The Role of Glutamine Oxoglutarate Aminotransferase and Glutamate Dehydrogenase in Nitrogen Metabolism in *Mycobacterium bovis* BCG

Albertus J. Viljoen*, Catriona J. Kirsten, Bienyameen Baker, Paul D. van Helden, Ian J. F. Wiid

DST/NRF Centre of Excellence for Biomedical Tuberculosis Research, MRC Centre for Molecular and Cellular Biology, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, Cape Town, South Africa

Abstract

Recent evidence suggests that the regulation of intracellular glutamate levels could play an important role in the ability of pathogenic slow-growing mycobacteria to grow in vivo. However, little is known about the in vitro requirement for the enzymes which catalyse glutamate production and degradation in the slow-growing mycobacteria, namely; glutamine oxoglutarate aminotransferase (GOGAT) and glutamate dehydrogenase (GDH), respectively. We report that allelic replacement of the *Mycobacterium bovis* BCG *gltBD-* operon encoding for the large (*gltB*) and small (*gltD*) subunits of GOGAT with a hygromycin resistance cassette resulted in glutamate auxotrophy and that deletion of the GDH encoding-gene (*gdh*) led to a marked growth deficiency in the presence of L-glutamate as a sole nitrogen source as well as reduction in growth when cultured in an excess of L-asparagine.

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* Email: ajvil@sun.ac.za

Introduction

The nitrogen metabolic pathways of pathogenic mycobacteria are factors which allow the bacteria to survive and replicate in host cells [1,2]. These pathways may be a potential source of novel target molecules that could be exploited in future drug development. Central nitrogen metabolism in slow growing mycobacteria mainly involves the biochemical pathways that fix inorganic ammonium and produce glutamine, glutamate and aspartate (Figure 1) [3]. These three amino acids act as precursors or nitrogen donors in the production of nearly all other nitrogenous molecules in the cell.

Glutamine synthetase (GS), which assimilates inorganic ammonium for the production of glutamine, has been studied extensively in *Mycobacterium tuberculosis* and related mycobacteria. The gene *glnA1*, which encodes the major isoform of GS in *M. tuberculosis* was shown to be essential for virulence in guinea pigs [2]. GS activity in *M. tuberculosis* is regulated at both the transcriptional and post-translational level. The latter is achieved by an adenylyl transferase.

Figure 1. Genes involved in nitrogen metabolism in the slow growing mycobacterium *M. bovis* BCG. Ammonia is assimilated in the production of L-glutamine and L-glutamate. Together, L-glutamine, L-glutamate, and L-aspartate act as precursors or nitrogen donors to most other nitrogenous compounds in the mycobacterium. The map was constructed from the combined PATRIC pathways for *M. bovis* BCGstr Pasteur 1743P2 nitrogen metabolism and alanine, aspartate and glutamate metabolism [3]. Genes were assigned to the EC numbers by PATRIC and/or Refseq and/or Legacy BRC. doi: 10.1371/journal.pone.0084452.g001
encoded by the gene glnE [4–7]. It has been shown that a glnE deletion mutant of _M. tuberculosis_ is only viable when GS is inhibited by methionine sulfoximine and the culture supplemented with glutamine [6]. Furthermore, the serine threonine protein kinase G (PknG) which is involved in the homeostatic regulation of glutamine and glutamate levels has been shown to be important to _in vivo_ growth of _M. tuberculosis_ [8]. This indicates that central nitrogen metabolism is tightly regulated in _M. tuberculosis_. Although an interaction between GlnE and PknG in the control of glutamine metabolism was suggested, it has not been investigated [8]. It was found, however, that PknG phosphorylates glycogen accumulation regulator A (GarA), thereby modulating its interaction with the glutamate producing enzyme, glutamate oxoglutarate aminotransferase (GOGAT) and the glutamate catabolizing enzyme, glutamate dehydrogenase (GDH) [9,10]. Phosphorylation of GarA at residue threonine 21 by PknG is thought to abrogate both its inhibition of GDH activity and its stimulation of GOGAT activity, leading to a decrease in glutamate levels [10]. These results suggest that the tight regulation of glutamate levels might be important to the survival and proliferation of _M. tuberculosis_ during infection of the host.

