A Genomic View on Nitrogen Metabolism and Nitrogen Control in *Mycobacteria*

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Key Words

Ammonium · *Mycobacterium* · Glutamine synthetase · Nitrogen control · Nitrogen regulation · GlnR

Abstract

Knowledge about nitrogen metabolism and control in the genus *Mycobacterium* is sparse, especially compared to the state of knowledge in related actinomycetes like *Streptomyces coelicolor* or the close relative *Corynebacterium glutamicum*. Therefore, we screened the published genome sequences of *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium avium* ssp. *paratuberculosis* and *Mycobacterium leprae* for genes encoding proteins for uptake of nitrogen sources, nitrogen assimilation and nitrogen control systems, resulting in a detailed comparative genomic analysis of nitrogen metabolism-related genes for all completely sequenced members of the genus. Transporters for ammonium, nitrate, and urea could be identified, as well as enzymes crucial for assimilation of these nitrogen sources, i.e. glutamine synthetase, glutamate dehydrogenase, glutamate synthase, nitrate reductase, nitrite reductase, and urease proteins. A reduction of genes encoding proteins for nitrogen transport and metabolism was observed for the pathogenic mycobacteria, especially for *M. leprae*. Signal transduction components identified for the different species include adenyl- and uridylyltransferase and a P_{II}-type signal transduction protein. Exclusively for *M. smegmatis*, two homologs of putative nitrogen regulato-

ry proteins were found, namely GlnR and AmtR, while in other mycobacteria, AmtR was absent and GlnR seems to be the nitrogen transcription regulator protein.

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Introduction

The genus *Mycobacterium* includes saprophytic soil bacteria like *Mycobacterium smegmatis* as well as human pathogens such as *Mycobacterium leprae* and, the most prominent member, *Mycobacterium tuberculosis*. The latter is responsible for an estimated eight million new infections and nearly two million deaths each year [Dye et al., 2005]. Due to the ability of *M. tuberculosis* to replicate within macrophages, these bacteria are able to escape the human immune system and can persist in the human lung for decades in a state of dormancy prior to reactivation. Medical treatment of tuberculosis is further impeded by the appearance of strains, which are highly resistant against almost all standard antibiotics. For the design of new drugs, a more thorough understanding of the molecular biology and physiology of mycobacteria becomes crucial. A milestone in this respect has been achieved by sequencing several mycobacterial genomes including *M. tuberculosis*, *M. leprae*, *Mycobacterium avium* and *Mycobacterium bovis* [Cole et al., 1998, 2001; Garnier et al., 2003; Li et al., 2005]. This is also the case for the fast-growing *M. smegmatis* (<http://www.tigr.org/>), which has

been used as the basis of mycobacterial genetics [Pelicic et al., 1998] and as a non-pathogen substitute to study the metabolic and regulatory pathways of *M. tuberculosis* [Bange et al., 1999; Titzgemeyer et al., 2007].

We are especially interested in nitrogen metabolism and regulation in mycolic acids-containing actinomycetes. Since almost all of the macromolecules in a bacterial cell, e.g. proteins, nucleic acids and cell wall components, contain nitrogen, prokaryotes have developed elaborate mechanisms to provide an optimal nitrogen supply for cell maintenance and growth, and to overcome and survive situations of nitrogen starvation. In actinomycetes, the uptake and assimilation of nitrogen sources has been studied intensively for the close relatives of mycobacteria *Corynebacterium glutamicum* [for reviews, see Burkovski, 2003a, b, 2005, 2007; Hänßler and Burkovski, 2008] and *Streptomyces coelicolor* [Reuther and Wohlleben, 2007], while for the genus *Mycobacterium*, work concentrated mainly on the glutamine synthetase GlnA1 and its adenylylase GlnE as potential drug targets [Carroll et al., 2008; Harth et al., 2005; Hotter et al., 2008; Nordqvist et al., 2008]. Using the information available for these well-studied model organisms, we compiled a list of nitrogen metabolism-related genes based on in silico analyses of the published genome sequence of *M. smegmatis* mc²155 and their respective homologs in various pathogenic mycobacteria to provide comprehensive information for further experimental studies.

