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Nitrogen starvation-induced transcriptome alterations and influence of transcription regulator mutants in *Mycobacterium smegmatis*

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Abstract

Background: As other bacteria, *Mycobacterium smegmatis* needs adaption mechanisms to cope with changing nitrogen sources and to survive situations of nitrogen starvation. In the study presented here, transcriptome analyses were used to characterize the response of the bacterium to nitrogen starvation and to elucidate the role of specific transcriptional regulators.

Results: In response to nitrogen deprivation, a general starvation response is induced in *M. smegmatis*. This includes changes in the transcription of several hundred genes encoding e.g. transport proteins, proteins involved in nitrogen metabolism and regulation, energy generation and protein turnover. The specific nitrogen-related changes at the transcriptional level depend mainly on the presence of GlnR, while the AmtR protein controls only a small number of genes.

Conclusions: *M. smegmatis* is able to metabolize a number of different nitrogen sources and nitrogen control in *M. smegmatis* is similar to control mechanisms characterized in streptomycetes, while the master regulator of nitrogen control in corynebacteria, AmtR, is plays a minor role in this regulatory network.

Keywords: AmtR, GlnR, Nitrogen control, Nitrogen metabolism, OmpR/EnvZ

Background

Nitrogen is one of the macro-elements of life and constituent of numerous cellular metabolites (e.g. amino acids, amino sugars, nucleotides) and macro-molecules (proteins, peptidoglycan, DNA, mRNA). Therefore, its supply is crucial for organisms. Bacteria are able to use a number of different nitrogen sources to cover their nitrogen requirements; however, in most species metabolism is biased in favor of ammonium. When ammonium becomes limiting, typical bacterial responses to this situation include the expression and activation of ammonium carriers and high affinity ammonium assimilation enzymes as well as expression of carriers and enzymes for the use of alternative nitrogen sources. This response, designated as nitrogen control, is governed by different types of

transcriptional regulators. In respect to these transcription control proteins, *Actinobacteria* are divided into two major groups, one comprising GlnR and the other AmtR as master regulators of nitrogen control. GlnR was described for example in *Streptomyces coelicolor*, *Streptomyces venezuelae*, *Amycolatopsis mediterranei* and *Mycobacterium tuberculosis*, while AmtR controls nitrogen regulation in corynebacteria such as *Corynebacterium diphtheriae*, *Corynebacterium efficiens* and *Corynebacterium glutamicum* [1].

In *S. coelicolor* the OmpR-type regulator GlnR acts as a transcriptional activator for at least 15 genes, encoding proteins related to nitrogen uptake, metabolism and regulation as well as proteins with unknown function [2,3]. Proteomics analyses indicated broader regulatory influence, e.g. on amino acid and carbon metabolism [4]. *S. coelicolor* exhibits a second GlnR-type regulator, designated GlnRII, which was shown to bind to the upstream regions of the *amtB-glnK-glnD* operons and the

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glnA and *glnII* gene [2,3,5]. The regulatory function of this protein is still unknown.

In corynebacteria, like the amino acid producing strains *C. glutamicum* and *C. efficiens* or the pathogen *C. diphtheriae*, expression of genes coding for proteins for uptake and assimilation of nitrogen sources is under control of the TetR-type regulator AmtR [6-8]. In *C. glutamicum* AmtR blocks transcription of at least 35 genes during sufficient ammonium supply [9,10]. The corresponding regulon comprises the *amtA* and *amtB* genes, which encode ammonium transporters, as well as *gdh*, *glnA* and *gltBD*, which code for enzymes crucial for ammonium assimilation, i.e. glutamate dehydrogenase, glutamine synthetase and glutamate synthase. Furthermore, transcription of the *crnT* and *codA* gene, responsible for creatinine transport and metabolism as well as the *urtABCDE* and *ureABCEFGD* operon, encoding a urea ABC transporter and urease, are controlled by AmtR in addition to a number of genes coding for biochemically uncharacterized enzymes and transport systems as well as signal transduction proteins.

In *M. smegmatis* transcription of *amt1,* encoding a putative ammonium permease, the *amtB-glnK-glnD* operon, which codes for an additional ammonium transporter and signal transduction proteins, as well as *glnA*, coding for glutamine synthetase, are positively controlled by GlnR [11]. Besides these genes encoding proteins for ammonium uptake and assimilation, the regulation of other genes and operons encoding various (putative) transporters of alternative nitrogen sources, e.g. *narK*, *narK3*, *urtABCDE*, as well as nitrogen assimilatory enzymes, e.g. *gdh*, *glnA2*, *glnA3*, *glnA4*, *gltBD*, *narIJHG*, *nirBD*, *ureABCFG*, *ureE-FABCGD*, was unclear until now (for review, see [1,12]). Furthermore, *M. smegmatis* belongs to a group of *Actinobacteria* including e.g. *Nocardia farcinica*, *Rhodococcus* sp., *Clavibacter michiganensis* and *Kineococcus radiotolerans* that exhibit both, GlnR and AmtR. In none of these species, the function of AmtR and its interaction with GlnR have been addressed until now.