Although it was found by Himar-1 based transposon mutagenesis that the genes _gltB_ (encodes for the large subunit of GOGAT), _gltD_ (encodes for the small subunit of GOGAT) and _gdh_ are essential to the _in vitro_ growth of _M. tuberculosis_ [1,11,12], we could disrupt _gltBD_ (BCG_3922c-BCG_3921c) and _gdh_ (BCG_2496c) in _Mycobacterium bovis_ BCG, a closely related slow growing mycobacterium. _M. bovis_ BCG is considered to be non-pathogenic to humans, but it does survive in macrophages and maintains a degree of virulence [13]. We show that while GOAT is required for the _de novo_ synthesis of glutamate, GDH is important for the utilization of glutamate as a sole nitrogen source and for growth with high levels of asparagine in the culture medium even in combination with glutamate and/or ammonium.

**Materials and Methods**

**Growth of bacteria**

All bacterial strains used are listed in Table S1. _Escherichia coli_ was cultured with shaking at 200 rpm in Lysogeny Broth (LB) and on LB agar at 37°C. _M. bovis_ BCG was cultured without agitation in Difco Middlebrook 7H9 liquid medium (Becton Dickinson, USA) supplemented with 10% (v/v) ADC (50 g/L bovine serum albumin fraction V, 20 g/L D-glucose, 15 mg/L catalase), 0.2% (v/v) glycerol, and 0.05% (v/v) Tween 80 in 25 cm² (5 – 10 ml) and 75 cm² (30 ml) cell culture flasks (Nunc, Denmark) and on BBL 7H11 agar base (Becton Dickinson, USA) supplemented with 0.5% (v/v) glycerol and 10% (v/v) BBL Middlebrook OADC (Becton Dickinson, USA). In order to investigate growth of bacteria in the presence of different nitrogen sources, we prepared modified Middlebrook 7H9 medium which lacked the nitrogen sources present in 7H9, namely L-glutamate, ammonium sulphate and ferric ammonium citrate (-N7H9; sodium citrate, 0.1 g/L; pyridoxine, 1 mg/L; biotin, 0.5 mg/L; disodium phosphate, 2.5 g/L; monopotassium phosphate, 1 g/L; ferric citrate, 40 mg/L; magnesium sulphate, 50 mg/L; CaCl₂, 0.5 mg/L; ZnSO₄, 1 mg/L; CuSO₄, 1 mg/L; glycerol, 0.2% v/v; Tween80, 0.05% v/v; ADC, 10% v/v), which was subsequently supplemented with different nitrogen sources as indicated in the text. _M. bovis_ BCG starter cultures maintained in 7H9 were washed twice with –N7H9 before they were used to inoculate –N7H9 medium supplemented with different nitrogen sources for growth curve determinations. Antibiotic concentrations used in _M. bovis_ BCG cultures were as follows: hygromycin, 50 μg/ml on solid medium and 25 μg/ml in liquid medium; kanamycin, 20 μg/ml; and gentamicin, 2.5 μg/ml. Antibiotic concentrations used in _E. coli_ cultures were: ampicillin, 50 μg/ml; hygromycin, 100 μg/ml; kanamycin, 50 μg/ml; and gentamicin, 5 μg/ml.

