolism under stress conditions. Icl and Gad thus may also be linked with fatty acid metabolism as well, which suggests the possibility that fatty acids may also be utilized as energy source during acidic stress in mycobacteria (43).

In the Mtb genome, the gene for Gab-T—the enzyme that catalyzes the first reaction of the GABA shunt is located adjacent to the gene coding for FadD9 (the enzyme that catalyzes the first step of the pathway, producing acetyl coA from fatty acids). This suggests the possibility that both enzymes may be required for a common pathway. It is also noteworthy to mention that the Mtb genome does not code for a GABA/glutamate antiporter which is required to exchange external glutamate for intracellular GABA and thereby reducing intracellular protons. This suggests that GABA produced by the action of Gad must be utilized by other pathways. One such probable pathway is the GABA shunt, which allows GABA to be consumed by the TCA cycle. This pathway requires GABA-AT which catalyzes transfer of an amino group from GABA to α-ketoglutarate to convert it into SSA and simultaneous regeneration of glutamate. SSA is then converted to succinate and enters the TCA cycle. In this way, glutamate can be continuously decarboxylated and regenerated leading to the consumption of protons and increase in intracellular pH. This hypothetical model explains the absence of GABA/glutamate antiporter in Mtb (Fig. 6). The existence of such mechanisms has been shown in other bacteria but not in mycobacteria (57–59).

The ability of Mtb to survive within the acidic and oxidative environment of host macrophages is critical for its pathogenesis and the establishment of TB. One key adaptation mechanism that has emerged from our work is the role of Gad in mediating acid resistance. Our results demonstrate that the overexpression of Gad in MS confers a significant survival advantage in acidic environments (pH 3.0). Similar, results were observed when Mtb Gad was expressed in MS, suggesting a conserved function of Gad in mycobacteria. Conversely, a *gad* deletion mutant of MS exhibited markedly reduced survival under acidic conditions, with the phenotype being rescued upon complementation with the *gad* gene from either MS or Mtb origin. Our results showed that Gad supports mycobacterial growth both at lethal (pH 3.0) and sub-lethal (pH 5.0) pH. These findings underscore the role of Gad in acid resistance and tolerance, indicating that this enzyme may be required for the survival of mycobacteria in acidic environments. These observations suggest that Gad may also support the intracellular survival of mycobacteria within the host. The *gad* deletion in MS reduced the survival within macrophages, which was restored by gene complementation. Interestingly, our data show that BCG, which lacks inherent Gad activity, exhibits enhanced survival within macrophages when Gad is ectopically overexpressed. This suggests that the Gad-mediated acid resistance mechanism not only enables mycobacteria to withstand the acidic pH of the phagosome but may also contribute to the pathogen's virulence by enhancing its ability to persist within the host. Previous studies have shown that PknG regulates multiple processes such as antibiotic sensitivity, glutamate metabolism, acid tolerance, and intracellular survival, it suggests a common link between these adaptive responses in mycobacteria (28–30, 60–62). Notably, *gad* expression has been shown to be increased in antibiotic-resistant *E. coli*, indicating a potential role of this gene in resistance mechanisms (39). Furthermore, altered levels of GABA and TCA cycle intermediates, have been associated with antibiotic sensitivity in mycobacteria (37, 63–65) thus raising the possibility that Gad, through its impact on glutamate metabolism and intracellular pH regulation, might modulate metabolic states that influence mycobacterial responses to antibiotics. Further investigations will be needed to understand if Gad contributes to antibiotic resistance in mycobacteria.

Conclusion

Our study highlights the important role of Gad in mycobacterial survival under acidic stress prevailing within host macrophages. By facilitating glutamate decarboxylation and maintaining intracellular pH homeostasis, Gad enables mycobacteria to survive in hostile environments. The previously reported upregulation of *gad* in antibiotic-resistant *E. coli* and the link between altered GABA/TCA cycle intermediates and antibiotic sensitivity in mycobacteria suggest that Gad may also contribute to antibiotic resistance. Further studies are required to elucidate the role of Gad in the antibiotic response in mycobacteria. These findings underscore the importance of Gad in mycobacterial physiology and suggest it as a potential target for developing novel

