Identification of the *Mycobacterium tuberculosis* GlnE promoter and its response to nitrogen availability

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**INTRODUCTION**

*Mycobacterium tuberculosis* is an extremely successful human pathogen that causes the largest number of deaths due to a single bacterial infection worldwide. The bacilli are able both to avoid and to subvert the host immune response, in part due to their ability to replicate within macrophages. In addition, these organisms have the remarkable ability to persist within the human lung for decades prior to reactivation. It has been estimated that one-third of the world’s population is latently infected. These figures suggest that *M. tuberculosis* is well adapted to its niche and reinforce the need for a greater understanding of its biology in order to eradicate this devastating disease.

Relatively little is known about nitrogen metabolism in *M. tuberculosis* and most work has focused on the study of the major glutamine synthetase (GS), GlnA1, the key enzyme in ammonia assimilation. This enzyme is responsible for the ATP-dependent incorporation of ammonia into L-glutamate to form L-glutamine. During growth in axenic culture, GlnA1 is expressed to a high level, resulting in its secretion into the culture filtrate (Harth et al., 1994; Tullius et al., 2001). Inhibition of this enzyme directly by enzyme inhibitors such as L-methionine-S-sulfoximine (Harth & Horwitz, 1999) or by antisense-oligonucleotide inhibition of expression results in reduced bacterial growth (Harth et al., 2000). In addition, a glnA1 mutant of *M. tuberculosis* is auxotrophic for L-glutamine and shows a reduced ability to multiply within macrophages (Tullius et al., 2003). These data indicate that the function of GS is essential for the viability and pathogenicity of *M. tuberculosis*.

Aside from GlnA1, *M. tuberculosis* has three other potential GSs encoded by glnA2, glnA3 and glnA4 (Cole et al., 1998), but to date only glnA1 has been shown to be essential for growth in vitro (Tullius et al., 2003). The enzymic function of the other three GS homologues has recently been confirmed (Harth et al., 2005) and it has also been shown that a glnA2 mutant of *Mycobacterium bovis* is avirulent in the guinea pig model (Collins et al., 2002).

Glutamine and glutamate are key precursors in many biosynthetic reactions, and in other bacteria the intracellular levels of these metabolites are strictly controlled. During ammonia shock, a quick response to reduce GS activity is required in order to preserve the intracellular pools of these key chemicals. If GS activity were not controlled, then the intracellular levels of these metabolites would quickly be depleted. GS activity is normally controlled by the product of the glnE gene, an adenylyltransferase, as well as by other mechanisms including feedback inhibition (reviewed by Merrick & Edwards, 1995; Reitzer & Magasanik, 1987). The role of GlnE in other bacteria is to inactive GS in response to ammonia levels; GlnE adenylylates GS on a conserved tyrosine residue, resulting in a loss of synthetase activity. GS exists as a dodecamer and the activity of GS is directly proportional to the number of subunits that are adenylylated, so that the enzyme can display a range of activities depending on its precise adenylation state. Thus GlnE controls the interconversion of active and inactive GS, which, in turn regulates the glutamate to glutamine transformation and the assimilation of ammonia. This

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**Abbreviations:** GS, glutamine synthetase; RACE, rapid amplification of cDNA ends; RT-qPCR, quantitative RT-PCR.
form of post-translational modification is thought to be used since it provides a much quicker response to ammonia levels than transcriptional control of GS expression. Indeed, a GlnE mutant of *Streptomyces coelicolor*, GS activity is not down-regulated after ammonium shock and the intracellular glutamate/glutamine balance is altered (Fink et al., 1999). Of the *M. tuberculosis* GSSs, *glnA1* and *glnA3* encode type I enzymes, which are usually controlled by GlnE, whereas *glnA2* and *glnA4* encode type II enzymes, which are not.

We have studied the role of GlnE in glutamine metabolism in *M. tuberculosis*, following our earlier observation that it is an essential gene in *M. tuberculosis* (Parish & Stoker, 2000; Parish et al., 2001). In other bacteria, including the closely related species *S. coelicolor*, GlnE is not essential since mutants can be obtained (Fink et al., 1999). Thus our results point to a key difference in glutamine metabolism between *M. tuberculosis* and other bacteria. We speculated previously that this may be because control of the glutamate/glutamine balance is critical to the pathogen’s survival, since L-glutamine is a major component of the cell wall, both in the form of D-isoglutamine in the peptidoglycan and as the major cell wall component poly-L-glutamine. In the present study we investigated the expression of glnE in response to nitrogen availability. We have identified the promoter region which directs the transcription of glnE and have shown that it is controlled in response to nitrogen availability.