**Generation of ΔgltBD::hyg and ΔgltBD::hyg attB::pGCgltBD strains**

All oligonucleotides and plasmids used are listed in Table S1. All molecular cloning procedures were carried out as described elsewhere [14]. The mycobacterial recombineering method developed by van Kessel et al. (2007) was used to replace the _gltB_ operon with a hygromycin cassette [15]. Briefly, the specific oligonucleotides _UgltBDF_ and _UgltBDR_ harbouring Spfl and NcoI restriction endonuclease recognition sites respectively, were used to generate a 499bp PCR fragment of the region directly upstream of the _gltB_ operon. The PCR fragment was cloned into pGEM-T Easy, which was subsequently digested with Spfl and NcoI to obtain a restriction fragment of the upstream (U) region. The restriction fragment was directionally cloned into the Spfl-NcoI sites of pMNFhyg to obtain pMNFhygU. The specific oligonucleotides _DgltBDF_ and _DgltBDR_, harbouring Spel and PstI restriction endonuclease recognition sites respectively, were used to generate a 511bp PCR fragment of the region directly downstream (D) of the _gltB_ operon. The PCR fragment was cloned into pGEM-T Easy, and subsequently digested with Spel and PstI to obtain a restriction fragment of the D region.

The restriction fragment was directionally cloned into the Spfl-NcoI sites of pMNFhygU to produce pAVΔgltBD. The linear allelic exchange substrate was obtained by digesting pAVΔgltBD with Spfl and PstI, and electro-transformed into _M. bovis_ BCG carrying the pJV53 recombineering plasmid (wt-BCG pJV53) as described previously [15]. Before electro-transformation, wt-BCG pJV53 was cultured in 7H9 supplemented with 0.05% Tween 80 and 0.2% (w/v) succinate to a density of approximately OD₆00 = 0.5 at which point acetamide was added to a final concentration of 0.2% (w/v). The culture was incubated overnight at 37°C without agitation and used to prepare electrocompetent cells as described previously [16,17]. Hygromycin resistant colonies were subjected to PCR and Southern blot analysis as described elsewhere (see section on Southern blot analysis). The ΔgltBD::hyg mutant strain (from here on referred to as ΔgltBD) was complemented with a functional _gltBD_ operon using the integrating vector pGINTO [18]. Briefly, the _gltB_ operon, along with a 525bp region upstream of the gene was amplified by PCR using Phusion High Fidelity PCR polymerase (Finnzymes, Finland) and the specific oligonucleotides CgltBDF and CgltBDR. The CgltBDF fragment was ligated to...
pGINTO that was linearized with Scal to produce pGCgltBD. Electro-competent **ΔgltBD** bacteria were transformed with pGCgltBD. Complemented colonies (referred to as **ΔgltBD attB**:pGCgltBD) were gentamicin resistant. The progenitor strain of the **ΔgltBD** mutant, wt-BCG pJV53, was included in analyses (as indicated in the text) to control for residual effects of the recombineering plasmid. All fragments generated by PCR within pAVΔgltBD and pGCgltBD were subjected to DNA sequencing (Stellenbosch University Central Analytical Facility) to confirm that no mutations were introduced by the polymerases.