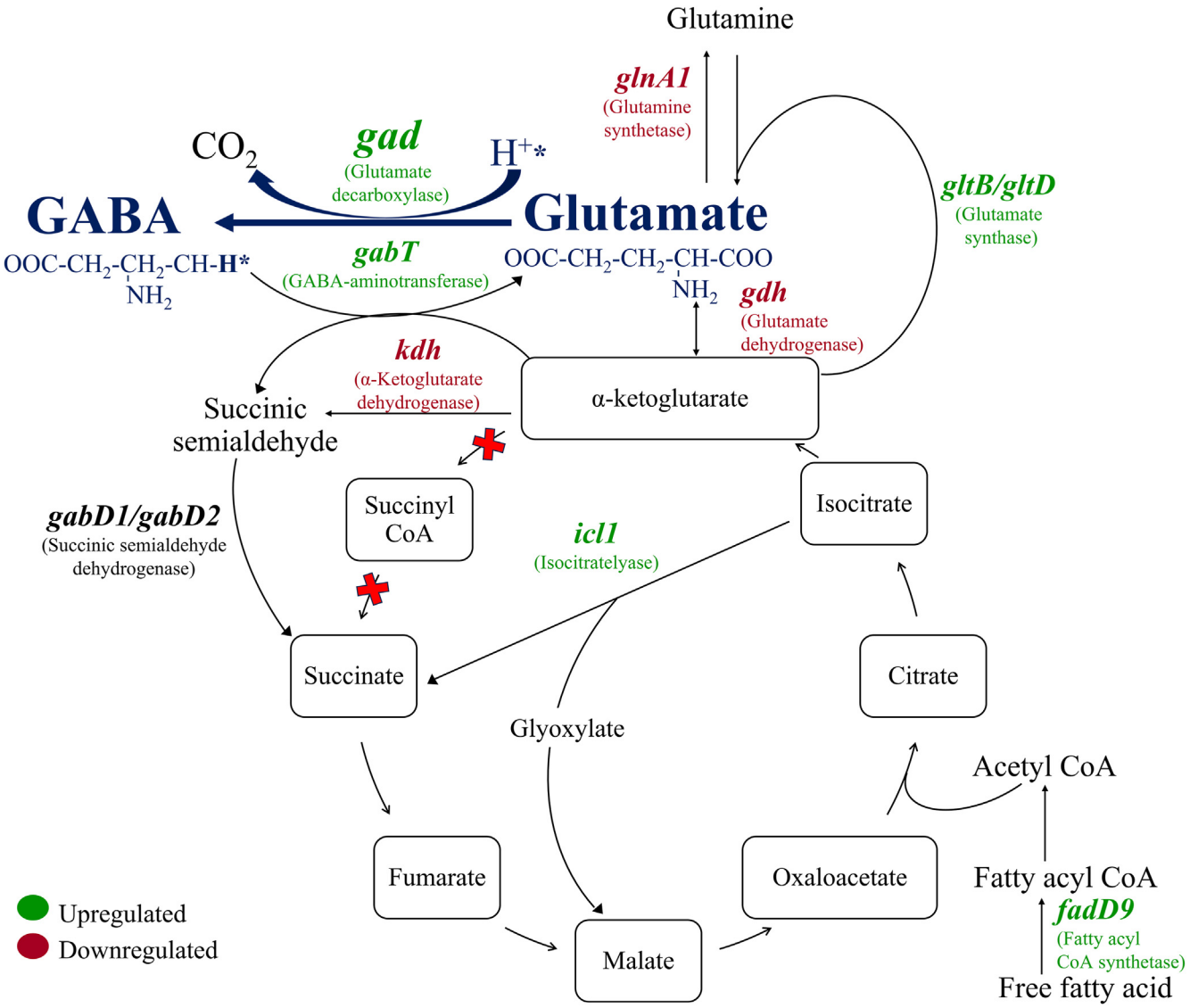

Figure 6. Proposed mechanism of glutamate decarboxylase (Gad)-mediated acid resistance in mycobacteria. The decarboxylation reaction catalyzed by Gad consumes a proton, helping to neutralize the intracellular acidity during acidic stress. Since the mycobacterial genome does not encode a glutamate-GABA antiporter, sustained glutamate decarboxylation relies on the availability of glutamate and the removal of GABA. Mtb lacks Kdh activity, preventing the conversion of α-ketoglutarate to succinyl-CoA (40). Instead, *kdh* encodes α-ketoglutarate decarboxylase, which converts α-ketoglutarate into succinic semialdehyde (SSA). Reduced expression of α-ketoglutarate decarboxylase may inhibit this conversion, potentially stalling the TCA cycle. Under these conditions, GABA produced by Gad can react with α-ketoglutarate *via* the GABA shunt, generating SSA and glutamate. This process restores TCA cycle activity and regenerates glutamate, enabling continuous decarboxylation and intracellular pH regulation. Genes involved in glutamate metabolism (*gdh, glnA1, gltD/gltB*) likely contribute to maintaining intracellular glutamate levels during acidic stress. The upregulation of *gltD/B* and downregulation of *gdh* and *glnA1* suggest a metabolic shift toward glutamate synthesis to sustain the glutamate pool. The upregulation of *fadD9*, encoding fatty acyl-CoA synthetase, may help maintain the carbon pool by channeling acetyl-CoA into the TCA cycle. When carbon is derived from acetyl-CoA through fatty acid oxidation, coupling TCA activity with glutamate decarboxylation may support acid tolerance by facilitating fatty acid oxidation. Under these conditions, acetyl-CoA provides the carbon input, while CO_2 released from glutamate decarboxylation serves as the output, preserving glutamate for other critical functions. The upregulation of *icl* and *gab-T* further highlights the importance of alternative TCA pathways in sustaining metabolism under acidic stress. Color Codes: *Green*: Genes encoding enzymes upregulated at acidic pH in Mtb. *Red*: Genes encoding enzymes downregulated at acidic pH in Mtb.

therapeutic strategies to combat TB, particularly the drug-resistant cases.

Materials and methods

Bacterial strains, culture media, and cell culture

MS strains were cultured in Luria-Bertani (LB) broth supplemented with 0.5% (v/v) glycerol and 0.05% (v/v) tween 80. Mtb H37Rv and BCG strain were cultured in Middlebrooks 7H9 broth (Himedia) supplemented with 10% albumin-dextrose-catalase (ADC) (Himedia), 0.5% glycerol (Himedia) and 0.05% Tween-80. Mtb H37Rv MC^{2}6206 (66) was cultured in Middlebrook 7H9 supplemented with 1 × oleic acid-albumin-dextrose-saline (OADS), 50 μg/ml leucine, 24 μg/ml pantothenate, 0.5% glycerol and 0.05% tyloxapol. *E. coli DH5α* cells was obtained from Himedia. All cultures were routinely tested for contamination. *E. coli* strains were cultured in LB broth, pH 7.0 ± 0.2 (Himedia). All cultures were grown at 37 °C with or without shaking at 220 rpm. Whenever required the pH of media was adjusted to 5.0 ± 0.2 and 3.0 ± 0.2 using