**METHODS**

**Growth of mycobacteria and RNA isolation.** *M. tuberculosis* H37Rv (ATCC 25618) was grown in Middlebrook 7H9 plus 10% (v/v) OADC supplement (Becton Dickinson) and 0.05% (w/v) Tween 80 or on Middlebrook 7H10 agar with 10% (v/v) OADC supplement. TSM medium was developed and contained 1·5 g K$_2$HPO$_4$·3H$_2$O l$^{-1}$, 0·5 g KH$_2$PO$_4$·H$_2$O l$^{-1}$, 0·5 g MgSO$_4$·7H$_2$O l$^{-1}$, 0·5 mg CaCl$_2$·2H$_2$O l$^{-1}$, 0·1 mg ZnSO$_4$ l$^{-1}$, 0·1 mg CuSO$_4$·5H$_2$O l$^{-1}$ and 50 mg FeCl$_3$·6H$_2$O l$^{-1}$ supplemented with 10% (v/v) OADC and 0.05% (w/v) Tween 80. For TSM-high ammonia, 30 mM (NH$_4$)$_2$SO$_4$ was added, and for TSM-low ammonia 0·1 mM (NH$_4$)$_2$SO$_4$ was added; l-amino acids (alanine, asparagine, glutamine and glutamate) were added as specified. Growth curves were determined with cultures in 12 mm diameter borosilicate tubes with 4·5 ml medium and stirring at 250 r.p.m. with an 8 mm fle. Cultures for RNA isolation were incubated rolling for 7 days in 100 ml medium, at which point they were in late exponential phase. RNA was isolated as previously described (Betts et al., 2002).

**Determination of co-transcription with glnA2 by RT-PCR.** RT-PCR was carried out using a two-step procedure using the Reverse Transcriptase kit (Promega) according to the manufacturer’s instructions. cDNA (20 ng) was amplified in the second step using the programme 95°C 2 min, 35 cycles of 95°C 45 s, 52°C 1 min, 72°C 1 min followed by a final extension step of 72°C for 7 min. Ten microliters of the 20 μl reaction was run on an agarose gel. Two pairs of primers were used to determine if co-transcription occurred: glnA2-Efor and glnA2-Erev should amplify the last 50 bp of glnA2, the intergenic region and the first 100 bp of glnE; Gap100 and Gap101 should amplify the last 200 bp of glnA2 and the first 400 bp of glnE (Table 1).

**Northern blotting.** For Northern analysis, 15 μg total RNA was separated by electrophoresis using precast agarose gels (Sigma) and transferred for 5·5 h using the Northern Blotting kit (Sigma). Hybridization probes were generated by PCR amplification using primers glnEF and gln1F (glnE) (Table 1). Labelling and detection were carried out using the AlkPhos Direct kit (Amerham).

**Quantitative RT-PCR (RT-qPCR).** Probes and primers were designed for quantitative PCR with molecular beacons for *sigA* (endogenous control), glnE and glnA2 using the software Primer Express (Table 1). cDNA was synthesized from RNA using RT and random hexamer primers using AMV reverse transcriptase. PCR was carried out in a Taqman 7900 using a standard PCR master mix. For glnA, glnE and *sigA*, the primer pairs were GlnA-R and GlnA-F, GlnE-F and GlnE-R and SigA-F and SigA-R respectively, and the probes used were GlnA-T, GlnE-T and SigA-T. The primer and probe concentrations were first optimized. The optimal primer concentration was 300 nM for all three genes; the probe concentration was 100 nM for *sigA*, 125 nM for glnE and 150 nM for glnA2. In order to measure relative gene expression levels, standard curves for each primer-probe set were generated using genomic DNA. C$	ext{t}$ values were converted into the equivalent of ng using the standard curve. Control reactions without RT were used to confirm that there was no significant contaminating genomic DNA present. C$	ext{t}$ values for genomic DNA were converted to ng and subtracted from the plus RT values. In order to standardize the samples to ensure that equal amounts of cDNA were used, each value was standardized to *sigA* to generate unit-less values. At least three independent RNA samples were assayed in triplicate for each gene.