To expand our knowledge of nitrogen starvation response in *M. smegmatis* and to shed light on the function and interplay of GlnR and AmtR in this organism, DNA microarray analyses were carried out, with the aim to characterize the nitrogen control network of *M. smegmatis* in more detail.

Results and discussion
Nitrogen starvation-dependent transcription of genes
For a global analysis of nitrogen-starvation-induced genes, wild-type cells were grown up to the mid-exponential growth phase, when nitrogen starvation was induced by washing the cells and transferring them to minimal medium without nitrogen source. Total RNA was isolated after 0.5 hours of starvation and hybridized together with

RNA from untreated control cultures to DNA microarrays. As shown by Amon and co-workers [11], this procedure is sufficient for full induction of genes encoding ammonium transporters and high affinity ammonium assimilation enzymes. In response to the induction of nitrogen starvation, transcripts of 231 genes were found to be decreased and of 284 genes to be increased by at least a factor of 3 in the wild-type (Additional file 1: Table S1 and S2). These were classified according to functional categories [13].
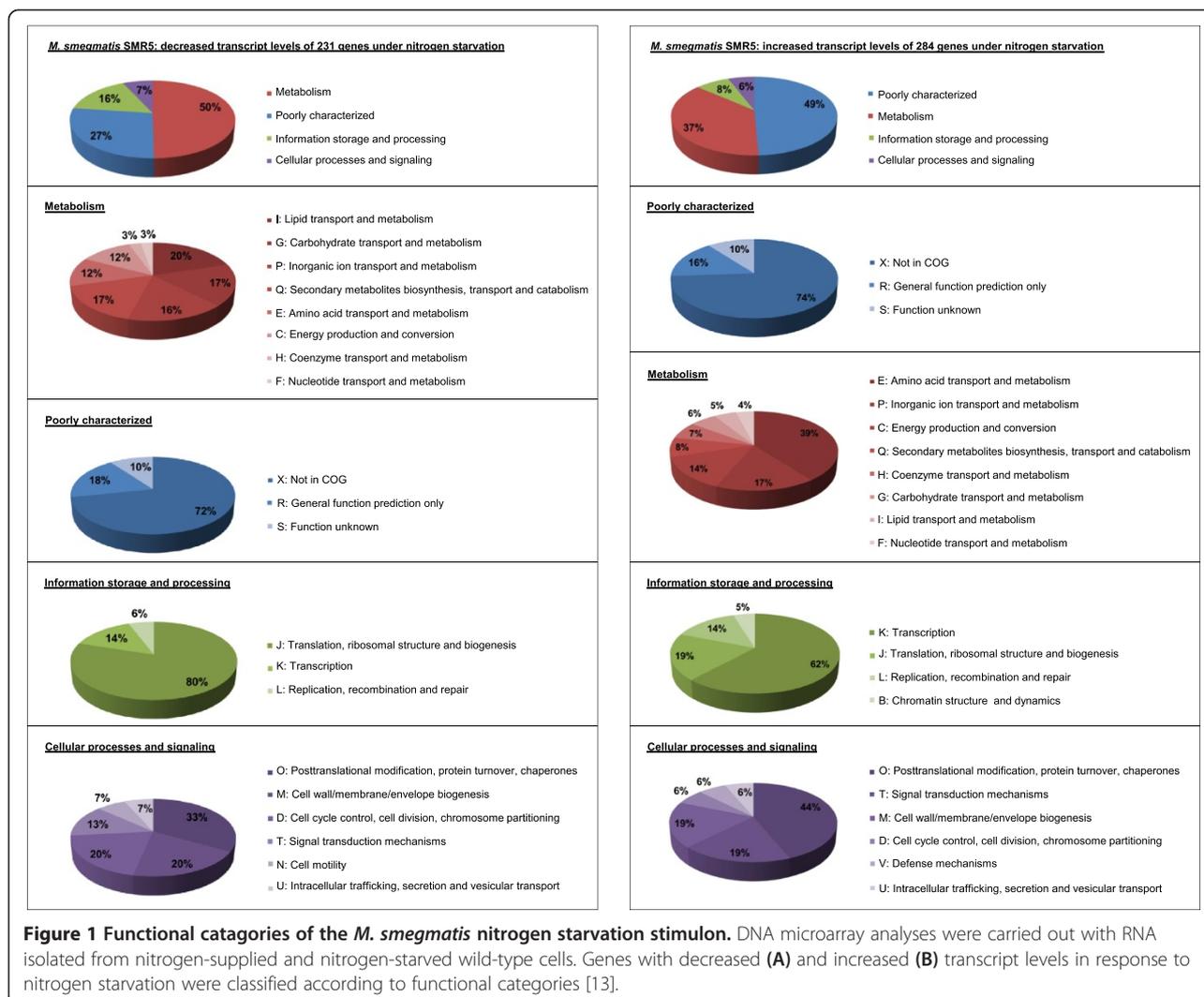
COG classification of genes with decreased mRNA level (Figure 1A) revealed that 50% of these were allocated to the major group *metabolism*, another 27% to the major category *poorly characterized*. 16% of the genes with decreased transcript amounts in the wild-type under nitrogen starvation belonged to the group *information storage and processing* and 7% correlated with the group *cellular processes and signaling*. Only 4% of these 231 genes are described as putatively involved in nitrogen metabolism.