either HCl or 20 mM sodium citrate buffer before autoclaving. Whenever needed, LB or LBGT (LB with 0.5% glycerol and 0.05% tween 80) medium was supplemented with 1.5% agar (Himedia) in order to make semi-solid media for culturing respective bacteria. Whenever needed 7H10 media was used supplemented with 10% ADC, 0.5% glycerol and 0.05% tween-80. 25 μg/ml and 50 μg/ml kanamycin (kan), 100 μg/ml ampicillin (amp), 50 μg/ml hygromycin (hyg), and 200 μg/ml amikacin were used, wherever needed. All the restriction enzymes were obtained from NEB. All reagents used in the study were procured from Sigma Aldrich unless mentioned otherwise. The primers used in the study are listed in Table S1. All the plasmids and bacterial strains used in the study are listed in Table S2.

The THP-1 human macrophage-like cell line (ATCC strain acquired from the National Centre of Cell Science (NCCS), Pune) were cultured in RPMI-1640 medium (Gibco) additionally adding 2.0 g/L sodium bicarbonate supplemented with 10% heat inactivated fetal bovine serum (FBS) (Hi-FBS, Gibco) (complete RPMI) at 37 °C, 5% CO_2. THP-1 cells were differentiated by treatment with 50 nM Phorbol 12- Myristate, 13-acetate (PMA) (Sigma) for 48 h (h) before using for the experiments. The J774.A.1 mouse derived macrophage cell line (ATCC strain acquired from the NCCS) were cultured in DMEM medium (Gibco) additionally adding 3.7 g/L sodium bicarbonate supplemented with 10% heat inactivated FBS (complete DMEM) at 37 °C, 5% CO_2. All cell lines were routinely verified for their characteristics and tested for contamination.

Cloning of gad *in MS and BCG strains*

The *gadB* gene was amplified from Mtb genomic DNA and *gadA* was amplified from MS genomic DNA using phusion polymerase enzyme. PCR amplicon amplified using gene specific oligonucleotides was cloned in pJET1.2/blunt end cloning vector using T4 DNA ligase enzyme (NEB). Both DNA fragments of ∼1.4 kb were obtained by EcoRI and HindIII digestion and cloned at the same sites in pMV361 vector separately. *E. coli* DH5α was used for propagation of the recombinant clones. The transformants were selected on LB agar supplemented with 50 μg/ml kan after overnight incubation at 37 °C. The integrity of clone was confirmed by restriction enzyme digestion and Sanger sequencing. The confirmed pMV361:*gadB*, pMV361:*gadA* plasmid and pMV361 empty vector (EV) were electroporated into MS MC^2155 at 2500 V using Eporator Electroporator (Eppendorf) and selected on LBGT agar plates with 25 μg/ml kan. The clone pMV361:*gadB* and pMV361 (EV) were also electroporated in BCG strain at 2500 V and selected on 7H10 agar plates with 25 μg/ml kan. Confirmation of recombinant was done by colony PCR using vector and gene specific primers.

Gad assay

The Gad assay was performed as described previously by Cotter *et al.* (33). The method was optimized for mycobacterial cultures as described previously (35). Briefly, the test solution containing 1 g of L-glutamic acid (MP Biomedicals), 0.3 ml of Triton X-100, 90 g of NaCl and 0.05 g of bromocresol green indicator (MP Biomedicals), in 1 l of water was prepared (Gad reagent). Gad reagent was adjusted to pH 4.5 ± 0.2 using NaOH. Mycobacterial cultures were grown till OD_{600} reached 0.8 to 1.0. 1 ml of cells were harvested by centrifugation at 6000 rpm for 10 min (minutes) followed by washing with phosphate-buffer saline (PBS). Cells were then resuspended in 500 μl Gad reagent followed by incubation at 37 °C. The appearance of a blue color within 4 h of incubation was considered positive for Gad activity. The bromocresol green dye serve as an indicator for change in pH of the solution. *E. coli* served as the positive control, while assays without cells, without L-glutamate, or with D-glutamate functioned as negative controls (D-glutamate is not a substrate but an inhibitor of Gad).

Generation of gadA *deletion mutant in MS*

An allelic exchange strategy was applied to generate *gadA* deletion mutant of MS. The 2407 bp sequence was amplified from MS genomic DNA containing 1380 bp of *gadA* with 502 bp upstream of the gene and 525 bp downstream of the gene as flanking regions using phusion polymerase enzyme (NEB). The PCR amplicon amplified using gene specific oligonucleotides was cloned in pJET1.2/blunt end cloning vector using T4 DNA ligase enzyme (NEB). *E. coli* DH5α was used for propagation of the recombinant clones. Transformants were selected in the presence of the amp on LB agar plates after overnight incubation at 37 °C. Plasmid was isolated and inverse PCR was performed using phusion polymerase enzyme with specific oligonucleotides, deleting 1236 bp of *gadA*. The purified inverse PCR product was ligated with *hyg* cassette of 1026 bp using T4 DNA ligase and transformed in *E. coli* DH5α cells. Before ligation *hyg* cassette was amplified using specific oligonucleotides, digested by EcoRV and purified. Transformants were selected in the presence of the amp and hyg on LB agar plates after overnight incubation at 37 °C. Plasmid isolation was performed and clone was confirmed by restriction enzyme digestion. The fragment of 2197 bp was obtained by XbaI and XhoI digestion and cloned at the same sites in pDrive vector. pDrive was chosen as delivery vector. It has AmpR, KanR, and *ori*E for propagation in *E. coli*. As vector does not have *ori*M, it works as a suicide vector in mycobacteria. The generated pDrive:*hyg*:*gadA* plasmid was electroporated in MS. Transformants were selected on LBGT agar plates with amp and hyg. Single colonies obtained were replica plated on hyg and kan plates to segregate colonies with single crossing over from the cells which had undergone double crossing over event *i.e.* Δ*gadA* mutant cells. Putative mutants screening for double recombinants generating MSΔ*gadA* were performed using colony PCR using specific confirmatory primers. One of the mutants was selected and designated as MSΔ*gadA* and used in the present study. The MSΔ*gadA* generated was further confirmed by Southern Blotting and Sanger sequencing. A complement strain of *gadA* deletion mutant was generated by electroporating pMV361:*gadA* into