Results and Discussion

Genes Related to Ammonium Uptake and Assimilation

Ammonium is the preferred nitrogen source of most microorganisms and consequently ammonium uptake systems and assimilating enzymes are widely distributed among bacteria. A highly conserved apparent operon was found in all mycobacterial genomes comprising the genes *amtB*-*glnK*-*glnD*. The *amtB* gene encodes a putative ammonium uptake system, which has been functionally characterized in enterobacteria, *Bacillus subtilis* [Detsch and Stülke, 2003] and *C. glutamicum* [Jakoby et al., 1999; Meier-Wagner et al., 2001; Walter et al., 2008]. The two other genes are crucial for nitrogen-dependent signal transduction. The *glnK* gene is coding for a P_{II}-type signal transduction protein and *glnD* encodes a GlnK modifying/demodifying enzyme, which works as a uridylyl-transferase in enterobacteria and acts as an adenylyl-transferase in *S. coelicolor* [Hesketh et al., 2002] and *C.*

glutamicum [Strösser et al., 2004]. In accordance with the massive gene decay observed in the leprosy bacillus, in *M. leprae* all genes of this operon are pseudogenes and carry mutations, which prevent synthesis of active proteins. For example, *amtB* exhibits a mutated start codon, thus preventing translation, and multiple frame shifts resulting in a nonfunctional reading frame.

In *M. smegmatis*, another two genes, designated *amtA* (*msmeg_4635*) and *amt1* (*msmeg_6259*) were found, which encode additional putative ammonium transporters. While for *amtA* no apparent operon was observed, the latter one is organized in a cluster together with genes that apparently encode different functional domains of a class III glutamine synthetase (*msmeg_6260*), a class II glutamine amidotransferase (*msmeg_6261*), and domains of a glutamate synthase (*msmeg_6262*, *msmeg_6263*). This conserved region seems to be of Pseudomonadales origin according to the BLAST results and is in the sequenced actinomycetes only found in the genomes of *M. smegmatis*, *Nocardia farcinica*, and *Rhodococcus* sp.

Once ammonium has entered the cell via diffusion across the cytoplasmic membrane or by protein-dependent transport, this nitrogen source has to be assimilated. Most bacteria have two primary pathways to facilitate the incorporation of ammonium into the key nitrogen donors for biosynthetic reactions, L-glutamate and L-glutamine, namely the glutamate dehydrogenase (GDH) and the glutamine synthetase/glutamate synthase pathways (GS/GOGAT). Assimilation via glutamate dehydrogenase is bioenergetically more favorable, as the GS/GOGAT pathway utilizes an additional ATP per assimilated ammonium. Consequently, GDH is often preferentially used in ammonium-rich medium. However, when the cells face nitrogen limitation, assimilation via GDH is not sufficient due to the low affinity of the enzyme and the GS/GOGAT pathway is recruited for ammonium assimilation. Interestingly, only the genome of *M. smegmatis* features a *gdhA* gene coding for an assimilatory NADPH-dependent glutamate dehydrogenase. This gene is missing in the other four mycobacterial species investigated here and consequently ammonium assimilation depends on the GS/GOGAT pathway in these bacteria.

The genes *glnA1* and *glnA2* are found in all mycobacterial genomes in a conserved region together with *glnE*, encoding an adenylyltransferase that is essential in *M. tuberculosis* [Parish et al., 2000] and was shown to regulate glutamine synthetase activity [Carroll et al., 2008]. Homologs of the GlnA3 and GlnA4 proteins were found in all mycobacterial genomes except in *M. leprae*, while for GlnA4 at least three homologous proteins were iden-