**Determination of transcriptional start site by rapid amplification of cDNA ends (RACE).** We used RACE to locate the 5’ end of the mRNA from glnE. RNA was extracted from *M. tuberculosis* H37Rv grown in TSM-high ammonia. cDNA was synthesized from 1 μg total RNA using reverse transcriptase at 42°C for 30 min (according to the manufacturer’s instructions) and the gene-specific primer ESP1 (Table 1) located approximately 500 bp downstream from the predicted 5’ end. A homopolymeric A-tail was added to the 3’ end of the cDNA using terminal transferase. The tailed cDNA was amplified using an oligo-dT primer and a second gene-specific primer (ESP2, Table 1) using the programme 95°C for 2 min, 10 cycles of 95°C 2 min, 55°C 45 s, 72°C 1 min, 30 cycles of 95°C 45 s, 55°C 1 min, 72°C 1 min 20 s (+20 s per cycle) and a final step of 72°C for 7 min. This product was further amplified using the same programme with nested primers ESP3 (Table 1; located approximately 250 bp downstream from the predicted 5’ ends) and an anchor primer to increase specificity. The products were cloned into pGEM-Easy T (Promega) and sequenced to identify the 5’ end.

**Analysis of promoter activity.** The predicted glnE promoter region was PCR amplified using glnA2-Efor and Elac4, which amplified a 0·6 kb product covering the 3’ end of the glnA2 gene, the intergenic region and the first 96 bp of the glnE gene (Table 1), and cloned into the vector pSM128, a mycobacteriophage L5-based integrating vector carrying a lacZ reporter (Dussurget et al., 1999). This vector is therefore present in only one copy, which is integrated into the chromosome. The predicted glnE2 promoter region (484 bp upstream of glnA2) was amplified using primers CAGE7 and CAGE8 and cloned into pSM128. The plasmids were electroporated into *M. tuberculosis* or *M. smegmatis* and transformants selected on 20 μg streptomycin ml$^{-1}$. For each plasmid three independent transformants were assayed in duplicate for promoter activity in TSM liquid medium containing high or low ammonia or 3 mM amino acid. Cell-free extracts were prepared and assayed for β-galactosidase activity (Miller, 1972).

**Site-directed mutagenesis.** Site-directed mutagenesis was carried out according to the Stratagene Quikchange II XL site-directed
mutagenesis instructions. The primer pairs used were: glnE sdm 10 F and glnE sdm 10 Rev for the –10 mutation; glnE sdm 35 F and glnE sdm 35 Rev for the –35 mutation; GSDM1 and GSDM2 for mutation 5A; GSDM7 and GSDM8 for mutation 5B; GSDM9 and GSDM10 for mutation 5C. Amplification reactions were carried out in 50 μl total volume containing 1× *Pfu* Ultra reaction buffer, 0.5 mM dNTPs, 10 pmol each primer, 10% DMSO, 10 ng template and 2.5 units *Pfu* Ultra. The thermocycling programme used was 94 °C for 1 min, followed by 18 cycles of 94 °C for 1 min, 56 °C for 1 min, 68 °C for 12 min and a final extension cycle of 68 °C for 20 min. Template was degraded using 10 units *Dpn* I at 37 °C for 1 h. Ten microlitres of each reaction was used to transform competent *E. coli*. Recombinant plasmids were isolated and sequence verified.

**RESULTS AND DISCUSSION**

GlnE is predicted to be an adenylyltransferase which controls the activity of GS in response to nitrogen availability. This modification prevents uncontrolled GS activity in the presence of excess ammonia, which is an energy-consuming process and leads to depletion of the intracellular glutamate pool. In *M. tuberculosis* this process is critical as GlnE is essential under normal culture conditions (Parish & Stoker, 2000).