When the 284 genes with enhanced transcript levels upon starvation were analyzed, 12.5% were associated with nitrogen metabolism. Classification of these genes into COGs (Figure 1B) shows that 49% of these genes were *poorly characterized*, 37% belonged to the major category *metabolism*, 8% of the genes with enhanced transcript amounts in the wild-type under nitrogen starvation were allocated to *information storage and processing*, while the remaining 6% of genes belonged to *cellular processes and signaling*.

All in all, the microarray results indicated a general adaptation of *M. smegmatis* wild-type to the situation of growth arrest induced by nitrogen starvation. This includes not only nitrogen control, but influences other regulatory networks such as carbon and energy metabolism as well and a similar adaptation strategy was also shown previously for the closely related actinomycete *C. glutamicum* [14].

Transcriptome analyses of wild-type and glnR mutant strain
In order to elucidate the specific nitrogen starvation response in more detail, the global transcription patterns of nitrogen-deprived wild-type and *ΔglnR* strain MH1 were compared. Transcripts of 125 genes were upregulated and of six genes down-regulated by at least a factor of 3 in the wild-type in comparison to MH1, when the two strains were starved for nitrogen. The classification of these genes into COGs (Figure 2) gave a first hint that GlnR is not only involved in the regulation of ammonium assimilation, which was further supported by a detailed inspection of the DNA microarray results (Table 1). Putative GlnR regulated genes included the genes *amtB* (*msmeg_2425*, 56 x), *glnK* (*msmeg_2426*, 53 x), *glnA* (*msmeg_4290*, 34 x), *glnD* (*msmeg_2427*, 18 x)

Figure 1 Functional catagories of the *M. smegmatis* nitrogen starvation stimulon. DNA microarray analyses were carried out with RNA isolated from nitrogen-supplied and nitrogen-starved wild-type cells. Genes with decreased **(A)** and increased **(B)** transcript levels in response to nitrogen starvation were classified according to functional categories [13].

and *amt1* (*msmeg_6259*, 12 x), which were described previously [11] besides others with obvious function in nitrogen metabolism. These include for example the *nirBD* gene cluster (*msmeg_0427-0428*) coding for nitrite reductase subunits, *msmeg_0781*, *msmeg_1052*, *msmeg_2184* and *msmeg_6735*, encoding different putative permeases and transporter subunits involved in amino acid uptake, *msmeg_1293*, *msmeg_2748*, *msmeg_4011*, *msmeg_5730* and *msmeg_6660*, encoding proteins putatively involved in purine and pyrimidine transport, as well as *msmeg_2187*, *msmeg_2981* (*urtB*), *msmeg_2982* (*urtA*) and *msmeg_3626*, encoding proteins for urea uptake and utilization. All in all, the results of transcriptome analyses of nitrogen-deprived wild-type and *ΔglnR* strain MH1 indicated a more global function of GlnR in the regulation of nitrogen metabolism. Similar results were obtained for *S. coelicolor* [3] and together indicate a more general role of GlnR in *Actinobacteria*.

The genes showing decreased transcript amounts (Table 2) had no obvious connection to nitrogen metabolism and were not further characterized. Four of these are encoding hypothetical proteins, two code for uncharacterized proteins with connection to hydrogenase expression.