tified in the genome of *M. smegmatis* via BLASTP (MSMEG_2595, MSMEG_3828, MSMEG_1116), of which MSMEG_2595 shares the highest identity to the *S. coelicolor* GlnA4 protein (62%, SCO1613 [Rexer et al., 2006]). Besides homologs of *glnA1*, *glnA2*, *glnA3* and *glnA4*, *M. smegmatis* possesses various additional genes that seem to encode homologs of glutamine synthetases (see below and table 1), and which are missing in other mycobacterial genomes. MSMEG_5374 shares with 51% the highest identity to glutamine synthetases-like proteins in the soil-dwelling α proteobacteria *Rhodopseudomonas palustris* CGA009 (RPA0984) and *Bradyrhizobium japonicum* USDA 110 (BLL1069). Furthermore, *msmeg_5374* features with 63% a GC-content significantly below the average 67% G+C content of the genome of *M. smegmatis*. For MSMEG_3827, only weak identities (about 30%) to various not further characterized glutamine synthetase-like proteins in proteobacteria were found. MSMEG_6260, which also shares 69% identity to a putative glutamine synthetase of *N. farcinica* (NFA21040), seems to be a type III glutamine synthetase with around 45% identity to GS III enzymes of various *Synechococcus* and *Pseudomonas* species, also indicated by the BLASTP results of the corresponding putative operon (*msmeg_6260–6264*). Another putative type III GS might be encoded by *msmeg_6693*, exhibiting 35–45% identical amino acids to various rhizobial GS III enzymes and 33% identity to the glutamine synthetase III of *Agrobacterium tumefaciens* (Atu4230). While it was already shown for *M. tuberculosis* that only GlnA1 is abundantly expressed and essential for bacterial homeostasis [Harth et al., 2005], the physiological role and function of the various other glutamine synthetase-encoding genes, especially in the genome of *M. smegmatis*, remain to be verified.

In all mycobacterial genomes, a highly conserved apparent operon encoding the large and small subunit of the glutamate synthase (GOGAT; *gltB* and *gltD*) was observed. In addition, the genome of *M. smegmatis* features several additional copies of *gltB* (*msmeg_5594*, *msmeg_6263*, *msmeg_6459*) and *gltD* (*msmeg_6262*, *msmeg_6458*) that are not found in other mycobacteria.

Genes Encoding Transporters of Alternative Nitrogen Sources and Assimilatory Enzymes

Ammonium cannot only be provided directly, but also generated from alternative nitrogen sources. A prominent example is urea, which is degraded to ammonium and carbon dioxide by ureases. For *M. smegmatis*, two putative urease-encoding operons were found, of which only one (*msmeg_3623–3627*) exhibits homology to the

ure gene clusters in *M. tuberculosis* and *M. bovis* based on gene identity and arrangement. The second one exhibits striking similarities to the urease subunits – encoding genes from α proteobacteria (e.g. *Pseudomonas syringae*, *Helicobacter pylori*, and various *Burkholderia* species) with DNA sequence identities between 60 and 70%, an identical operon arrangement and a *ureAB* fusion gene. No urease operon or urease-related genes were found in *M. avium* and *M. leprae*. Also, *M. smegmatis* is the only mycobacterial species to feature a distinct operon (*msmeg_2978–2982*) encoding the subunits of a putative urea ABC transporter, emphasizing – together with the presence of the different urease-encoding operons – the importance of urea as a nitrogen source for this species.

Furthermore, we found genes encoding the necessary components for the complete reduction of nitrate to ammonium via nitrate reductase (NarGHJI, NarX) and nitrite reductase (NirBD), including putative nitrite/nitrate transporters (homologs of *Escherichia coli* NarK and NarU), in the genomes of all screened mycobacteria except *M. leprae*, which seems to possess a nitrate/nitrite antiporter (ML0844) but is missing genes encoding the corresponding enzymes for reduction and assimilation.

Signal Transduction

Among mycobacteria, proteins involved in signalling and posttranslational modification are best characterized in *M. tuberculosis* [Parish and Stoker, 2000]. Since glutamine synthetase in *M. tuberculosis* is essential for this bacterium and consequently an important drug target [Harth et al., 1994, 2005; Nordqvist et al., 2008; Tullius et al., 2003], work concentrated first on the *glnE* gene product adenylyltransferase, which is involved in posttranslational modification and regulation of this enzyme. It was shown that *glnE* is essential in this organism [Parish and Stoker, 2000], an observation that is in agreement with the crucial function of adenylyltransferase in regulation of GS activity [Carroll et al., 2008]; furthermore, the *M. tuberculosis* *glnE* promoter is upregulated in ammonia- or glutamine-containing media, at least in the heterologous host *M. smegmatis* [Pashley et al., 2006], while the transcriptional organization of the *glnA1–glnE–glnA2* gene cluster remains to be elucidated further [Hotter et al., 2008].