MSΔ*gadA* and selected on kan hyg plates. Integration of gene was confirmed by PCR using vector and *gadA* specific primers. One of colony was picked and designated as MSΔ*gadA::gadA*. A complement of *gadA* mutant was also generated by electroporating pMV361:*gadB* into MSΔ*gadA* and selected on kan hyg plates. Integration of the gene was confirmed by colony PCR using vector and *gadB* specific primers. One of colony was picked and designated as MSΔ*gadA::gadB*.

Southern blotting

The MSΔ*gadA* strain was confirmed by Southern blotting. Equal quantities of Genomic DNA of MS wild type (MS wt) and putative *gadA* deletion mutant were digested with using EcoRI and XhoI. The digested DNAs were and resolved on 0.8% agarose gel and Southern blotting was performed as described previously for MS with few modifications (67) The probe was labeled using Dig DNA labeling mix (Sigma-Roche) as per manufacturer's instructions. Blots were developed using Dig detection kit according to the manufacturer's instructions.

Acid survival assay

Methods as described previously for MS were adopted with few modifications (28, 29). Mycobacterial cultures were grown until the OD_{600} reached 0.8 to 1.0. Equal no. of cells from each culture were pelleted by centrifugation at 6000 rpm for 10 min. Pellet was then washed with PBS and then resuspended in LBGT media of either 3.0 ± 0.2 pH or 5.0 ± 0.2 pH and further incubated for 4 h and 6 h respectively at 37 °C with shaking at 200 rpm. Mycobacterial culture pellet resuspended in pH 7.0 ± 0.2 LBGT media was taken as control under similar conditions. The viability of cultures was determined using dilution plating which was done before and after acid challenge on LBGT agar plates. Plates were incubated at 37 °C for 3 days. CFUs were counted and the CFU survival percentage was calculated of each recombinant strain by comparing CFU of mycobacterial culture plated before and after acid challenge. Survival at each time point was expressed as a percentage of the 0-h CFU. The 4 h or 6 h survival percentage of the wild-type strains was used as the reference (normalized to 1.0), with the survival rates of the other strains expressed relative to this standard.

Intracellular survival assay

Methods as described previously for MS and BCG were adopted with few modifications for infection (26, 29).

Preparation of mycobacterial cells for infection

Mycobacterial cells were grown until the OD_{600} reached 0.8 to 1.0. Equal no. of cells from each culture was pelleted by centrifugation at 6000 rpm for 10 min. Pellet was then washed with complete DMEM/RPMI medium and resuspended in fresh complete DMEM/RPMI medium. A single cell bacterial cell suspension was prepared by vortexing the culture with 3 mm sterile glass beads five times (2 min each) then suspension was passed through 26-gauge needle several times to disaggregate any remaining clumps. After homogenization with a needle, the bacterial suspension was centrifuged for 3 min at 100*g* to pellet remaining clumps. The total no. of bacteria was ascertained by measuring the OD_{600} of the bacterial suspension and further by counting the CFUs on LBGT agar/MB7H10-agar plates.

Preparation of mammalian cells for infection

A total of 1×10^5 THP-1 cells were seeded in 12 well plates and incubated with 50 nM PMA at 37 °C in 5% CO_2 for 48 h. A total of 1×10^5 J774.A.1 cells were seeded in 12 well plates and incubated for 24 h at 37 °C in 5% CO_2.

Method for infection

Differentiated THP-1 cells or J774.A.1 macrophage cells were infected with the single-cell bacterial suspension at a multiplicity of infection (MOI) of 1:10 and incubated for 2 h. To eliminate extracellular bacteria, amikacin treatment was applied for 2 h, followed by multiple PBS washes. The cells were lysed with 0.05% SDS, and CFU counts were taken from lysates to determine intracellular bacterial counts, marking this as the 0-h time point. For cells incubated beyond 0 h, PBS was used to remove residual amikacin, and fresh medium (without amikacin) was added. The intracellular survival of mycobacterial strains was assessed using CFU assays at 0 h, 24 h, and 48 h post-infection. Infections with each mycobacterial strain were set at MOI 1:10, and CFU counts at 0 h served as the baseline (100%) to account for minor variations in bacterial counts at infection onset. After a 24 h incubation (for MS) or 48 h incubation (for BCG), the macrophages were lysed for CFU determination. Survival at each time point was expressed as a percentage of the 0-h CFU. The 24 h survival percentage of the wild-type strains was used as the reference (normalized to 1.0), with the survival rates of the other strains expressed relative to this standard.