**Generation of **Δgdh** and **Δgdh attB**:pGCgdh strains**

All oligonucleotides and plasmids used are listed in Table S1 and all molecular cloning procedures were carried out as described elsewhere [14]. The **gdh** gene was disrupted by allelic-exchange using the plasmids p2Nil and pGOAL17 [19]. Briefly, a fragment that spans the GDH domain of **gdh**, as well as approximately 1kb of the 5’ and 3’ sequences flanking the GDH domain, was amplified by PCR with long PCR enzyme mix (Fermentas, USA) using the specific oligonucleotides gdhF and gdhR. The gdh PCR amplicon was cloned into the pGEM-T Easy vector to generate pGEMgdh. The central in-frame NruI fragment spanning the GDH domain (see Figure 2A) was subsequently excised to obtain the vector pGEMΔgdh containing adjacent 5’ and 3’ flanking sequences of the GDH domain. The KpnI fragment containing the GDH domain flanking regions was excised from pGEMΔgdh and cloned into the KpnI site of p2Nil to produce p2NilΔgdh. Finally, a PacI cassette containing the genes sacB and lacZ from pGOAL17 [19] was excised and cloned into the PacI site of p2NilΔgdh to produce pAVΔgdh. The pAVΔgdh deletion construct was treated with 100 mJ UV irradiation prior to electro-transformation into *M. bovis* BCG as described previously [20]. An exponentially growing *M. bovis* BCG culture (OD<sub>600</sub> 0.5-0.8) was made electro-competent and electro-transformed with pAVgdh (see section on generation of **ΔgltBD** strain). Bacteria in which pAVΔgdh was integrated into the chromosome by homologous recombination were resistant to kanamycin (kan<sup>R</sup>) and coloured blue in the presence of 50 μg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal). Hence, blue kan<sup>R</sup> colonies were transferred to liquid culture without antibiotics, and all molecular cloning procedures were carried out as described previously [14]. The **gdh** PCR amplicon was cloned into the pGEM-T Easy vector to generate pGEMgdh. The central in-frame NruI fragment spanning the GDH domain (see Figure 2A) was subsequently excised to obtain the vector pGEMΔgdh containing adjacent 5’ and 3’ flanking sequences of the GDH domain. The KpnI fragment containing the GDH domain flanking regions was excised from pGEMΔgdh and cloned into the KpnI site of p2Nil to produce p2NilΔgdh. Finally, a PacI cassette containing the genes sacB and lacZ from pGOAL17 [19] was excised and cloned into the PacI site of p2NilΔgdh to produce pAVΔgdh. The pAVΔgdh deletion construct was treated with 100 mJ UV irradiation prior to electro-transformation into *M. bovis* BCG as described previously [20]. An exponentially growing *M. bovis* BCG culture (OD<sub>600</sub> 0.5-0.8) was made electro-competent and electro-transformed with pAVgdh (see section on generation of **ΔgltBD** strain). Bacteria in which pAVΔgdh was integrated into the chromosome by homologous recombination were resistant to kanamycin (kan<sup>R</sup>) and coloured blue in the presence of 50 μg/ml 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal). Hence, blue kan<sup>R</sup> colonies were transferred to liquid culture without antibiotics, and all molecular cloning procedures were carried out as described elsewhere [14]. The **gdh** gene was disrupted by allelic-exchange using the plasmids p2Nil and pGOAL17 [19]. Briefly, a fragment that spans the GDH domain of **gdh**, as well as approximately 1kb of the 5’ and 3’ sequences flanking the GDH domain, was amplified by PCR with long PCR enzyme mix (Fermentas, USA) using the specific oligonucleotides gdhF and gdhR. The gdh PCR amplicon was cloned into the pGEM-T Easy vector to generate pGEMgdh. The central in-frame NruI fragment spanning the GDH domain (see Figure 2A) was subsequently excised to obtain the vector pGEMΔgdh containing adjacent 5’ and 3’ flanking sequences of the GDH domain. The **Δgdh** mutant strain was generated by PCR using the specific oligonucleotides PgdhF and PgdhR (Table S1) and the pGEMΔgdh construct as template.

**Statistical analysis**

Statistical analyses were carried out with the statistics software GraphPad Prism version 5.01. All differences in colony forming unit (cfu) data for the growth curve analyses were evaluated as randomised block design experiments by two-way repeated measures ANOVA and Bonferroni post-tests. In each analysis, the matching of subjects (strains) between experimental repeats was efficient (p < 0.05). Probabilities of < 0.05 were considered significant.

**Results**

**Generation of **ΔgltBD** and **Δgdh** strains**

Putative **ΔgltBD** colonies and **Δgdh** colonies were initially screened by PCR (data not shown) before analysis by Southern blot (Figure 2). When Southern blot analysis was done with probes that hybridised in the region directly upstream of **gltBD** (a 1639 bp SphI fragment spanning the start codon of the **gltBD** operon) or directly downstream of **gltBD** (a 6305 bp SphI fragment which spans the 3’ portion of **gltBD**, the entire **gltBD** and two genes downstream of **gltD**). The corresponding fragments were observed for wt-BCG (Figure 2B). Neither of these fragments were observed for the **ΔgltBD** mutant (Figure 2B). However a single fragment corresponding to the expected 4065 bp was observed for the **ΔgltBD** mutant when either probe was used (Figure 2B). A fragment corresponding to the expected 6261 bp KpnI fragment spanning **gldh** was observed for wt-BCG, whereas a fragment corresponding to the 3774 bp KpnI fragment spanning **gldh**, containing a 2487 bp deletion, was detected for the **Δgdh** mutant (Figure 2D). Growth of the **ΔgltBD** mutant was markedly slower on both 7H11 and 7H10
slower than on 7H11) than growth of wt-BCG or the complemented ΔgltBD strain (Figure S1). However, growth of the Δgdh mutant was comparable to that of wt-BCG on both 7H10 and 7H11.