Validation of DNA microarray results

The dependence of transcription changes of selected genes on the presence of GlnR was validated by RNA hybridization experiments (data not shown) and quantitative RT-PCR (qPCR; see Figure 3). For qPCR, the transcript of *msmeg_3084* was used as internal control (Figure 3A). An increase of mRNA level in response to nitrogen starvation in the wild-type compared to *glnR* deletion strain MH1 was verified for all targets tested, i.e. *nirD, msmeg_0779, msmeg_1293, msmeg_2184*, the *amtB-glnK-glnD* operon, *msmeg_2522, msmeg_2526*,
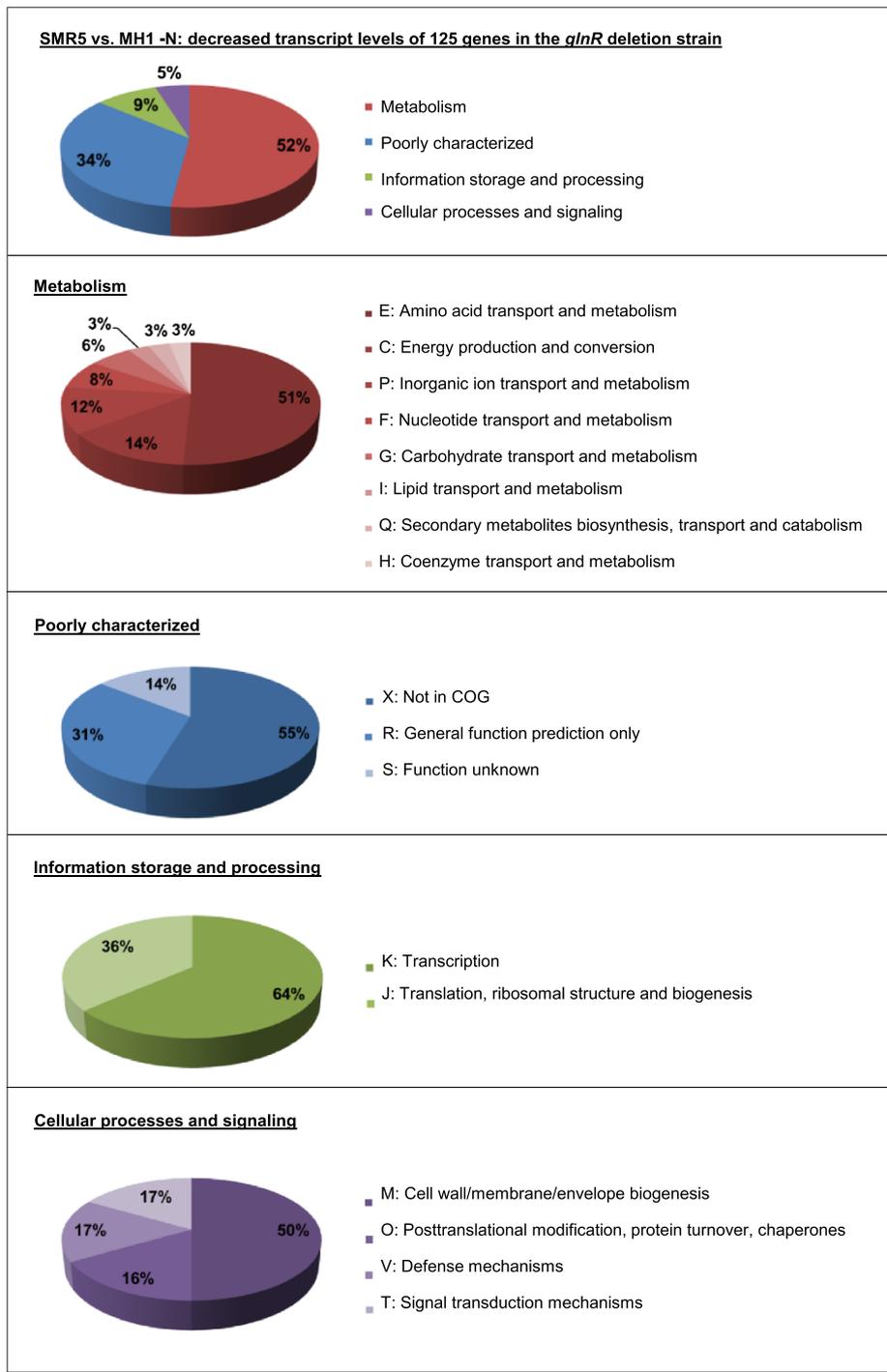
Figure 2 The GlnR modulon of *M. smegmatis*. DNA microarray analyses were carried out with RNA isolated from nitrogen-starved wild-type SMR5 and *glnR* deletion strain MH1. Genes with decreased transcript levels in *glnR* deletion strain MH1 were classified according to functional categories [13].

urtA, *msmeg_3400*, *glnA*, *glnA2*, *amtA*, *msmeg_5730*, *amt1*, *msmeg_6261* and *msmeg_6816* (Figure 3B).

The real-time RT-PCR experiments validated the DNA microarray analyses; however, from the transcriptome analyses, no conclusion about direct or indirect influences of GlnR can be drawn, since for example changing metabolite concentrations might influence transcription patterns of genes.