Isolation of RNA, cDNA synthesis, and quantitative PCR

Methods as described previously for mycobacteria were adopted with few modifications (29, 68). For the gene expression analysis after exposure to acidic medium, RNA from mycobacterial cultures exposed to acidic medium of pH 5.0 ± 0.2 for 4 h was isolated. Cultures incubated at pH 7.0 ± 0.2 were taken as control. For gene expression analysis of mycobacterial cultures after infection in macrophages, RNA was isolated from mycobacterial cultures infected within macrophages for 4 h. To isolate RNA from intracellular mycobacteria, macrophages were subjected to osmotic lysis and released bacteria were pelleted and total RNA was isolated using TRiZol reagent (Thermo-fisher scientific). Mycobacterial cell lysis was done using bead beating 5 times each for 45 s pulse and 60 s rest. The isolated RNA was then further purified using RNAeasy purification kit (Qiagen). Purified RNA was quantified using Nanodrop and to confirm the integrity of RNA, the samples were subjected to agarose gel electrophoresis. 2 μg of total RNA was digested with RNase free DNase (Ambion) and used for cDNA synthesis with random hexamer primers using HI capacity reverse cDNA synthesis kit

(Thermo-fisher scientific). Equal amount of cDNA was used in each reaction. To confirm absence of genomic DNA in the RNA samples, cDNA reactions without Reverse Transcriptase were performed. Reverse Transcriptase Quantitative PCR (qRT-PCR) was performed in 96 well plates on qTOWER^3 G (Analytik jena) using SYBR Green 1 master mix (Thermo-fisher scientific) and results were analyzed. Specificity of amplicons was confirmed by Melt curve analysis. Further, after the completion, the reactions were subjected to agarose gel electrophoresis for confirmation of integrity and specificity of reactions. Change in gene expression was analyzed by $2^{-\Delta\Delta Ct}$ method. Primer pairs used in the study are listed in Table S1.

Data availability

The datasets generated during and/or analyzed during the current study are available in this manuscript.

Supporting information—This article contains supporting Information.

Acknowledgments—We thank Dr William Jacobs at the Albert Einstein College of Medicine, USA for providing MC^2 6206 strain of Mtb. We sincerely thank Dr Nisheeth Agarwal at the Translational Health Science and Technology Institute, Faridabad for providing access to his laboratory and logistical support to carry out the acid treatment of Mtb.

Author contributions—R. R., R. C., A. K. S., and S. K. C. writing–review & editing; R. R. writing–original draft; R. R., A. K. S., and S. K. C. visualization; R. R. and S. K. C. validation; R. R. and S. K. C. project administration; R. R., B. J. M., R. C., A. K. S., and S. K. C. methodology; R. R. investigation, R. R., B. J. M., and S. K. C. formal analysis; R. R. data curation. R. C. and S. K. C. conceptualization; S. K. C. supervision; S. K. C. resources; S. K. C. funding acquisition.

Funding and additional information—This study was supported by grants received from SERB, India (Grant ID: CRG/2020/000520) and MANIT, Bhopal (Grant ID: Dean R&C 19/1021 dated 14–08–2019) both sanctioned to Dr Shivendra K. Chaurasiya. Rupal Rai is recipient of Teaching Assistantship from MANIT, Bhopal. Lab of Dr Anirudh K. Singh has been funded by DBT-Ramalingaswami Fellowship (BT/RLF/Re-Entry/17/2016) and ANRF, Startup research grant, India (Grant ID: SRG/2023/001498).

Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: ADC, albumin-dextrose-catalase; amp, ampicillin; CDRI, Central Drug Research Institute; *E.coli*, *Escherichia coli*; EV, empty vector; *fadD9*, Fatty acyl CoA synthetase; *gaba-at*, GABA-aminotransferase; *gabD2*, Succinic semialdehyde dehydrogenase; Gad, glutamate decarboxylase; *gdh*, Glutamate dehydrogenase; *glnA1*, glutamine synthetase; *gltD/gltD6468*, Glutamate synthase; hyg, hygromycin; *icl1*, Isocitratelyase; kan, kanamycin; *kdh*, α-Keto-glutarate Dehydrogenase; LB, Luria-Bertani; LBGT, LB with 0.5% glycerol and 0.05% tween 80; MDR, multidrug-resistant; MS, *Mycobacterium smegmatis*; Mtb, *Mycobacterium tuberculosis*; NCCS, National Centre of Cell Science; OADS, 1 × oleic acid-albumin-dextrose-saline; PBS, phosphate buffer saline; PknG, Protein kinase G; PMA, Phorbol 12-Myristate 13-acetate; qRT-PCR, Quantitative real time PCR; SSA, succinic semialdehyde; *sucA*, 2-oxoglutarate dehydrogenase; TB, Tuberculosis; TCA, tricarboxylic acid; TDR, totally drug-resistant; TraDIS, transposon-directed insertion site sequencing; V-ATPase, vacuolar proton-ATPase; wt, wild type; XDR, extensively drug-resistant.