**Growth of wt-BCG in 7H9 containing different nitrogen sources**

In order to investigate the *in vitro* growth requirements of the ΔgltBD and Δgdh mutants, we modified both 7H9 (containing 3.4 mM L-Glu and 3.8 mM ammonium sulphate, see materials and methods) and -N7H9 (see materials and methods) by supplementation with different nitrogen sources (see Table S2). wt-BCG growth in all formulations was comparable to that in 7H9, except in the case of -N7H9 without nitrogen source supplementation (Figure 3, ) or with 3 mM L-Ala (Figure 3, ). This result is in line with a previous finding that *M. bovis* BCG cannot utilize alanine as a sole nitrogen source because of a frame-shift mutation in the gene that encodes for alanine dehydrogenase (*ald*) and inhibition of GS by non-catabolised alanine [22].

**In vitro requirement of gltBD**

The growth of the ΔgltBD mutant was markedly impaired in standard 7H9, which contains 3.4 mM L-Glu and 3.8 mM (NH₄)₂SO₄ (Figure 4A, ), but was restored to wt-BCG levels when an additional 10 mM L-Glu was introduced in the medium (Figure 4B, ). Moreover, no growth of the ΔgltBD mutant was observed when ammonium was used as sole nitrogen source (Figure 4C, and D, ). Growth of the ΔgltBD mutant was comparable to that in 7H9 when L-Glu (Figure 4E, ) or L-Asn (Figure 4F, ), but not when L-Gln (Figure 4G, ) was added as sole nitrogen sources. In addition, growth of the ΔgltBD mutant was better in medium containing aspartate as a sole nitrogen source (Figure 4H, ) or in combination with glutamine (Table S2) than in 7H9. L-asparagine is deaminated to L-aspartate by the L-asparaginase encoded by *ansA* [23,24]. Production of L-
glutamate from L-aspartate could possibly be done by an aspartate aminotransferase (EC 2.6.1.1) (Figure 1). Although not assigned to EC 2.6.1.1 in the KEGG pathways for alanine, glutamate and asparagine metabolism in *M. bovis* BCG, two genes were annotated with this EC number in the PATRIC version of the KEGG pathway, namely aspB and BCG_3782c [3,25,26]. Glutamate synthesis from glutamine could be catalysed by the asparagine synthetase encoded by asnB, but this would require aspartate as a substrate which may explain the suppression of the ΔgltBD mutant’s growth when L-Gln is the sole nitrogen source [25,26]. While the other strains investigated in this study grew exponentially for a short duration when cultured in 7H9 media stripped of the nitrogen sources glutamate, ammonium sulphate and ferric ammonium citrate (the ADC supplement may be a source of trace nitrogen which may be utilized by the bacteria), growth of the ΔgltBD mutant (Figure 4I,) was inhibited, suggesting that glutamate production by GOGAT may support some growth under these conditions. The importance of the GS/GOGAT system to nitrogen assimilation during limiting nitrogen conditions is well documented for several organisms including *M. tuberculosis* and *M. smegmatis* (for a review, see 27,28). Since GDH can catalyse the reductive amination of 2-oxoglutarate to produce production by GOGAT may support some growth under these conditions. The importance of the GS/GOGAT system to nitrogen assimilation during limiting nitrogen conditions is well documented for several organisms including *M. tuberculosis* and *M. smegmatis* (for a review, see 27,28). Since GDH can catalyse the reductive amination of 2-oxoglutarate to produce glutamate, we hypothesised that excess ammonium would complement glutamate auxotrophy through GDH activity. However, supplementation of 7H9 with 30 mM ammonium sulphate (Figure 4J,) did not ameliorate the poor growth of the mutant, suggesting that GDH does not produce glutamate in *M. bovis* BCG.