GlnD seems to have no crucial function in the regulation of ammonium assimilation in *M. tuberculosis* [Read et al., 2007]. This is in agreement with observations made in *S. coelicolor* and *C. glutamicum* [Hesketh et al., 2002; Strösser et al., 2004]. In contrast to the signal transfer via GlnD, GlnK and GlnE to GS as shown in *E. coli*, enzyme

Table 1. Nitrogen metabolism-related genes in mycobacteria: the given references were selected to provide information for representative, well-studied homologs

Enzyme/function	Gene name	<i>M. smegmatis</i> mc ² 155	<i>M. tuberculosis</i> H37rv	<i>M. leprae</i> TN	<i>M. avium</i> ssp. <i>para-tuberculosis</i>	<i>M. bovis</i> ssp. <i>bovis</i> AF2122/97	Reference/nearest homologs
Assimilatory nitrite reductase	<i>nirB</i> <i>nirD</i>	<i>msmeg_0427</i> <i>msmeg_0428</i>	Rv0252 Rv0253	n/a n/a	MAP_3702 MAP_3703	Mb0258 Mb0259	<i>E. coli</i> nitrite reductase operon [Wang and Gunsalus, 2000]
Hydrolysis of urea	<i>ureE</i> <i>ureF</i> <i>ureAB</i> <i>ureC</i> <i>ureG</i> <i>ureD</i>	<i>msmeg_1091</i> <i>msmeg_1092</i> <i>msmeg_1093</i> <i>msmeg_1094</i> <i>msmeg_1095</i> <i>msmeg_1096</i>	n/a n/a n/a n/a n/a n/a	n/a n/a n/a n/a n/a n/a	n/a n/a n/a n/a n/a n/a	n/a n/a n/a n/a n/a n/a	<i>Pseudomonas syringae</i> urease operon (<i>psyr_2200-2195</i>)
Unknown	<i>glnA*</i>	<i>msmeg_1116</i>	n/a	n/a	n/a	n/a	[Harth et al., 2005]
Ammonium uptake	<i>amtB</i>	<i>msmeg_2425</i>	Rv2920c	(ML1627)	MAP_2988c	Mb2944c	<i>amtB-glnK-glnD</i> <i>S. coelicolor</i> [Fink et al., 2002]
Signal transduction	<i>glnK</i>	<i>msmeg_2426</i>	Rv2919c	(ML1626)	MAP_2987c	Mb2943c	
Post-translational regulation of GlnK	<i>glnD</i>	<i>msmeg_2427</i>	Rv2918c	(ML1625)	MAP_2986c	Mb2942c	
Unknown	<i>glnA4</i>	<i>msmeg_2595</i>	Rv2860c	n/a	MAP_2931c	Mb2885c	[Harth et al., 2005]
Urea uptake	<i>urtE</i> <i>urtD</i> <i>urtC</i> <i>urtB</i> <i>urtA</i>	<i>msmeg_2978</i> <i>msmeg_2979</i> <i>msmeg_2980</i> <i>msmeg_2981</i> <i>msmeg_2982</i>	n/a n/a n/a n/a n/a	n/a n/a n/a n/a n/a	n/a n/a n/a n/a n/a	n/a n/a n/a n/a n/a	<i>C. glutamicum</i> urease transport operon [Beckers et al., 2004]