References

1. Huang, L., Nazarova, E. V., and Russell, D. G. (2019) *Mycobacterium tuberculosis*: bacterial fitness within the host macrophage. *Microbiol. Spectr.* **7**. https://doi.org/10.1128/microbiolspec.bai-0001–2019
2. Stutz, M. D., Clark, M. P., Doerflinger, M., and Pellegrini, M. (2018) *Mycobacterium tuberculosis*: rewiring host cell signaling to promote infection. *J. Leukoc. Biol.* **103**, 259–268
3. Abraham, A. O., Nasiru, A. U., Abdulazeez, A. K., Seun, O. O., and Ogonna, D. W. (2020) Mechanism of drug resistance in *Mycobacterium tuberculosis*. *Am. J. Biomed. Sci. Res.* **7**, 378
4. Bañuls, A.-L., Sanou, A., Van Anh, N. T., and Godreuil, S. (2015) *Mycobacterium tuberculosis*: ecology and evolution of a human bacterium. *J. Med. Microbiol.* **64**, 1261–1269
5. Boritsch, E. C., and Brosch, R. (2016) Evolution of *Mycobacterium tuberculosis*: new insights into pathogenicity and drug resistance. *Microbiol. Spectr.* **4**. https://doi.org/10.1128/microbiolspec.tbtb2-0020–2016
6. Salgame, P. (2005) Host innate and Th1 responses and the bacterial factors that control *Mycobacterium tuberculosis* infection. *Curr. Opin. Immunol.* **17**, 374–380
7. Russell, D. G., Cardona, P.-J., Kim, M.-J., Allain, S., and Altare, F. (2009) Foamy macrophages and the progression of the human tuberculosis granuloma. *Nat. Immunol.* **10**, 943–948
8. Hayman, J. (1984) *Mycobacterium ulcerans*: an infection from Jurassic time? *Lancet Lond. Engl.* **2**, 1015–1016
9. Prasanthi, K. (2014) A brief review on ecology and evolution of mycobacteria. *Mycobact. Dis.* https://doi.org/10.4172/2161-1068.1000172
10. Adams, L. B., Dinauer, M. C., Morgenstern, D. E., and Krahenbuhl, J. L. (1997) Comparison of the roles of reactive oxygen and nitrogen intermediates in the host response to *Mycobacterium tuberculosis* using transgenic mice. *Tuber. Lung Dis.* **78**, 237–246
11. Schaible, U. E., Sturgill-Koszycki, S., Schlesinger, P. H., and Russell, D. G. (1950) (1998) Cytokine activation leads to acidification and increases maturation of *Mycobacterium avium*-containing phagosomes in murine macrophages. *J. Immunol. Baltim. Md.* **160**, 1290–1296
12. Vandal, O. H., Nathan, C. F., and Ehrt, S. (2009) Acid resistance in *Mycobacterium tuberculosis*. *J. Bacteriol.* **191**, 4714–4721
13. Chaurasiya, S. K. (2018) Tuberculosis: smart manipulation of a lethal host. *Microbiol. Immunol.* **62**, 361–379
14. Bouvier, G., Benoliel, A. M., Foa, C., and Bongrand, P. (1994) Relationship between phagosome acidification, phagosome-lysosome fusion, and mechanism of particle ingestion. *J. Leukoc. Biol.* **55**, 729–734
15. Hackam, D. J., Rotstein, O. D., and Grinstein, S. (1999) Phagosomal acidification mechanisms and functional significance. *Adv. Cell. Mol. Biol. Membr. Organelles.* **5**, 299–319
16. Sun-Wada, G.-H., Tabata, H., Kawamura, N., Aoyama, M., and Wada, Y. (2009) Direct recruitment of H+-ATPase from lysosomes for phagosomal acidification. *J. Cell Sci.* **122**, 2504–2513
17. Vandal, O. H., Pierini, L. M., Schnappinger, D., Nathan, C. F., and Ehrt, S. (2008) A membrane protein preserves intrabacterial pH in intraphagosomal *Mycobacterium tuberculosis*. *Nat. Med.* **14**, 849–854
18. MacMicking, J. D., Taylor, G. A., and McKinney, J. D. (2003) Immune control of tuberculosis by IFN-gamma-inducible LRG-47. *Science* **302**, 654–659
19. Levitte, S., Adams, K. N., Berg, R. D., Cosma, C. L., Urdahl, K. B., and Ramakrishnan, L. (2016) Mycobacterial acid tolerance enables phagolysosomal survival and establishment of tuberculous infection in vivo. *Cell Host. Microbe.* **20**, 250–258