In vitro requirement of gdh

Growth of the Δgdh mutant (Figure 5A,) was comparable to that of wt-BCG (Figure 5A,) in the standard 7H9 formulation, but markedly impaired when L-Glu was the sole nitrogen source (Figure 5B,). Supplementation of 7H9 with as high concentration (30 mM) of L-Glu lead to a slight repression of growth of the Δgdh mutant (Figure 5C,) in comparison to wt-BCG (Figure 5C,) and the complemented strain (Figure 5C,).

However, growth of the Δgdh mutant was markedly impaired when L-Asn was present in standard 7H9 (Figure 5D,) or – N7H9 (Figure 5E,). Greater suppression of the Δgdh mutant’s growth was observed in the presence of 30 mM L-Asn (Figure 5D and E,) than in the presence of 3 mM L-Asn (Figure 5F and G,) and it was not suppressed by supplementation of 7H9 with 30 mM L-Asp (Figure 5H,). Addition of 1mM ammonium sulphate to the -N7H9 + 3 mM L-Glu markedly improved the growth of the Δgdh mutant (Figure 5I,). After approximately 3 weeks of culture in medium containing 3 mM L-Glu as only nitrogen source, the optical density of Δgdh cultures started to increase (Figure 5B,). Contaminating micro-organisms as a source of the increase in turbidity were not detected by Ziehl-Neelsen staining as previously described [29] or by spreading out 100 μl of the cultures on blood agar plates (Becton Dickinson, USA). When aliquots of the three week old Δgdh mutant -N7H9 + 3 mM L-Glu cultures were washed and used to inoculate fresh -N7H9 + 3 mM L-Glu, immediate growth was observed (Figure 5J,). Aliquots of the 3 week old -N7H9 + 3 mM L-Glu Δgdh cultures were also spread onto 7H11 plates and single colonies obtained. The single colonies were grown in 7H9 to mid-log phase and frozen stocks were prepared. Frozen stock cultures were thawed and cultured to mid-log phase and growth curve determinations were subsequently performed in -N7H9 + 3 mM L-Glu. Out of 7 colonies analysed, 6 colonies had similar growth profiles to that of wt-BCG (Figure 5K; A1, ; A2, ; B1, ; B2, ; C2, ; C3, ). These colonies did not have a severe growth defect in 7H9 + 30 mM L-Asn either (Figure S2). To exclude wt-BCG and the Δgdh complement strain as contaminating sources of the increase in turbidity observed, the colonies were analysed by PCR (Figure S3). These results suggest that a currently unknown genetic adaptation compensates for the loss of GDH activity in these colonies.