**Nitrogen-source utilization**

We first developed a new medium formulation (TSM) for the growth of *M. tuberculosis*, which allowed us to add single nitrogen sources in the absence of glutamate or ferric ammonium citrate, which are present in many commonly used media. For example Middlebrook 7H9 has 0.5 g glutamate l⁻¹ and 40 mg ferric ammonium citrate l⁻¹. We tested the ability of TSM with different nitrogen sources to support growth of *M. tuberculosis*. We used four different L-amino acids (alanine, asparagine, glutamate and glutamine) and high- and low-ammonia states (Fig. 1).
Growth curves were determined in media with single nitrogen sources. All L-amino acid sources supported growth at 3 mM, as did ammonium sulfate at 0-1 mM and 30 mM. There were no major differences in the growth kinetics between the nitrogen sources. Growth was not limited in the low-ammonia medium. In media containing 30 mM amino acids, only alanine supported growth whereas asparagine, glutamine and glutamate did not. However, in the latter case this was most likely due to the effect on the pH of the media, which became acidic. Since good growth was obtained with 3 mM amino acids, we used this concentration for further work.

**Genomic organization**

The region of the chromosome where *glnE* is located is shown in Fig. 2(a). Immediately upstream of *glnE* is the *glnA2* gene, which encodes a class II GS. Although the genes do not overlap there is a very short intergenic region (51 bp) that is suggestive of an operon. Downstream, but transcribed divergently, is the major GS gene (*glnA1*). This genomic organization is conserved in *M. bovis* as well as in the related actinomycetes *Corynebacterium glutamicum* (Nolden et al., 2001) and *S. coelicolor* (Fink et al., 1999). The reason for the co-localization of *glnE* and *glnA2* is not immediately apparent, as GlnA2 is not predicted to be adenylated by GlnE.

**Transcription of *glnA2* and *glnE***

In *C. glutamicum* *glnA2* and *glnE* are co-transcribed (Nolden et al., 2001), whereas in *M. bovis*, there appears to be a separate promoter for *glnE* (Collins et al., 2002). We determined whether these genes were co-expressed in *M. tuberculosis* using both Northern blotting and RT-PCR. First we used RT-PCR to determine whether we could identify a co-transcript for *glnA2* and *glnE*. Using primers designed to amplify the junction between the two genes we could detect transcripts spanning the junction in all three media tested (7H9, low and high ammonia) (Fig. 2b). This suggested that the two genes are co-transcribed and that the bicistronic message is produced regardless of ammonia availability. RT-PCR analysis with primers designed to amplify either gene independently also confirmed expression of *glnA2* and *glnE*. Although RT-PCR is not quantitative, the results suggested that the co-transcript was present at the same level as the *glnA2* transcript (Fig. 2b), whereas *glnE* was present at a lower level (not shown).

Northern analysis was carried out on total RNA isolated from cells grown in 7H9 or TSM-high ammonia (Fig. 2c). We used the *glnE* gene as a probe and a dominant band of approximately 3 kb which represents a monocistronic transcript was seen (Fig. 2). A faint smear from approximately 4 kb was seen in the 7H9 medium which could indicate the presence of a bicistronic transcript as well. This was not seen in the TSM-high ammonia medium. The fact that the RT-PCR indicates that a bicistronic message is formed, whereas the Northern analysis indicates that the major band is monocistronic, could imply either that the mRNA had been processed to give rise to two independent transcripts or that the major transcript was monocistronic for each gene and that co-transcription only occurred at a
Identification of transcriptional start site

In order to determine whether the glnE band in the Northern analysis was a primary transcript or arose from RNA processing, we mapped the 5' end of the mRNA and assayed for promoter activity upstream of the gene. The region upstream of glnE was examined for potential start sites using RACE analysis. This technique is widely used to find the 5' end of mRNA species and relies on amplification by PCR. We obtained one major PCR product and cloned this for sequencing, which identified the end of the PCR product. The major 5' end was at base +4 relative to the annotated translational start site. Therefore the predicted translational start site is probably incorrect and the GTG codon at position 7 is the correct start site, making the untranslated 5' region only 3 bp in length. This is not unusual in the actinomycetes and in fact in many cases the transcriptional and translational start sites are the same nucleotide (Kamalakannan et al., 2002; Timm et al., 1994).

