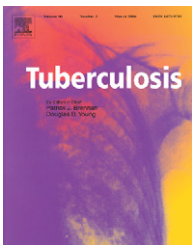
Tuberculosis (2007) **87**, 384–390

Available at www.sciencedirect.com

ScienceDirect

journal homepage: http://intl.elsevierhealth.com/journals/tube



The role of GlnD in ammonia assimilation in *Mycobacterium tuberculosis*

Rose Read[a], Carey A. Pashley[a], Debbie Smith[b], Tanya Parish[a,*]

[a]*Centre for Infectious Disease, Institute for Cell and Molecular Science, Barts and the London, 4 Newark Street, London E1 2AT, UK*
[b]*Department of Infectious and Tropical Diseases, London School of Hygiene & Tropical Medicine, Keppel Street, London WC1E 7HT, UK*

Received 28 September 2006; received in revised form 4 December 2006; accepted 12 December 2006

KEYWORDS
Nitrogen metabolism;
Glutamine synthetase;
Gene regulation

Summary
The control of ammonia assimilation in *Mycobacterium tuberculosis* is poorly understood. We have been investigating a regulatory cascade predicted to control the activity of glutamine synthetase (GS). We previously demonstrated that the GS-modifying protein, GlnE (an adenylyl transferase), is essential for *M. tuberculosis* growth. GlnD, a uridylyl transferase, is involved in the control of GlnE activity in other bacteria. In *M. tuberculosis*, *glnD* is arranged in an apparent operon with *amt* and *glnB*; all three genes are up-regulated in a low-ammonia medium. We constructed an in-frame deletion of *glnD* by homologous recombination. The mutant had no growth defect in media containing different nitrogen sources. Total GS activity in culture filtrates was markedly reduced in the mutant, although activity in cell-free extracts remained normal. Virulence was unaffected in both in vitro and in vivo model systems of infection, indicating that the presence of extra-cellular GS is not critical for virulence and that the residual intra-cellular GS activity is sufficient. Thus although GlnD does play a role in the control of ammonia assimilation, it is not required for virulence.

Introduction

Mycobacterium tuberculosis is responsible for the largest number of human deaths from a single bacterial agent. Nearly two million people die from tuberculosis each year and more than eight million are newly infected.[1] A better understanding of the basic metabolism of this pathogen could lead to new strategies for eradication. Although progress has been made in understanding some of the nutritional requirements of this organism both in vitro and in vivo, in particular its carbon source acquisition,[2,3] little is known about nitrogen metabolism.

M. tuberculosis has four enzymes with glutamine synthetase (GS) activity (GlnA1-4).[4,5] Of these, GlnA1, GlnA3 and GlnA4 synthesise L-glutamine, whereas GlnA2 synthesises the D-glutamine and D-isoglutamine required for cell wall biosynthesis.[5] The major GS, GlnA1, is expressed to a high

*Corresponding author. Tel.: +44 20 7882 2306; fax: +44 20 7882 2189.
E-mail address: t.parish@qmul.ac.uk (T. Parish).

1472-9792/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tube.2006.12.003

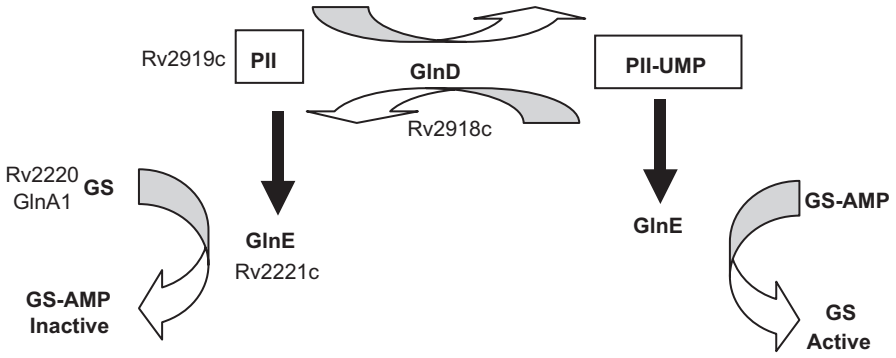


Figure 1 Control of glutamine synthetase activity by a regulatory cascade as defined in the model organism *E. coli*. The GlnD protein modifies the PII protein (encoded by GlnB) by uridylylation and deuridylylation. PII controls the activity of GlnE, either by promoting its adenylylating activity or by promoting its deadenylylating activity. GlnE controls the activity of GS by adenylylation (inactive enzyme) and deadenylylation (active enzyme). The gene homologues identified in *M. tuberculosis*[4] are indicated.

level and is exported.[6] The role of the secreted enzyme is not clear, but it has been suggested that it may play a role in pH modulation, although the biosynthetic reaction does require a source of ATP.