20. Dragotakes, Q., Stouffer, K. M., Fu, M. S., Sella, Y., Youn, C., Yoon, O. I., *et al.* (2020) Macrophages use a bet-hedging strategy for antimicrobial activity in phagolysosomal acidification. *J. Clin. Invest.* **130**, 3805–3819
21. Baker, J. J., Dechow, S. J., and Abramovitch, R. B. (2019) Acid Fasting: modulation of *Mycobacterium tuberculosis* metabolism at acidic pH. *Trends Microbiol.* **27**, 942–953
22. Rai, R., Singh, V., Mathew, B. J., Singh, A. K., and Chaurasiya, S. K. (2022) Mycobacterial response to an acidic environment: protective mechanisms. *Pathog. Dis.* **80**, ftac032
23. Cotter, P. D., and Hill, C. (2003) Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiol. Mol. Biol. Rev.* **67**, 429–453
24. Lund, P. A., De Biase, D., Liran, O., Scheler, O., Mira, N. P., Cetecioglu, Z., *et al.* (2020) Understanding how microorganisms respond to acid pH is central to their control and successful exploitation. *Front. Microbiol.* **11**, 556140
25. Booth, I. R. (1985) Regulation of cytoplasmic pH in bacteria. *Microbiol. Rev.* **49**, 359–378
26. Chaurasiya, S. K., and Srivastava, K. K. (2009) Downregulation of protein kinase C-α enhances intracellular survival of Mycobacteria: role of PknG. *BMC Microbiol.* **9**, 271
27. Walburger, A., Koul, A., Ferrari, G., Nguyen, L., Prescianotto-Baschong, C., Huygen, K., *et al.* (2004) Protein kinase G from pathogenic mycobacteria promotes survival within macrophages. *Science* **304**, 1800–1804
28. Wolff, K. A., Nguyen, H. T., Cartabuke, R. H., Singh, A., Ogwang, S., and Nguyen, L. (2009) Protein kinase G is required for intrinsic antibiotic resistance in mycobacteria. *Antimicrob. Agents Chemother.* **53**, 3515–3519
29. Paroha, R., Chourasia, R., Mondal, R., and Chaurasiya, S. K. (2018) PknG supports mycobacterial adaptation in acidic environment. *Mol. Cell. Biochem.* **443**, 69–80
30. O'Hare, H. M., Durán, R., Cerveñansky, C., Bellinzoni, M., Wehenkel, A. M., Pritsch, O., *et al.* (2008) Regulation of glutamate metabolism by protein kinases in mycobacteria. *Mol. Microbiol.* **70**, 1408–1423
31. Maksymiuk, C., Balakrishnan, A., Bryk, R., Rhee, K. Y., and Nathan, C. F. (2015) E1 of α-ketoglutarate dehydrogenase defends *Mycobacterium tuberculosis* against glutamate anaplerosis and nitroxidative stress. *Proc. Natl. Acad. Sci.* **112**, E5834–E5843
32. Lee, J. J., Lim, J., Gao, S., Lawson, C. P., Odell, M., Raheem, S., *et al.* (2018) Glutamate mediated metabolic neutralization mitigates propionate toxicity in intracellular *Mycobacterium tuberculosis*. *Sci. Rep.* **8**, 8506
33. Cotter, P. D., Gahan, C. G. M., and Hill, C. (2001) A glutamate decarboxylase system protects *Listeria monocytogenes* in gastric fluid. *Mol. Microbiol.* **40**, 465–475
34. Capitani, G. (2003) Crystal structure and functional analysis of *Escherichia coli* glutamate decarboxylase. *EMBO J.* **22**, 4027–4037
35. Rai, R., Paroha, R., Rai, S., Singh, A. K., Chaurasia, R., Agarwal, N., *et al.* (2025) Cloning, expression, purification and characterization of glutamate decarboxylase (*Rv3432c*) from *Mycobacterium tuberculosis*. *Int. Microbiol.* https://doi.org/10.21203/rs.3.rs-4932796/v1
36. Opoku-Temeng, C., Onyedibe, K. I., Aryal, U. K., and Sintim, H. O. (2019) Proteomic analysis of bacterial response to a 4-hydroxybenzylidene indolinone compound, which re-sensitizes bacteria to traditional antibiotics. *J. Proteomics* **202**, 103368
37. Nandakumar, M., Nathan, C., and Rhee, K. Y. (2014) Isocitrate lyase mediates broad antibiotic tolerance in mycobacterium tuberculosis. *Nat. Commun.* **5**, 4306
38. Mueller, E. A., Egan, A. J., Breukink, E., Vollmer, W., and Levin, P. A. (2019) Plasticity of *Escherichia coli* cell wall metabolism promotes fitness and antibiotic resistance across environmental conditions. *eLife* **8**, e40754
39. Adam, M., Murali, B., Glenn, N. O., and Potter, S. S. (2008) Epigenetic inheritance based evolution of antibiotic resistance in bacteria. *BMC Evol. Biol.* **8**, 52
40. Nguyen, L. (2016) Antibiotic resistance mechanisms in *M. tuberculosis*: an update. *Arch. Toxicol.* **90**, 1585–1604
41. Tian, J., Bryk, R., Itoh, M., Suematsu, M., and Nathan, C. (2005) Variant tricarboxylic acid cycle in *Mycobacterium tuberculosis:* identification of α-ketoglutarate decarboxylase. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 10670–10675
42. Rizvi, A., Shankar, A., Chatterjee, A., More, T. H., Bose, T., Dutta, A., *et al.* (2019) Rewiring of metabolic network in *Mycobacterium tuberculosis* during adaptation to different stresses. *Front. Microbiol.* **10**, 2417
43. Serafini, A., Tan, L., Horswell, S., Howell, S., Greenwood, D. J., Hunt, D. M., *et al.* (2019) *Mycobacterium tuberculosis* requires glyoxylate shunt and reverse methylcitrate cycle for lactate and pyruvate metabolism. *Mol. Microbiol.* **112**, 1284–1307
44. Hubert, C. B., and de Carvalho, L. P. S. (2021) Convergence and divergence in the metabolic network of *Mycobacterium tuberculosis*. *Curr. Opin. Syst. Biol.* **28**, 100384
45. Borah, K., Mendum, T. A., Hawkins, N. D., Ward, J. L., Beale, M. H., Larrouy-Maumus, G., *et al.* (2021) Metabolic fluxes for nutritional flexibility of *Mycobacterium tuberculosis*. *Mol. Syst. Biol.* **17**, e10280
46. BERNEY, M., and BERNEY-MEYER, L. (2017) *Mycobacterium tuberculosis* in the face of host-imposed nutrient limitation. *Microbiol. Spectr.* **5**. https://doi.org/10.1128/microbiolspec.TBTB2-0030–2016
47. Agapova, A., Serafini, A., Petridis, M., Hunt, D. M., Garza-Garcia, A., Sohaskey, C. D., *et al.* (2019) Flexible nitrogen utilisation by the metabolic generalist pathogen Mycobacterium Tuberculosis. *Elife.* **8**, e41129
48. Borah, K., Girardi, K. D. C. V., Mendum, T. A., Lery, L. M. S., Beste, D. J. V., Lara, F. A., *et al.* (2019) Intracellular *Mycobacterium leprae* utilizes host glucose as a carbon source in schwann cells. *mBio* **10**, e02351-19
49. Gallant, J. L., Viljoen, A. J., Helden, P. D. van, and Wiid, I. J. F. (2016) Glutamate dehydrogenase is required by *Mycobacterium bovis BCG* for resistance to cellular stress. *PLoS One* **11**, e0147706
50. Viljoen, A. J., Kirsten, C. J., Baker, B., Helden, P. D. van, and Wiid, I. J. F. (2013) The role of glutamine oxoglutarate aminotransferase and glutamate dehydrogenase in nitrogen metabolism in *Mycobacterium bovis BCG*. *PLoS One* **8**, e84452
51. Harth, G., Masleša-Galić, S., Tullius, M. V., and Horwitz, M. A. (2005) All four *Mycobacterium tuberculosis glnA* genes encode glutamine synthetase activities but only GlnA1 is abundantly expressed and essential for bacterial homeostasis. *Mol. Microbiol.* **58**, 1157–1172
52. Harper, C. J., Hayward, D., Kidd, M., Wiid, I., and van Helden, P. (2010) Glutamate dehydrogenase and glutamine synthetase are regulated in response to nitrogen availability in *Myocbacterium smegmatis*. *BMC Microbiol.* **10**, 138
53. Tullius, M. V., Harth, G., and Horwitz, M. A. (2003) Glutamine synthetase GlnA1 is essential for growth of *Mycobacterium tuberculosis* in human THP-1 macrophages and Guinea pigs. *Infect. Immun.* **71**, 3927–3936
54. Chandra, H., Basir, S. F., Gupta, M., and Banerjee, N. (2010) Glutamine synthetase encoded by glnA-1 is necessary for cell wall resistance and pathogenicity of *Mycobacterium bovis*. *Microbiology* **156**, 3669–3677
55. Choudhary, E., Sharma, R., Pal, P., and Agarwal, N. (2022) Deciphering the proteomic landscape of *Mycobacterium tuberculosis* in response to acid and oxidative stresses. *ACS Omega* **7**, 26749–26766
56. Baker, J. J., and Abramovitch, R. B. (2018) Genetic and metabolic regulation of *Mycobacterium tuberculosis* acid growth arrest. *Sci. Rep.* **8**, 4168
57. Karatzas, K.-A. G., Suur, L., and O'Byrne, C. P. (2012) Characterization of the intracellular glutamate decarboxylase system: analysis of its function, transcription, and role in the acid resistance of various strains of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* **78**, 3571–3579
58. Lyu, C., Zhao, W., Peng, C., Hu, S., Fang, H., Hua, Y., *et al.* (2018) Exploring the contributions of two glutamate decarboxylase isozymes in *Lactobacillus brevis* to acid resistance and γ-aminobutyric acid production. *Microb. Cell Factories.* **17**, 180
59. Sezgin, E., and Tekin, B. (2023) Molecular evolution and population genetics of glutamate decarboxylase acid resistance pathway in lactic acid bacteria. *Front. Genet.* **14**, 1027156
60. Cowley, S., Ko, M., Pick, N., Chow, R., Downing, K. J., Gordhan, B. G., *et al.* (2004) The *Mycobacterium tuberculosis* protein serine/threonine kinase PknG is linked to cellular glutamate/glutamine levels and is important for growth in vivo. *Mol. Microbiol.* **52**, 1691–1702
61. Khan, M. Z., and Nandicoori, V. K. (2021) Deletion of pknG abates reactivation of latent *Mycobacterium tuberculosis* in mice. *Antimicrob. Agents Chemother.* **65**, e02095-20