Ammonium assimilation	<i>gltB</i> <i>gltD</i>	<i>msmeg_3225</i> <i>msmeg_3226</i>	Rv3859 Rv3858	ML0061 ML0062	MAP_0172 MAP_0173	Mb3888c Mb3889c	<i>C. glutamicum</i> glutamate synthase [Beckers et al., 2001; Schulz et al., 2001]
Unknown	<i>glnA3</i>	<i>msmeg_3561</i>	Rv1878	n/a	MAP_1599	Mb1910	[Harth et al., 2005]
Hydrolysis of urea	<i>ureD</i> <i>ureG</i> <i>ureF</i> <i>ureC</i> <i>ureB</i> <i>ureA</i>	n/a <i>msmeg_3623</i> <i>msmeg_3624</i> <i>msmeg_3625</i> <i>msmeg_3626</i> <i>msmeg_3627</i>	Rv1853 Rv1852 Rv1851 Rv1850 Rv1849 Rv1848	n/a n/a n/a n/a n/a n/a	n/a n/a n/a n/a n/a n/a	Mb1884 Mb1883 Mb1882 Mb1881 Mb1880 Mb1879	<i>M. tuberculosis</i> urease operon [Clemens et al., 1995]
Unknown	<i>glnA*</i>	<i>msmeg_3827</i>	n/a	n/a	n/a	n/a	<i>E. coli</i> K12 putative glutamine synthetase (<i>b1297</i>)
Unknown	<i>glnA*</i>	<i>msmeg_3828</i>	n/a	n/a	n/a	n/a	[Harth et al., 2005]
Ammonium assimilation	<i>glnA1</i>	<i>msmeg_4290</i>	Rv2220	ML0925	MAP_1962	Mb2244	[Harth et al., 2005]
Post-translational regulation of GSI	<i>glnE</i>	<i>msmeg_4293</i>	Rv2221c	ML1630	MAP_1965c	Mb2245c	[Carroll et al., 2008]
Unknown	<i>glnA2</i>	<i>msmeg_4294</i>	Rv2222c	ML1631	MAP_1966c	Mb2246c	[Harth et al., 2005]
Transcriptional regulation	<i>amtR</i>	<i>msmeg_4300</i>	n/a	n/a	n/a	n/a	<i>amtR</i> <i>C. glutamicum</i> [Jakoby et al., 2000]
Ammonium uptake	<i>amtA</i>	<i>msmeg_4635</i>	n/a	n/a	n/a	n/a	<i>Silicibacter pomeroyi</i> (<i>spo_1578</i>)
Assimilatory nitrate reductase	<i>narI</i> <i>narJ</i> <i>narH</i> <i>narG</i> <i>narX</i>	<i>msmeg_5137</i> <i>msmeg_5138</i> <i>msmeg_5139</i> <i>msmeg_5140</i> n/a	Rv1164 Rv1163 Rv1162 Rv1161 Rv1736c	(ML1499) (ML1500) (ML1501) (ML1502) n/a	MAP_2617c MAP_2618c MAP_2619c MAP_2620c n/a	Mb1196 Mb1195 Mb1194 Mb1193 Mb1765c	[Khan et al., 2008; Sohaskey, 2008; Sohaskey and Wayne, 2003]
Nitrite/nitrate transporter	<i>narK</i> <i>narK1</i> <i>narK2</i> <i>narK3</i> <i>narU</i>	<i>msmeg_5141</i> n/a n/a <i>msmeg_0433</i> n/a	n/a Rv2329c Rv1737c Rv0261c Rv0267	n/a n/a ML0844 n/a n/a	n/a MAP_2102c n/a MAP_3707c MAP_3712	n/a Mb2356c Mb1766c Mb0267c Mb0273	
Unknown	<i>glnA*</i>	<i>msmeg_5374</i>	n/a	n/a	n/a	n/a	<i>Rhodopseudomonas palustris</i> <i>glnA</i> (RPA0984)