GS catalyses the production of glutamine from glutamate and ammonia. Since this reaction requires ATP, it needs to be strictly regulated in the presence of excess ammonia to conserve both energy and glutamate pools in the cell. GS activity can be controlled by several mechanisms, including feedback inhibition, transcriptional control of gene expression and by post-translational modification. In *Escherichia coli* a regulatory cascade of three proteins, GlnD, PII and GlnE, is involved in the latter mechanism (Fig. 1). GlnD is a uridylyl transferase which modifies the PII protein. PII in turn controls the activity of GlnE. GlnE is an adenylyl transferase which controls the interconversion of GS and GS-AMP. Transfer of the AMP moiety to the GS enzyme reduces its glutamine synthetic activity. GlnE is also able to deadenylylate GS with the predominant reaction being determined by interaction with the PII protein; PII promotes the adenylylation reaction, whereas PII-UMP promotes the deadenylylation reaction. In this way the cells can rapidly control GS biosynthetic activity in response to ammonia availability. An *M. tuberculosis* GlnA1 mutant is auxotrophic for glutamine, and attenuated in macrophages and guinea pigs,[7] suggesting that the assimilation of ammonia via this pathway is required in vivo.

We have previously shown that, in contrast to other bacteria including closely related organisms such as *Streptomyces coelicolor*, GlnE is an essential gene in *M. tuberculosis*.[8] Thus the control of GS activity seems critical to normal growth. Here we show that *glnD* is not essential and that although it has an effect on GS activity in the cells, it is not required for virulence.

Materials and methods

Culture

M. tuberculosis H37Rv (ATCC25618) was grown in Middlebrook 7H9 plus 10% v/v OADC supplement (Becton Dickinson) and 0.05% w/v Tween 80, Middlebrook 7H10 agar with 10% v/v OADC supplement or TSM media (1.5 g/L K_2HPO_4, 0.5 g/L KH_2PO_4, 0.5 g/L $MgSO_4$, 0.5 mg/L $CaCl_2$, 0.1 mg/L $ZnSO_4$, 0.1 mg/L $CuSO_4$ and 50 mg/L ferric chloride) supplemented with 10% v/v OADC and 0.05% w/v Tween 80.[9] Working pH 7.2. For TSM-high ammonia, 30 mM $(NH_4)_2SO_4$ was added; for TSM-low ammonia, 0.1 mM $(NH_4)_2SO_4$ was added, L-amino acids (alanine asparagine, glutamine and glutamate) were added to 3 mM. Growth curves were obtained in 12 mm diameter borosilicate tubes with 4–5 ml media and stirring at 250 rpm with an 8 mm flea. Hygromycin was used at 100 μg/ml and kanamycin at 20 μg/ml.

Quantitative RT-PCR

Probes and primers were designed for quantitative PCR for *sigA* (endogenous control), *amt*, *glnB* and *glnD* using the software Primer Express (Table 1). cDNA was synthesised 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 *sigA*, *amt*, *glnB* and *glnD*, the primer pairs were SigA-R and SigA-F, Amt-R and Amt-F, GlnB-R and GlnB-F, and GlnD-F and GlnD-R, respectively, and the probes used were SigA-T, Amt-T, GlnB-T and GlnD-T. The primer and probe concentrations were first optimised. The optimal primer concentration was 300 nM for all four genes, the probe concentration was 100 nM for *sigA* and *glnB*, 125 nm for *glnD* and 200 nm for *amt*. In order to measure relative gene expression levels, standard curves for each primer-probe set were generated using genomic DNA. CT 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. CT values for genomic DNA were converted to ng and subtracted from the plus RT values. In order to standardise the samples to ensure that equal amounts of cDNA were used, each value was standardised to *sigA* to generate unit-less values. At least three independent RNA samples were assayed in triplicate for each gene.

Construction of *glnD* mutant

We used our previous method for generating delivery vectors with a marker cassette.[10] The delivery vector was constructed by amplifying two regions flanking *glnD* such that an