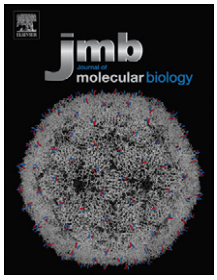

# Structure and Function of AmtR in *Mycobacterium smegmatis*: Implications for Post-Transcriptional Regulation of Urea Metabolism through a Small Antisense RNA

**Michael Petridis[1,†], Chelsea Vickers[2,†], Jennifer Robson[1], Joanna L. McKenzie[2], Magdalena Bereza[2], Abigail Sharrock[2], Htin Lin Aung[1], Vickery L. Arcus[2] and Gregory M. Cook[1,3]**

*1 - **Department of Microbiology and Immunology,** Otago School of Medical Sciences, University of Otago, Dunedin 9054, New Zealand*
*2 - **Department of Biological Sciences,** University of Waikato, Hamilton 3240, New Zealand*
*3 - **Maurice Wilkins Centre for Molecular Biodiscovery,** The University of Auckland, Private Bag 92019, Auckland 1042, New Zealand*

***Correspondence to Vickery L. Arcus and Gregory M. Cook:*** *varcus@waikato.ac.nz; gregory.cook@otago.ac.nz*
http://dx.doi.org/10.1016/j.jmb.2016.09.009
***Edited by M Gottesman***

## Abstract

Soil-dwelling bacteria of the phylum actinomycetes generally harbor either GlnR or AmtR as a global regulator of nitrogen metabolism. *Mycobacterium smegmatis* harbors both of these canonical regulators; GlnR regulates the expression of key genes involved in nitrogen metabolism, while the function and signal transduction pathway of AmtR in *M. smegmatis* remains largely unknown. Here, we report the structure and function of the *M. smegmatis* AmtR and describe the role of AmtR in the regulation of nitrogen metabolism in response to nitrogen availability. To determine the function of AmtR in *M. smegmatis*, we performed genome-wide expression profiling comparing the wild-type *versus* an *ΔamtR* mutant and identified significant changes in the expression of 11 genes, including an operon involved in urea degradation. An AmtR consensus-binding motif (CTGTC-$N_4$-GACAG) was identified in the promoter region of this operon, and ligand-independent, high-affinity AmtR binding was validated by both electrophoretic mobility shift assays and surface plasmon resonance measurements. We confirmed the transcription of a *cis*-encoded small RNA complementary to the gene encoding AmtR under nitrogen excess, and we propose a post-transcriptional regulatory mechanism for AmtR. The three-dimensional X-ray structure of AmtR at 2.0 Å revealed an overall TetR-like dimeric structure, and the alignment of the *M. smegmatis* AmtR and *Corynebacterium glutamicum* AmtR regulatory domains showed poor structural conservation, providing a potential explanation for the lack of *M. smegmatis* AmtR interaction with the adenylylated $P_{II}$ protein. Taken together, our data suggest an AmtR (repressor)/GlnR (activator) competitive binding mechanism for transcriptional regulation of urea metabolism that is controlled by a *cis*-encoded small antisense RNA.



## Introduction

Nitrogen is an essential component of macromolecules (e.g., nucleic acids, proteins, and cell wall components), and bacteria have developed elaborate mechanisms for the uptake, assimilation, and regulation of nitrogen source utilization. Actinomycetes inhabit diverse environments, and some members of the soil-dwelling actinomycetes, for example, *Streptomyces coelicolor, Corynebacterium glutamicum*, and *Mycobacterium smegmatis* harbor two distinct global nitrogen regulatory proteins, GlnR and AmtR [1–3]. GlnR is an OmpR-like transcriptional regulator, which has been characterized in *S. coelicolor* [1,4]. The global regulator of nitrogen metabolism in *C. glutamicum* is AmtR, which mediates the expression of at least 35 genes in response to changing nitrogen levels, including genes implicated in ammonium uptake (*amtA, amtB*), ammonium assimilation (*glnA, gltBD*), urea metabolism (*ureABCEFGD*), and regulatory proteins (*glnK, glnD*) [2,5,6].

*M. smegmatis* has homologs for both GlnR and AmtR, whereas members of the *Mycobacterium tuberculosis* complex encode only for GlnR [7,8]. In *M. smegmatis*, GlnR was reported to regulate the expression of over 100 genes in response to changing

 

nitrogen levels, including multiple nitrogen uptake systems, and ammonium assimilation mechanisms [3]. Transcription of the urease-encoding operon is not under the regulatory control of GlnR, whereas an operon encoding proteins involved in a putative, alternative ATP-dependent urea-degrading metabolic pathway (*msmeg_2184-msmeg_2189)* was shown to be regulated by GlnR, and the expression of this operon was upregulated in response to nitrogen limitation [3]. While the genome-wide AmtR regulon of *M. smegmatis* is unknown, this operon has also been shown to be upregulated in a *M. smegmatis* Δ*amtR* mutant in response to nitrogen limitation [9].