Table 1 Genes with increased mRNA amounts in the wild-type compared to *glnR* deletion strain MH1 in response to nitrogen starvation

Gene identifier	Fold change	Annotation
msmeg_3358	3.02	YaeQ protein
msmeg_4965	3.07	Hypothetical protein
msmeg_6507	3.07	Glycogen debranching enyme GlgX (*glgX*)
msmeg_1792	3.12	Conserved hypothetical protein
msmeg_4567	3.14	Conserved hypothetical protein
msmeg_4382	3.15	Dehydrogenase-reductase SDR family member 10
msmeg_4011	3.26	Putative pyrimidine permease RutG
msmeg_2116	3.33	PTS system, glucose-specific IIBC component
msmeg_4570	3.34	Conserved hypothetical protein
msmeg_2187	3.36	Urea amidolyase
msmeg_4569	3.48	Conserved hypothetical protein
msmeg_1292	3.48	FAD binding domain in molybdopterin dehydrogenase protein
msmeg_4171	3.58	Ribose transport system permease protein RbsC
msmeg_3994	3.64	Short chain dehydrogenase
msmeg_5648	3.68	Hypothetical protein
msmeg_2189	3.68	Allophanate hydrolase (*atF*)
msmeg_0393	3.73	Fmt protein
msmeg_1153	3.73	FAD dependent oxidoreductase
msmeg_6659	3.74	Hypothetical protein
msmeg_6332	3.79	Amino acid ABC transporter, permease protein
msmeg_3626	3.89	Urease, beta subunit (*ureB*)
msmeg_5083	4.00	Conserved hypothetical protein
msmeg_1152	4.01	Citrate-proton symporter
msmeg_5331	4.04	UDP-glucoronosyl and UDP-glucosyl transferase family
msmeg_1151	4.08	DNA-binding protein
msmeg_5783	4.10	Acetyltransferase, GNAT family
msmeg_1155	4.16	Carnitinyl-CoA dehydratase
msmeg_1157	4.17	Short chain dehydrogenase
msmeg_3912	4.20	Acetoacetyl-CoA reductase
msmeg_0565	4.33	Putative glycosyl transferases group 1
msmeg_1184	4.40	Serine esterase, cutinase family
msmeg_1156	4.44	Dihydrodipicolinate synthetase
msmeg_1185	4.46	Transcriptional regulator, AsnC family
msmeg_1089	4.61	Hypothetical protein
msmeg_1088	4.63	Glutamyl-tRNA(Gln)-aspartyl-tRNA(Asn) amidotransferase
msmeg_0505	4.72	Probable sugar ABC transporter, substrate-binding protein
msmeg_6880	4.92	Hydrophobic amino acid ABC transporter, putative
msmeg_6879	4.97	Nat permease for neutral amino acids NatD
msmeg_3722	5.04	Bifunctional coenzyme PQQ synthesis protein C-D
msmeg_1596	5.05	Transcriptional regulator
msmeg_6264	5.06	Putative oxidoreductase
msmeg_2523	5.11	Efflux ABC transporter, permease protein, putative
msmeg_4381	5.14	Amidase

Table 1 Genes with increased mRNA amounts in the wild-type compared to *glnR* deletion strain MH1 in response to nitrogen starvation *(Continued)*

msmeg_1090	5.24	Amidase
msmeg_1508	5.54	Amino acid permease-associated region
msmeg_5729	5.75	Hydantoin racemase
msmeg_6733	6.32	Hydrolase, carbon-nitrogen family
msmeg_2748	6.36	Soluble pyridine nucleotide transhydrogenase (*sthA*)
msmeg_1085	6.41	Dipeptide transport system permease protein DppB
msmeg_2569	6.61	Oxidoreductase, 2OG-Fe(II) oxygenase family
msmeg_0429	6.64	Putative ferric uptake regulator
msmeg_6878	6.97	Inner-membrane translocator
msmeg_6263	7.01	Glutamate synthase family protein
msmeg_6262	7.40	FwdC-FmdC family protein
msmeg_1295	7.42	Transthyretin
msmeg_3402	7.82	Cytosine permease, putative
msmeg_5356	8.15	Hypothetical protein
msmeg_1296	8.73	Uricase
msmeg_0780	8.79	Phosphotransferase enyme family protein
msmeg_6877	8.90	Branched-chain amino acid transport system ATP-binding protein
msmeg_0566	8.94	Aliphatic amidase
msmeg_3403	9.26	Formamidase
msmeg_1990	9.45	Conserved hypothetical protein
msmeg_1086	9.94	ABC transporter permease protein
msmeg_0778	10.22	Putative transcriptional regulator
msmeg_6261	10.36	Glutamine amidotransferase, class II
msmeg_6660	10.75	Permease, cytosine-purines, uracil, thiamine, allantoin family
msmeg_6817	11.34	RNA polymerase sigma factor, sigma-70 family
msmeg_1052	11.63	Amino acid carrier protein
msmeg_6259	11.67	Ammonium transporter (*amt1*)
msmeg_6881	11.75	Transcriptional regulator, GntR family
msmeg_2185	12.81	Conserved hypothetical protein
msmeg_2525	14.19	Amino acid permease superfamily
msmeg_1293	14.73	Xanthine-uracil permeases family protein
msmeg_2978	15.78	ABC transporter ATP-binding protein
msmeg_6260	16.11	Glutamine synthetase, type III (*glnT*)
msmeg_4206	16.44	Molybdopterin oxidoreductase
msmeg_3400	17.66	Glutamyl-tRNA(Gln) amidotransferase subunit A
msmeg_0572	17.72	Conserved hypothetical protein
msmeg_2427	18.22	Protein P-II uridylyltransferase (*glnD*)
msmeg_4637	18.43	Conserved hypothetical protein
msmeg_3401	18.68	LamB-YcsF family protein
msmeg_1988	18.75	Conserved hypothetical protein
msmeg_1597	18.80	Transcription factor WhiB
msmeg_2979	18.88	ABC transporter ATP-binding protein
msmeg_2981	19.17	Branched-chain amino acid ABC-type transport system
msmeg_6115	19.21	Phosphoglycerate dehydrogenase