Since RACE only detects the 5' ends of mRNA, it cannot distinguish between ends arising from true transcriptional start sites or those arising from processing. In addition, secondary structure in mRNA can give rise to artefactual ends, so we also looked for promoter consensus sequences upstream of our identified 5' end. There is a predicted promoter upstream of the identified glnE start site in the correct location (Fig. 3b), suggesting strongly that the start site identified by RACE represents a 5' end arising from transcription rather than processing. The −35 region of the predicted promoter is immediately downstream of the glnA2 stop codon within the short intergenic region.

Promoter activity analysis

In order to confirm both the start site prediction from the RACE analysis and the sequence prediction of the −10 region, we looked for promoter activity in the region upstream of the glnE gene. We cloned the region containing the promoter element into the pSM128 vector carrying a lacZ reporter gene. β-Galactosidase assays from cells grown in either high- or low-ammonia media showed the presence of promoter activity (Table 2). There was no significant

Expression of glnE

We tested whether there was any control of expression at the transcriptional level. We used Taqman RT-qPCR to measure levels of glnE mRNA in comparison to levels of sigA mRNA, which remain constant (Manganelli et al., 1999). As glnE is immediately downstream of glnA2, encoding the type II GS, we determined expression of that gene as well, from cells grown in TSM with high or low ammonia. There was no significant change in the levels of mRNA for either gene in response to ammonia levels [glnA2: 23 ± 16 (low ammonia) vs 24 ± 14 (high ammonia); glnE: 8 ± 2 (low ammonia) vs 11 ± 5 (high ammonia); arbitrary values (means ± SD, n ≥ 3) expressed as percentage of sigA expression values]. We conclude that both genes are expressed in M. tuberculosis irrespective of ammonia concentration. The expression level of glnA2 was higher then that of glnE, which was in agreement with the relative levels of expression seen with the RT-PCR (Fig. 2).

low level, possibly due to lack of efficient termination (Wernisch et al., 2003).

In order to determine if the co-transcript detected arose from a lack of transcriptional termination, we repeated the RT-PCR with a second set of primers which were located further towards the middle of glnA2 and glnE (Fig. 1). These primers should amplify a region of 691 bp extending approximately 200 bp into glnA2 and 400 bp into glnE. No product was detected (data not shown), confirming the Northern results that a full-length co-transcript was not detectable.

Expression of glnE

We tested whether there was any control of expression at the transcriptional level. We used Taqman RT-qPCR to
Fig. 3. Promoter identification and site-directed mutagenesis. (a) The 0.6 kb region labelled was amplified by PCR and assayed for promoter activity. (b) Sequence of the predicted glnE promoter. The transcriptional start site (+1) identified by RACE and the proposed translational start site are underlined. Potential −10 and −35 promoter consensus sequences are indicated in bold capitals. The stop site of the glnA2 gene is indicated in bold and underlined. The T nucleotides indicated as large letters were changed to G residues by site-directed mutagenesis. (c) Promoter activity, β-Galactosidase activity was measured for each plasmid in the liquid media indicated after subculturing from liquid medium. pSM128, control, no promoter; pLUSH5, glnE promoter region; pLUSH10, mutagenized −10 region; pLUSH35, mutagenized −35 region. Results are the mean ± SD of three individual transformants each assayed in duplicate. Units are given in nmol o-nitrophenyl galactoside produced per min per mg total protein.

Table 2. Promoter activity

<table>
<thead>
<tr>
<th>Species</th>
<th>Low ammonia</th>
<th>High ammonia</th>
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</thead>
<tbody>
<tr>
<td>M. tuberculosis P_glnE</td>
<td>41 ± 22</td>
<td>56 ± 13</td>
</tr>
<tr>
<td>M. smegmatis P_glnE</td>
<td>70 ± 7</td>
<td>92 ± 24</td>
</tr>
<tr>
<td>M. tuberculosis P_glnA2</td>
<td>197 ± 117</td>
<td>206 ± 80</td>
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</tbody>
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Sequence analysis of the promoter region reveals an easily identifiable −10 region located at −10 to −5 (TAcggT), but the −35 region is less obvious. We used site-directed mutagenesis to investigate both of these regions. The −10 region was mutated from TACggT to GACGGG. This mutation abolished promoter activity (pLUSH10; Fig. 3) confirming that this is indeed a promoter for glnE with a functional −10 region. The region we predicted to be the −35 region was also mutated from TTGATC to GTGAGC and assayed. Promoter activity was not lost (pLUSH35; Fig. 3), demonstrating that this region is not a true −35 region. This is not unexpected as most mycobacterial promoters do not possess a −35 region in the same way that Escherichia coli promoters do. Lack of a −35 region is often a sign that a promoter is controlled by a regulatory protein. Mutation of the −35 region also led to significant up-regulation in glutamate and under high-ammonia conditions, and down-regulation in low-ammonia conditions, suggesting that there may be an element of control of glnE expression, with a regulatory factor binding in the −35 region. However, the assays indicate that the promoter is always active.