AmtR belongs to the TetR-like protein family, which is abundant in bacteria that are exposed to environmental changes, such as those found in soil. The members of TetR family are DNA-binding proteins and function generally as transcriptional repressors. This family of proteins are involved in regulating cellular processes such as antibiotic resistance, efflux pumps, osmotic stress, and pathogenicity [10]. Members of the TetR family usually bind small effector molecules; however, in *C. glutamicum*, AmtR interacts with the $P_{II}$ protein GlnK to regulate the expression of the genes within its regulon in response to changing nitrogen levels [2,5,6]. In this model, an AmtR:GlnK interaction depends on the nitrogen supply of the cell, which has an immediate effect on the state of GlnK (unmodified form or adenylylated form). GlnK is adenylylated (AMP) in response to nitrogen limitation, and in this form, AmtR interacts with GlnK-AMP, which results in abolishment of the repression of the genes in the AmtR regulon [2]. In regard to the mechanism of AmtR regulation in *M. smegmatis*, it is still unknown whether the signaling pathway involves an effector molecule, another protein, or a novel mode of regulation. Here, we report the structure and function of AmtR in *M. smegmatis* and propose a model of post-transcriptional regulation of urea metabolism mediated by a *cis*-encoded small antisense RNA (asRNA) that is complementary to *msmeg_4300 (amtR)*.

## Results and Discussion

### Molecular analysis of mycobacterial AmtR and phenotypic characterization of a *M. smegmatis* Δ*amtR* mutant

The AmtR protein encoded by *msmeg_4300* is 225 aa in length and belongs to the TetR/AcrR family of transcriptional regulators (Fig. S1). Bioinformatic analysis demonstrated that AmtR shares the highest amino acid sequence identity with AmtR homologs from other *Mycobacterium* species such as *Mycobacterium mageritense* (81%; Uniprot X5L5Y0) and *Mycobacterium vulneris* (79%; Uniprot X5LSM6), but it shares only 41% sequence identity with the well-studied AmtR from *C. glutamicum* (Uniprot Q79VH8; Fig. S1). The region of highest sequence similarity (~65%) is observed in the N-terminal DNA-binding domain, while the sequence similarity in the C-terminal ligand-binding domain is only ~20% to AmtR from *C. glutamicum*. A BLAST search using *C. glutamicum* AmtR and *M. smegmatis* AmtR against the *M. tuberculosis* complex shows no homologs with an amino acid identity over 33%, suggesting the lack of AmtR-like homologs in bacteria that belong to the *M. tuberculosis* complex [8].

In *M. smegmatis*, the *amtR* gene is flanked by genes that encode for an acyl-CoA synthase (*msmeg_4301*) and an enoyl-CoA hydratase/isomerase (*msmeg_4299*) with proposed roles in fatty acid and phospholipid metabolism, respectively (Fig. S2). To investigate the role of AmtR in *M. smegmatis*, we first confirmed whether the *amtR* gene was polycistronic with the flanking genes. Reverse transcriptase PCR and DNA sequencing of amplicons with primers that bind within the *amtR* gene alone (Fig. S2, PCR product II) and on either side of the gene of interest (Fig. S2, PCR products I and III) confirmed that *amtR* was a single, independent transcript under the conditions tested. We then created a markerless *amtR* deletion mutant (*M. smegmatis* JR258 Δ*amtR*; Table S1) and confirmed the deletion of 65% of the internal region of *amtR* with Southern hybridization and PCR (Fig. S3). To identify a phenotype of the JR258 Δ*amtR* mutant, we compared the growth between the wild-type strain and the JR258 Δ*amtR* mutant in Hartmans-de Bont (HdB) minimal medium with different nitrogen sources. Growth of the wild-type and JR258 Δ*amtR* mutant on the Phenotypic Micro-Array plate PM3 from Biolog Omnilog Systems™ (i.e., 95 different sole nitrogen sources tested) was comparable for the majority of the nitrogen sources tested [e.g., $(NH_4)_2SO_4$ shown in Fig. 1, and for other nitrogen sources tested, see Fig. S4]. We did observe a growth phenotype between the wild-type and JR258 Δ*amtR* mutant when lysine was added as the sole nitrogen source (Fig. 1b). Under these growth conditions, the JR258 Δ*amtR* mutant revealed a slower doubling time compared to the wild-type strain (wild-type 18.6 ±1.4 h *versus* JR258 Δ*amtR* 28.4 ± 2.7 h), and we confirmed this slow growth rate phenotype with colony-forming unit (CFU $ml^{-1}$) measurements (Fig. 1c). To validate the growth rate phenotype of the JR258 Δ*amtR* mutant, we successfully performed a complementation of JR258 Δ*amtR* with a tetracycline-inducible vector carrying the *amtR* gene (pMind-*amtR*) of *M. smegmatis* (Fig. 1d). To determine the metabolic basis for the lower growth rate of the JR258 Δ*amtR* mutant, we measured the cellular dry weight and molar growth yield ($Y_{glycerol}$) when cells were grown under these conditions (Fig. S5a and b). This analysis revealed that the cell dry weights for the wild-type (0.93 ± 0.02 g) and the