62. Khan, M. Z., Bhaskar, A., Upadhyay, S., Kumari, P., Rajmani, R. S., Jain, P., *et al.* (2017) Protein kinase G confers survival advantage to *Mycobacterium tuberculosis* during latency-like conditions. *J. Biol. Chem.* **292**, 16093–16108
63. Baek, S.-H., Li, A. H., and Sassetti, C. M. (2011) Metabolic regulation of mycobacterial growth and antibiotic sensitivity. *PLoS Biol.* **9**, e1001065
64. Samuels, A. N., Wang, E. R., Harrison, G. A., Valenta, J. C., and Stallings, C. L. (2022) Understanding the contribution of metabolism to *Mycobacterium tuberculosis* drug tolerance. *Front. Cell. Infect. Microbiol.* **12**, 958555
65. Quinonez, C. G., Lee, J. J., Lim, J., Odell, M., Lawson, C. P., Anyogu, A., *et al.* (2022) The role of fatty acid metabolism in drug tolerance of *Mycobacterium tuberculosis*. *mBio* **13**, e03559-21
66. Mouton, J. M., Heunis, T., Dippenaar, A., Gallant, J. L., Kleynhans, L., and Sampson, S. L. (2019) Comprehensive characterization of the attenuated double auxotroph *Mycobacterium tuberculosisΔleuDΔpanCD* as an alternative to H37Rv. *Front. Microbiol.* **10**, 1922
67. Singh, A. K., Dutta, D., Singh, V., Srivastava, V., Biswas, R. K., and Singh, B. N. (2015) Characterization of *Mycobacterium smegmatis sigF* mutant and its regulon: overexpression of SigF antagonist (MSMEG_1803) in *M. smegmatis* mimics sigF mutant phenotype, loss of pigmentation, and sensitivity to oxidative stress. *MicrobiologyOpen* **4**, 896–916
68. Singh, A. K., and Singh, B. N. (2009) Differential expression of sigH paralogs during growth and under different stress conditions in *Mycobacterium smegmatis*. *J. Bacteriol.* **191**, 2888–2893