Discussion

Despite previous reports that gltB, gltD, and gdh, are required for the in vitro growth of *M. tuberculosis* [1,11,12], we successfully generated a gene replacement mutant of the entire gltBD operon as well as an unmarked deletion mutant of gdh in *M. bovis* BCG. *M. bovis* BCG gltB, gltD and gdh each share 99% protein sequence identity with their homologues in *M. tuberculosis* (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Our success in generating the ΔgltBD mutant is possibly due to our use of 7H11 agar which has a very similar formulation to 7H10 but also contains casitone. The additional casitone in 7H11 enhances growth of fastidious mycobacteria [30]. It is possible that transposon-insertion mutants of gltB and gltD were under-represented in the transposon libraries used in the transposon site hybridisation (TraSH) experiments because of an inadequate concentration of glutamate in the culture media [1,11]. We found that growth of the GOGAT-deficient mutant was only fully restored to wt-BCG levels when 10 mM L-Glu was added to 7H9 medium already containing 3.4 mM L-Glu. Interestingly, the Δgdh mutant grew at the same rate as wt-BCG in 7H9 and on 7H10, suggesting that gdh is dispensable for optimal growth in *M. bovis* BCG. This may represent a
Figure 4. Growth of ΔgltBD in 7H9 with different nitrogen sources. Growth of wt-BCG, the ΔgltBD mutant and the ΔgltBD complemented strain in (A) standard 7H9 containing approximately 4 mM ammonium sulphate (AS, (NH₄)₂SO₄) and 3 mM L-Glu, (B) 7H9 + 10 mM L-Glutamate, or (C) -N7H9 (nitrogen-depleted 7H9) + 4 mM AS. Growth of the ΔgltBD mutant in (D) -N7H9 + 4 mM AS supplemented with increasing concentrations of glutamate. Cultures for cfu/ml determinations were inoculated to OD₆₀₀ = 0.0005 (cfu/ml of approximately 10⁵). Log₁₀(cfu/ml) of ΔgltBD cultured in -N7H9 + 4 mM AS supplemented with 10 mM L-Glu was different from log₁₀(cfu/ml) of ΔgltBD cultured in -N7H9 + 4 mM AS at every time point after and including 3 days (P < 0.001). Log₁₀(cfu/ml) of ΔgltBD cultured in -N7H9 + 4 mM AS supplemented with 3 mM L-Glu was different from log₁₀(cfu/ml) of ΔgltBD cultured in -N7H9 + 4 mM AS at 3 days (P < 0.01) and every following time point (P < 0.001). Growth of wt-BCG, the ΔgltBD mutant and the ΔgltBD complement strain in (E) -N7H9 + 3 mM L-Glu, (F) -N7H9 + 3 mM L-Asn, (G) -N7H9 + 3 mM L-Gln, (H) -N7H9 + 3 mM L-Asp, (I) unmodified -N7H9, or (J) 7H9 + 30 mM AS. Mean OD measurements with standard deviations presented in panels A-C and E-J and mean log₁₀(cfu/ml) with standard errors presented in panel D were calculated with growth curve data obtained from three independent experiments performed for each condition tested. In some instances error bars were smaller than the symbols used to depict the means. AS, ammonium sulphate.

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Growth of Δgdh in 7H9 with different nitrogen sources. Growth of wt BCG, the Δgdh mutant and the Δgdh complemented strain in (A) standard 7H9 containing approximately 4 mM ammonium sulphate (AS, (NH₄)₂SO₄) and 3 mM L-Glu, (B) -N7H9 (nitrogen-depleted 7H9) + 3 mM L-Glu, (C) 7H9 + 30 mM L-Glu, (D) 7H9 + 30 mM L-Asn, (E) -N7H9 + 30 mM L-Asn, (F) 7H9 + 3 mM L-Asn, (G) -N7H9 + 3 mM L-Asn, or (H) 7H9 + 30 mM L-Asp. Growth of the Δgdh mutant in (I) -N7H9 + 3 mM L-Glu supplemented with increasing concentrations of AS. Cultures for cfu/ml determinations were inoculated to OD₆₀₀ = 0.0005 (cfu/ml of approximately 10⁵). Log₁₀(cfu/ml) of Δgdh cultured in -N7H9 + 3 mM Glu was different from log₁₀(cfu/ml) of Δgdh cultured in -N7H9 + 3 mM Glu supplemented with 1 mM AS at day 9 and 14 (p < 0.01). Log₁₀(cfu/ml) of Δgdh cultured in 7H9 + 30 mM L-Asn was different from log₁₀(cfu/ml) of Δgdh cultured in -N7H9 + 3 mM Glu supplemented with 1 mM AS at day 6, 9 and 14 (p < 0.01). (J) Growth of the Δgdh mutant cultured in -N7H9 + 3 mM L-Glu for three weeks when sub-cultured in fresh 7H9 or -N7H9 + 3 mM L-Glu. Aliquots of three week old Δgdh mutant -N7H9 + 3 mM L-Glu cultures were washed once with -N7H9 and used to inoculate fresh 7H9 or -N7H9 + 3 mM L-Glu to an OD₆₀₀ = 0.020. (K) Determination of growth of single colonies obtained from three week old Δgdh mutant -N7H9 + 3 mM L-Glu cultures in fresh -N7H9 + 3 mM L-Glu. A1 and B1 were obtained from the first growth curve experiment, B1 and B2 from the second and C1-C3 from the third. Mean OD measurements with standard deviations presented in panels A-H and J and mean log₁₀(cfu/ml) with standard errors presented in panel I were calculated with growth curve data obtained from three independent experiments performed for each condition tested. In some instances error bars were smaller than the symbols used to depict the means. AS, ammonium sulphate.