Table 1 Genes with increased mRNA amounts in the wild-type compared to *glnR* deletion strain MH1 in response to nitrogen starvation (Continued)

msmeg_6735	19.26	Amino acid permease, putative
msmeg_1087	19.33	Oligopeptide ABC transporter ATP-binding protein
msmeg_6116	19.89	Conserved hypothetical protein
msmeg_5084	20.59	Glycosyl transferase, group 2 family protein
msmeg_5359	21.35	Cyanate hydratase (*cynS*)
msmeg_5360	22.01	Formate-nitrate transporter
msmeg_4501	22.33	Sodium:dicarboxylate symporter
msmeg_4635	22.90	Ammonium transporter family protein
msmeg_2980	23.33	Putative membrane protein
msmeg_2524	24.34	ABC transporter, ATP-binding protein
msmeg_2522	24.38	Efflux ABC transporter, permease protein
msmeg_5358	25.10	Acetamidase-Formamidase family
msmeg_4294	26.01	Glutamine synthetase, type I (*glnA*)
msmeg_5765	29.55	Globin
msmeg_4638	30.14	Vanillate O-demethylase oxidoreductase
msmeg_4636	31.81	Hypothetical protein
msmeg_2186	32.35	Conserved hypothetical protein
msmeg_0570	32.58	Conserved hypothetical protein
msmeg_6816	33.03	Molybdopterin oxidoreductase
msmeg_0432	34.03	Uroporphyrinogen-III synthetase
msmeg_4290	34.13	Glutamine synthetase, type I (*glnA*)
msmeg_0569	34.95	Flavoprotein involved in K$^+$ transport
msmeg_0779	35.16	Short-chain dehydrogenase-reductase SDR
msmeg_0781	36.20	Amino acid permease
msmeg_1987	42.51	Conserved hypothetical protein
msmeg_5730	45.31	Permease for cytosine-purines, uracil, thiamine, allantoin
msmeg_1082	47.68	Putative response regulator
msmeg_0571	48.04	Hydrolase, carbon-nitrogen family
msmeg_2184	48.27	Amino acid permease
msmeg_0428	48.97	Nitrite reductase [NAD(P)H] small subunit (*nirD*)
msmeg_2426	51.56	Nitrogen regulatory protein P-II (*glnK*)
msmeg_1084	51.81	Peptide-opine-nickel uptake family ABC transporter
msmeg_0427	52.06	Nitrite reductase [NAD(P)H], large subunit (*nirB*)
msmeg_0433	54.53	Nitrite extrusion protein
msmeg_2425	56.21	Ammonium transporter (*amtB*)
msmeg_6734	62.84	Dibenzothiophene desulfurization enzyme A
msmeg_2526	66.62	Copper methylamine oxidase
msmeg_2982	136.17	Putative periplasmic binding protein (*urtA*)

Binding of GlnR upstream of putative target genes

In order to test binding of GlnR to selected putative target DNAs, 200 to 300 bp DNA fragments located upstream of different genes were amplified by PCR and used together with purified MBP-GlnR fusion protein in gel retardation experiments (Figure 4). Specific binding of GlnR to the upstream DNA of *msmeg_0572*, *msmeg_0781*, *msmeg_2184*, *amtB*, *msmeg_2526*, *urtA*, *msmeg_3400*, *glnA*, *glnA2*, *amtA* and *amt1* was shown (Figure 4A).

Binding was not detected for the *nirB*, *msmeg_1052*, *msmeg_1084*, *msmeg_1090*, *msmeg_1293*, *msmeg_2748*, *msmeg_2981*, *msmeg_4638*, *narK*, *msmeg_5734*, *glnR*,

Table 2 Genes with decreased mRNA amounts in the wild-type compared to *glnR* deletion strain in response to nitrogen starvation

Gene identifier	Fold change	Annotation
msmeg_6498	3.96	Hypothetical protein
msmeg_2274	4.24	Hydrogenase assembly chaperone HypC-HupF (*hypC*)
msmeg_2275	4.37	Hydrogenase expression-formation protein HypD (*hypD*)
msmeg_1738	4.46	Probable conserved transmembrane protein
msmeg_3680	4.61	Hypothetical protein
msmeg_1999	10.08	Hypothetical protein

msmeg_6258, *msmeg_6734* and *msmeg_6816* upstream region (Figure 4B). In these cases, longer DNA fragments spanning from 300 to 500 bps of the respective upstream region were also tested; however, without positive result (data not shown).