Table 1 (continued)

Enzyme/function	Gene name	<i>M. smegmatis</i> mc ² 155	<i>M. tuberculosis</i> H37rv	<i>M. leprae</i> TN	<i>M. avium</i> ssp. <i>paratuberculosis</i>	<i>M. bovis</i> ssp. <i>bovis</i> AF2122/97	Reference/nearest homologs
Glutamate dehydrogenase	<i>gdh</i>	<i>msmeg_5442</i>	n/a	n/a	n/a	n/a	[Börmann et al., 1992]
Ammonium fixation	<i>gltB</i>	<i>msmeg_5594</i>	n/a	n/a	n/a	n/a	<i>C. glutamicum</i> glutamate synthase [Beckers et al., 2001; Schulz et al., 2001]
Transcriptional regulation	<i>glnR</i>	<i>msmeg_5784</i>	Rv0818	(ML2194)	MAP_0649	Mb0841	[Fink et al., 2002; Wray and Fisher, 1993; Wray et al., 1991]
Ammonium transporter	<i>amt1</i>	<i>msmeg_6259</i>	n/a	n/a	n/a	n/a	<i>Pseudomonas syringae</i> (<i>psyr_2277-2273</i>)
Similar to subunits of glutamate synthetase and glutamine synthase; for details, see text	<i>glxA</i>	<i>msmeg_6260</i>	n/a	n/a	n/a	n/a	<i>C. glutamicum</i> glutamate synthase [Beckers et al., 2001; Schulz et al., 2001]
	<i>glxB</i>	<i>msmeg_6261</i>	n/a	n/a	n/a	n/a	
	<i>glxC</i>	<i>msmeg_6262</i>	n/a	n/a	n/a	n/a	
	<i>glxD</i>	<i>msmeg_6263</i>	n/a	n/a	n/a	n/a	
Ammonium fixation	<i>gltD</i>	<i>msmeg_6458</i>	n/a	n/a	n/a	n/a	<i>C. glutamicum</i> glutamate synthase [Beckers et al., 2001; Schulz et al., 2001]
	<i>gltB</i>	<i>msmeg_6459</i>	n/a	n/a	n/a	n/a	
Unknown	<i>glnA</i> *	<i>msmeg_6693</i>	n/a	n/a	n/a	n/a	<i>Agrobacterium tumefaciens glnA</i> (<i>Atu4230</i>)

activity measurements in *glnK* deletion strains showed that the ATase GlnE works independently from GlnK in *S. coelicolor* [Hesketh et al., 2002] and *C. glutamicum* [Strösser et al., 2004].

Transcription Control

In parallel to the bioinformatics analysis of genes encoding transporters and enzymes related to nitrogen metabolism, a search was carried out for mycobacterial homologs of known transcriptional regulators of nitrogen control in actinomycetes, namely AmtR of *C. glutamicum* and GlnR of *S. coelicolor* (table 1). Interestingly, both a homolog to the corynebacterial regulator AmtR (42% identity) and a protein with high identity to the regulator of streptomycetes, GlnR (60%) were found in *M. smegmatis*, while in all other examined mycobacterial species, only a homolog for GlnR was found (fig. 1). A phylogenetic tree shows that GlnR homologs of pathogenic mycobacteria form a cluster, while *M. smegmatis* GlnR is more closely related to the corresponding *N. farcinica* and *Rhodococcus* sp. RHA1 protein (fig. 2a). The *M. smegmatis* AmtR is more isolated, only AmtR homologs of corynebacteria are clustered and in summary AmtR proteins are less distributed in actinomycetes (fig. 2b).

When all mycobacterial genomes were screened for known *cis*-acting elements for AmtR and for GlnR, AmtR binding sites could not be identified in any of the mycobacterial genomes. In contrast, using the GlnR motifs of *S. coelicolor*, *Streptomyces avermitilis*, and *Streptomyces*

Fig. 1. Distribution of putative nitrogen-dependent transcription regulators GlnR and AmtR in actinomycetes. Bold species names indicate experimental evidence for nitrogen control by the corresponding protein. In *M. smegmatis* (indicated by an asterisk), only the function of GlnR is characterized [Amon et al., 2008], while AmtR function remains unclear [for references of experimentally verified systems see Fink et al., 2002; Jakoby et al., 2000; Nolden et al., 2002; Yu et al., 2006]. Full species names are as follows: *Actinomyces naeslundii*, *Amycolatopsis mediterranei*, *Arthrobacter aureus*, *Bifidobacterium longum*, *Corynebacterium diphtheriae*, *Corynebacterium efficiens*, *Corynebacterium glutamicum*, *Frankia alni*, *Leifsonia xyli*, *Mycobacterium avium*, *Mycobacterium bovis*, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, *Nocardia farcinica*, *Propionibacterium acnes*, *Streptomyces avermitilis*, *Streptomyces coelicolor*, and *Thermobifida fusca*.

Fig. 2. Phylogenetic trees of the (a) GlnR and (b) AmtR family of proteins. Unrooted phylogenetic trees were computed with the CLUSTALW software making use of the implemented neighbor joining method with the function for evolutionary distance correction. Evolutionary distances are proportional to the branch length. 17 protein sequences for GlnR and 8 for AmtR homologs were selected as indicated in the figure. For full species names, see figure legend 1.

scabies as query sequences, putative binding sites were detected in the available mycobacterial genomes, including three highly conserved *cis* elements in *M. smegmatis* (fig. 3). These putative binding motifs were located upstream of the *glnA* gene, coding for glutamine synthetase,

as well as upstream of *amtB* and *amt1*, ammonium permease-encoding genes.