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physiological difference between *M. bovis* BCG and *M. tuberculosis* which could possibly be as a result of the regions of difference in the *M. bovis* BCG genome.

We showed that GDH is important for the deamination of glutamate in *M. bovis* BCG. A physiological activity favouring the deamination of glutamate is also characteristic of other members of the L180 class of GDHs [31–34]. To our knowledge this is the first study to show that a member of the L180 class of GDHs is required for optimal growth of an organism when L-glutamate is the sole nitrogen source. Interestingly, it was previously reported that supplementation of Sauton medium containing L-Glu as nitrogen source with L-Asn resulted in an increase in extracellular Glu concentration and decreased growth of *M. tuberculosis* [8].

M. tuberculosis Strains were complement strain on 7H10 and 7H11 agar. A fresh –N7H9 + 3 mM L-Glu or (B) fresh 7H9 + 30 mM L-Asn. day old glutamate in dilution series was made in 7H9 and each dilution spotted (10 μl) onto the agar, which was then incubated at 37°C. A) Differential PCR amplification patterns obtained using the specific oligonucleotides used in this study. B) Arrangement of genes in the Δgdh mutant chromosome region where the disrupted *gdh* is located and at the *attB* locus where pGCgdh is integrated into the chromosome. D) Gel image showing differential amplification patterns obtained when PCR was performed using the specific oligonucleotides gdhHR3R and gdhcomp10 which amplified a 1029bp product form wt-BCG template DNA (lane 1), but not from Δgdh mutant (lane 2), Δgdh complement (lane 3) or from template DNA prepared from seven single colonies obtained from 22 day old Δgdh –N7H9 + 3mM L-Glu cultures (lanes 4 - 10). E) Differential PCR amplification patterns obtained using the specific oligonucleotides gdhHR3R and gdhcomp10 which amplified a 3213bp product form wt-BCG template DNA (lane 1), but a 726bp product from Δgdh mutant (lane 2). Δgdh complement (lane 3) and from template DNA prepared from seven single colonies obtained from 22 day old Δgdh –N7H9 + 3mM L-Glu cultures (lanes 4 - 10). F) Differential PCR amplification patterns obtained using the specific oligonucleotides gdhHR8R, gdhcomp6 and gdhcomp10 which amplified a 670bp product from wt-BCG template DNA (lane 1), a 373bp product from Δgdh mutant template DNA (lane 2) and both a 670bp and a 373bp product from Δgdh complement DNA template DNA (lane 3). This primer combination only amplified a 373bp from template DNA prepared from the seven single colonies obtained from 22 day old Δgdh –N7H9 + 3mM L-Glu cultures. Lane 11 (D, E and F) - negative control.

Supporting Information

Table S1. Bacterial Strains, plasmids, and oligonucleotides used in this study.

(DOCX)

Table S2. Growth parameters of ΔgltBD and Δgdh mutant and complemented strains relative to wt-BCG in 7H9 and –N7H9 supplemented with different nitrogen sources.

(DOCX)

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Author Contributions

Conceived and designed the experiments: AJV CJK BB PDvH IJFW. Performed the experiments: AJV. Analyzed the data: AJV. Wrote the manuscript: AJV CJK BB PDvH IJFW. Interpreted the data: AJV CJK BB PDvH IJFW.
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