To validate GlnR binding and to localize the GlnR binding site in more detail, a 220 bp fragment upstream of *amtB* was chosen and competitive gel retardation assays were performed (Figure 5). When 50 bp overlapping DNA fragments covering the whole promoter sequence were added, fragments 1, 3, 7 and 8 did not lead to any inhibition of the DNA shift caused by GlnR binding. Addition of fragments 4, 5 and 6 led to strong inhibition, indicating that the GlnR binding site is located in this 100 bp fragment. An additional weak binding site might exist in fragment 2, as a very slight inhibition was also spotted here (Figure 5A and B).

To further specify the localization, the 50 bp fragments 4 and 6 which caused inhibition of GlnR-binding to the 220 bp promoter fragment were used as digoxigenin-labeled DNA probes and 25 as well as 15 bp DNA fragments were used as competitor DNA. While no inhibition of binding by the 25 bp fragments was observed for fragment 4 (data not shown) addition of fragment 6.1 inhibited GlnR-binding to fragment 6 (Figure 5C and D). Thus, for *amtB*, two binding sites were experimentally verified, one located 75–100 bp upstream (fragment 6.1) and the other 100–150 bp upstream of the gene's start codon (fragment 4).

The binding sequences show similarity to the proposed binding motif [11]; however, for a more detailed characterization of GlnR binding sites in *M. smegmatis*, further analyses are necessary. Besides competitive gel retardation assays, especially ChIP and ChIP-chip analyses
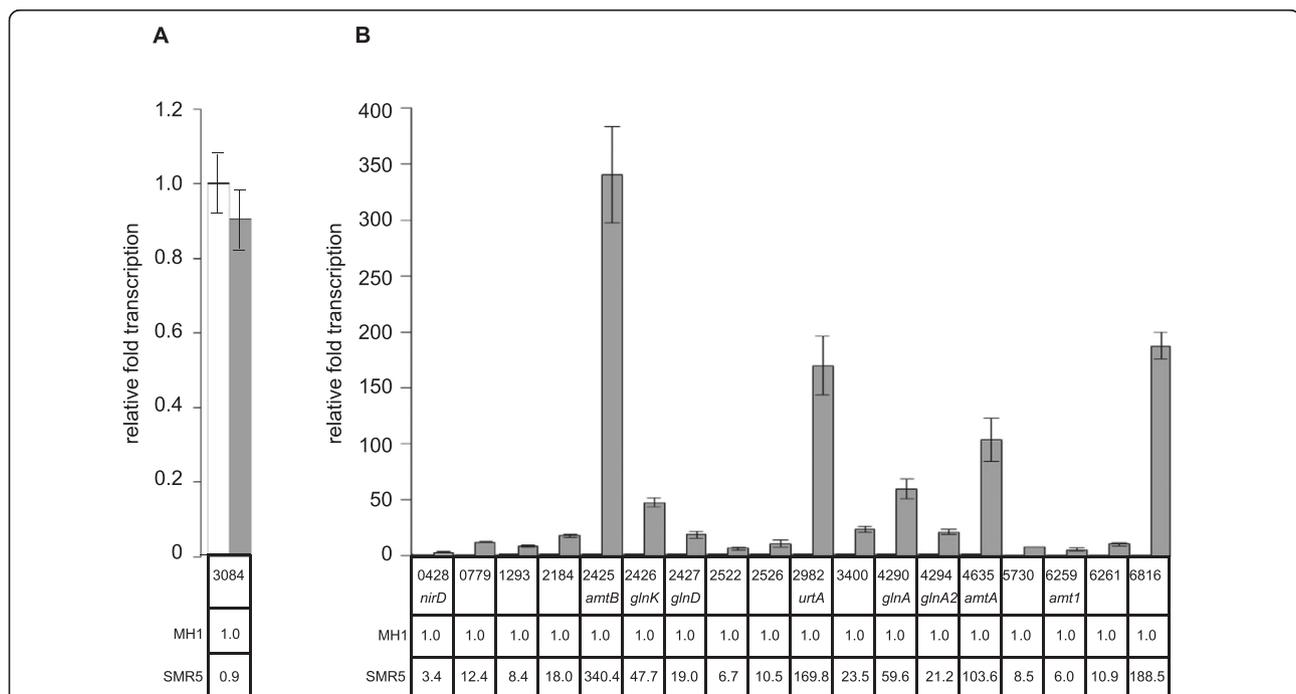