First experimental evidence supports the idea that GlnR is the crucial regulator of nitrogen control in mycobacteria. As shown by in vitro experiments, purified *S.*

Fig. 3. GlnR-binding motif in mycobacteria. Sequence logo [Crooks et al., 2004] of putative GlnR *cis* elements identified upstream of the *M. smegmatis* (msmeg), *M. tuberculosis* (H37rv), *M. bovis* (bovis), and *M. avium* (avium) *glnA*, *amtB*, and *amt1* genes (*amt1* is exclusively encoded in the *M. smegmatis* genome).

coelicolor GlnR is able to bind to the *M. tuberculosis glnA* promoter region in electrophoretic mobility shift assays [Tiffert et al., 2008]. Furthermore, deletion mutant analyses showed that transcription of *M. smegmatis amt1*, *amtB*, *glnK*, *glnD* and *glnA* is controlled by GlnR and that the corresponding mutant strain is unable to respond to nitrogen limitation [Amon et al., 2008].

Comparison of the Nitrogen Metabolism-Related Gene Repertoire of Mycobacteria

M. smegmatis is equipped with a variety of genes enabling the uptake and assimilation of nitrogen sources. Compared to the fast-growing *M. smegmatis*, all slow-growing pathogenic members of the genus exhibit a reduced number of genes encoding proteins for nitrogen uptake and assimilation (see table 1 for details and fig. 4 for comparison of *M. smegmatis* and *M. tuberculosis*). This is also due to the fact that *M. smegmatis* seems to have acquired an astonishingly wide range of nitrogen-related genes and gene regions via horizontal gene transfer from a variety of other bacteria, such as *Agrobacterium*, *Burkholderia*, and *Pseudomonas* species. This includes among others a second urease operon, additional ammonium transporters, and a broad variety of glutamine synthetases of various classes and origins (table 1). According to the genomic data, *M. smegmatis* is capable of the active uptake and assimilation of a comparatively wide range of substrates for the extraction of ammonium and further assimilation into central metabolites such as glutamate and glutamine, which is in good concordance to the situation found for the uptake and assimilation of

carbohydrates in *M. smegmatis* [Titgemeyer et al., 2007] and thus exhibits a similar repertoire of nitrogen-related genes to that of *C. glutamicum* [Burkovski, 2007; Hänssler and Burkovski, 2008]. Another interesting fact is the co-occurrence of homologs of both regulators of the nitrogen metabolism in actinomycetes in the genome of *M. smegmatis*, namely AmtR and GlnR. While the transcriptional repressor AmtR is the global regulator of nitrogen metabolism in corynebacteria [Walter et al., 2007], this function was only recently shown for the *M. smegmatis* GlnR and its respective target genes *amt1*, *amtB*, and *glnA* [Amon et al., 2008]; as only homologs of GlnR are found in the genomes of other mycobacteria (fig. 1), the role of AmtR for *M. smegmatis* remains to be further explored.

All mycobacteria investigated exhibit the subunits for a respiratory nitrate reductase (which are nonfunctional pseudogenes in *M. leprae*), while especially the tubercle members possess multiple homologs of the *E. coli* nitrite/nitrate antiporters, NarK and NarU. For *M. tuberculosis* it has already been shown that nitrate respiration plays an important role during hypoxia [Sohaskey, 2008], but the additional occurrence of a nitrite reductase, besides its role in detoxification by reduction of nitrite, points to a possible involvement of the enzyme in the complete reduction of nitrate to ammonium and following assimilation, which has been demonstrated for *M. smegmatis* [Khan et al., 2008].