The promoter region was analysed for the presence of potential nitrogen-regulatory motifs. GlnA1 of M. tuberculosis has a σ54 motif and is regulated in response to ammonia concentration (Harth et al., 2005), but no such motif is found in P_glnE. There is no σ54 in M. tuberculosis and no AmtR or GlnR regulator motifs (Fink et al., 2002; Jakoby et al., 2000) are found.

In order to characterize the promoter region more fully, we tested other mutated versions for activity in high ammonia conditions (Fig. 4). Mutation of two regions close to the −35 area had no effect on promoter activity (pLUSH5A and pLUSH5B). Mutation of the region immediately upstream of the −10 (TT to GG; pLUSH5C) had a significant effect on activity, reducing it approximately eightfold. Previous work has demonstrated that a subset of mycobacterial promoters have no −35 region, but have an extended −10 region instead (Agarwal & Tyagi, 2003; Bashyam & Tyagi, 1998). The extended −10 promoter region normally has a TGN motif immediately upstream of the −10 box, making a nonameric consensus sequence (TGNTATAAT). The two
bases mutated in pLUSH5C could disrupt a TGN motif, although there is a 2 bp distance from the −10 region. The role of the TGN motif is to facilitate transition from the closed to the open promoter complex (Agarwal & Tyagi, 2003); since the mutated bases form part of a short string of three Ts, it may be that these have the same function. Alternatively, the mutated bases could be part of an alternative −10 region TgcgcT located at −15 to −10, although this sequence has fewer matches to the TATAAT consensus sequence.

We also determined promoter activity from the glnA2 upstream region (Table 3). Promoter activity was approximately four-fold higher than the glnE promoter, confirming the previous RT-PCR data. No difference in promoter activity was seen between high- and low-ammonia conditions.

Expression of glnE from alternative promoters

Although we detected a co-transcript of glnA2 and glnE by RT-PCR, Northern analysis showed that it was not the major transcript. Since there is no predicted terminator downstream of glnA2, it seems likely that co-transcription would occur, and in fact this is also the case in C. glutanicum (Nolden et al., 2001). There is conflicting evidence for members of the M. tuberculosis complex, where independent transcription of glnE was seen in M. bovis (Collins et al., 2002) and co-transcription of glnA2 and glnE was seen in M. tuberculosis (Harth et al., 2005). Our data show that independent transcription of glnE from a functional promoter can occur in M. tuberculosis. Two possibilities could account for these differences: either there are strain-specific expression patterns, since Harth et al. (2005) used Erdman, whereas we analysed H37Rv, or alternatively there may be different expression patterns depending on the media used. We looked at 7H9 (1-glutamate) alone, whereas Harth et al. (2005) used 7H9 plus ammonia.

We have previously seen that transcription does not always terminate downstream of genes in M. tuberculosis and can often lead to run-through transcription into other genes on the same or opposite reading strand (Wernisch et al., 2003). Whether these transcripts are translated has not been investigated, but this type of run-through transcription does have the potential to drive changes in protein levels. However, recruitment of RNA polymerase to PglnE would likely cause premature termination of this transcript and so the amount of a bicistronic message may be dependent on the strength of PglnE. Thus while the potential for transcription from PglnA2 is there, we believe it is not the major transcriptional start site for glnE.

Regulatory control of GlnE

The finding that glnE is expressed in all nitrogen conditions supports the essentiality data, since GlnE activity is constantly required. However, there is control of glnE expression, since up-regulation of promoter activity is seen when cells are grown on ammonia, even at low concentrations, as compared to some amino acids. This increased expression suggests that M. tuberculosis is particularly sensitive to the presence of ammonia as a nitrogen source, again possibly because of the danger that large amounts of active GS in the cell would convert all the glutamate into glutamine.

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