M. leprae unsurprisingly reveals the strongest reduction of nitrogen metabolism-related genes as a process of gene decay termed ‘*reductive evolution*’ [Gómez-Valero

Fig. 4. Comparison of proteins involved in nitrogen uptake, metabolism and control in (a) *M. smegmatis* and (b) *M. tuberculosis*. Given are the protein names of the respective transporters and enzymes as derived from the in silico analyses (for details, see text). Question marks indicate putative protein-protein interactions and unknown signals. Multiple copies of enzymes are indicated by asterisks.

et al., 2007], resulting in a minimal set of genes required for a functional nitrogen metabolism. This set comprises the genes of the GS/GOGAT pathway (*gltBD*, *glnA*) as well as the GS ATase (*glnE*). While we did not find any nitrogen-specific transport systems besides one nitrite/nitrate antiporter (NarK1), the genome of *M. leprae* features putative amino acid and oligopeptide transporters and permeases (data not shown); these substrates could very well represent the main nitrogen sources, taking into account the lifestyle of *M. leprae* as an obligate in-

tracellular parasite [Sasseti et al., 2003; Vissa and Brennan, 2001].

Concluding Remarks

A growing number of genome sequences of different actinomycetes and useful tools and databases for whole genome screening and comparison are already available for research. Due to different studies based on these databases in combination with experimental approaches the transcriptional regulation of nitrogen control in acti-

nomycetes is now well understood. However, there are still a number of open questions regarding the post-translational interactions and modifications between the key players of nitrogen control (fig. 4). For example, the sensory protein for the nitrogen status of the cell that putatively controls the GlnR regulator as well as the signal sensed by this protein is still unknown, and also the function of the P_{II}-type signal transduction proteins is unclear. Furthermore, the existence of two different nitrogen-dependent transcription regulators in the actinomycetes, namely GlnR and AmtR, is still a mystery. The here compiled overview of nitrogen-related genes might thus serve as a blueprint to direct future studies.

Experimental Procedures

In silico Analysis of Mycobacterial Genome Repertoire

A bioinformatic screen of the available genome sequences of *M. smegmatis*, *M. tuberculosis*, *M. leprae*, *M. avium*, and *M. bovis* was performed. For prediction of genes encoding proteins involved in uptake and assimilation of nitrogen sources as well as in nitrogen signal transduction and nitrogen control, genome data obtained from The Institute for Genomic Research (<http://www.tigr.org>) were screened. To find the most representative mycobacterial homologs of *B. subtilis*, *E. coli*, *C. glutamicum* and *S. coelicolor* proteins, we used single genome protein and nucleotide BLAST available at the following genome server sites: <http://genolist.pasteur.fr>, TigrBLAST (<http://tigrblast.tigr.org/cmr-blast/>), CoryneRegNet (<http://www.cebitec.uni-bielefeld.de> [Baumbach, 2007]) and ScoDB (<http://streptomyces.org.uk>). Prediction of the possible function was based on the following criteria:

(1) protein identity of the mycobacterial protein of more than 30% to the homologous protein of *E. coli* or *B. subtilis*, more than 50% to *S. coelicolor* or *C. glutamicum*, or more than 60% to other mycobacterial proteins, (2) when more than one gene of the corresponding operon was conserved, and (3) most importantly, a solid biochemical analysis of the homologous protein was available. Sequence alignments were conducted with CLUSTALW applying pre-defined algorithms of The European Bioinformatics Institute (EBI) at The European Molecular Biology Laboratory (EMBL) (<http://www.ebi.ac.uk/clustalw>). Phylogenetic trees were calculated with the neighbour joining method as implemented in CLUSTALW and graphically visualized by importing phylip (*.ph) files into the TREEVIEW software (<http://taxonomy.zoology.gla.ac.uk/rod/treeview.html>).

Screening for Putative Transcriptional Regulator Binding Sites

All mycobacterial genomes were screened for conserved *cis* elements using the already published binding motifs of the corynebacterial AmtR [Beckers et al., 2005; Walter et al., 2007] and the *Streptomyces* GlnR [Reuther and Wohlleben, 2007] as query sequences using the PreDetector program [Hiard et al., 2007]. The screening for putative *cis* elements was performed by first creating positional weight matrices with different lengths (in bp) for the known *cis* elements of AmtR and GlnR with the Weight Matrix Creation module of PreDetector, then using these weight matrices in the Regulon Prediction module with the genomes of interest. All genomes to be examined were obtained automatically from the NCBI GenBank and downloaded to hard disk for later use.

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