Figure 3 Verification of DNA affinity microarray results by quantitative RT-PCR. Total RNA of strains SMR5 and MH1 incubated for 30 min under nitrogen starvation was prepared and used as template for reverse transcription and PCR reaction. Specific primers were used for amplification of 100 bp fragments of target genes. **(A)** The gene *msmeg_3084* was used as control; transcription of this housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase was not significantly different in wild-type (grey bar) and *glnR* deletion strain (white bar). **(B)** Relative fold transcription of 20 target genes in the wild-type SMR5 was calculated (grey bars), while the transcription in the *glnR* deletion strain was set one (white bars). Relative fold transcription was calculated in normalization to the reference gene *msmeg_3084*. Genes are sorted according to their msmeg numbers.
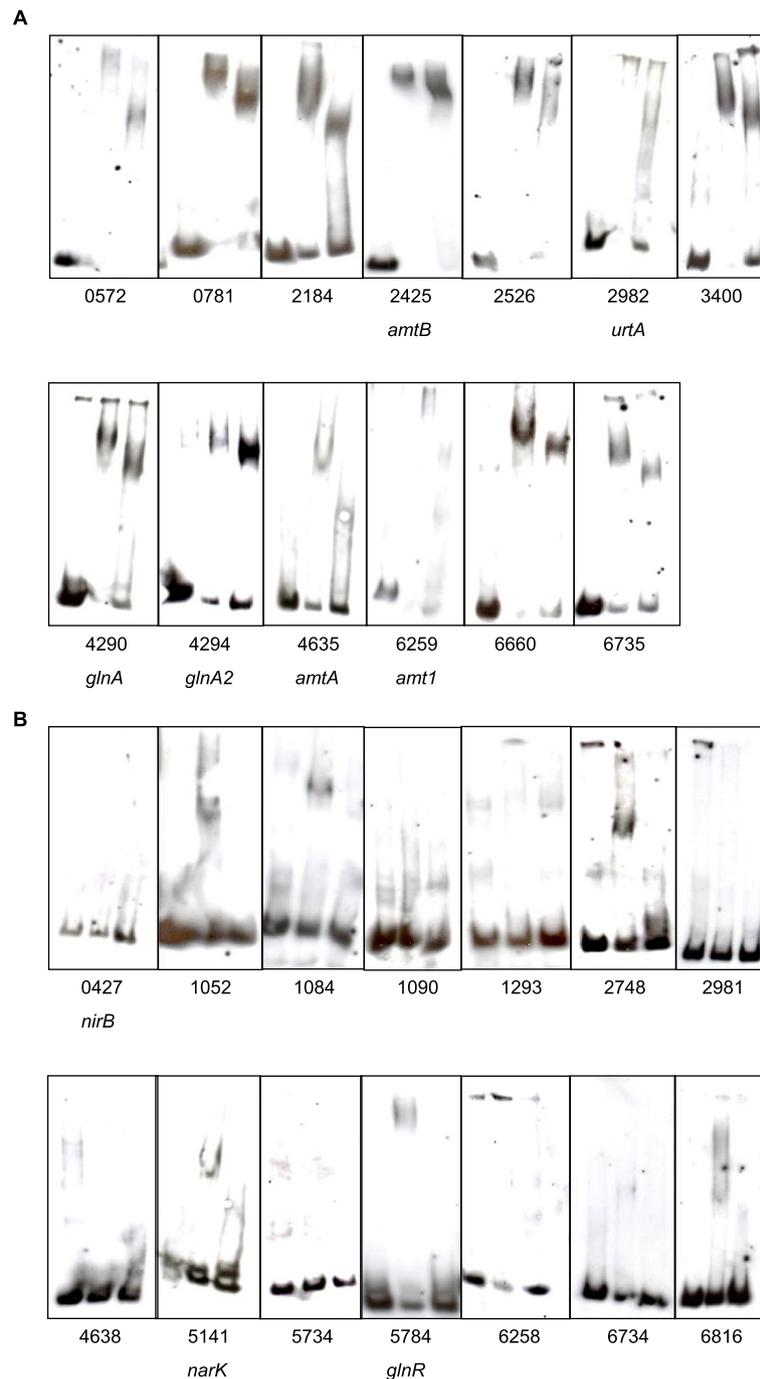
Figure 4 Test of GlnR binding by gel retardation assays. 200–300 bp DNA fragments upstream of GlnR target genes labeled with digoxigenin were used. For each gene free DNA (lane 1), DNA plus 400 ng MBP-GlnR (lane 2) and DNA plus 400 ng MBP-GlnR plus 3 µg competitor DNA polyd[I-C] (lane 3) was tested. **(A)** Target genes with specific binding; **(B)** unspecific or no binding.

as presented recently for *S. venezuelae* [15] might be carried out.

Growth of M. smegmatis on different nitrogen sources
Deduced from the increased transcript levels of several genes in the wild-type compared to *ΔglnR* strain MH1

(Table 1), a number of new substances were proposed as nitrogen sources for *M. smegmatis.* For ammonium, alanine, asparagine, glutamic acid, glutamine, nitrate, nitrite and urea a function as nitrogen source was already shown [16-18]; however, due to the experimental set-up, the experiments carried out here allowed conclusions
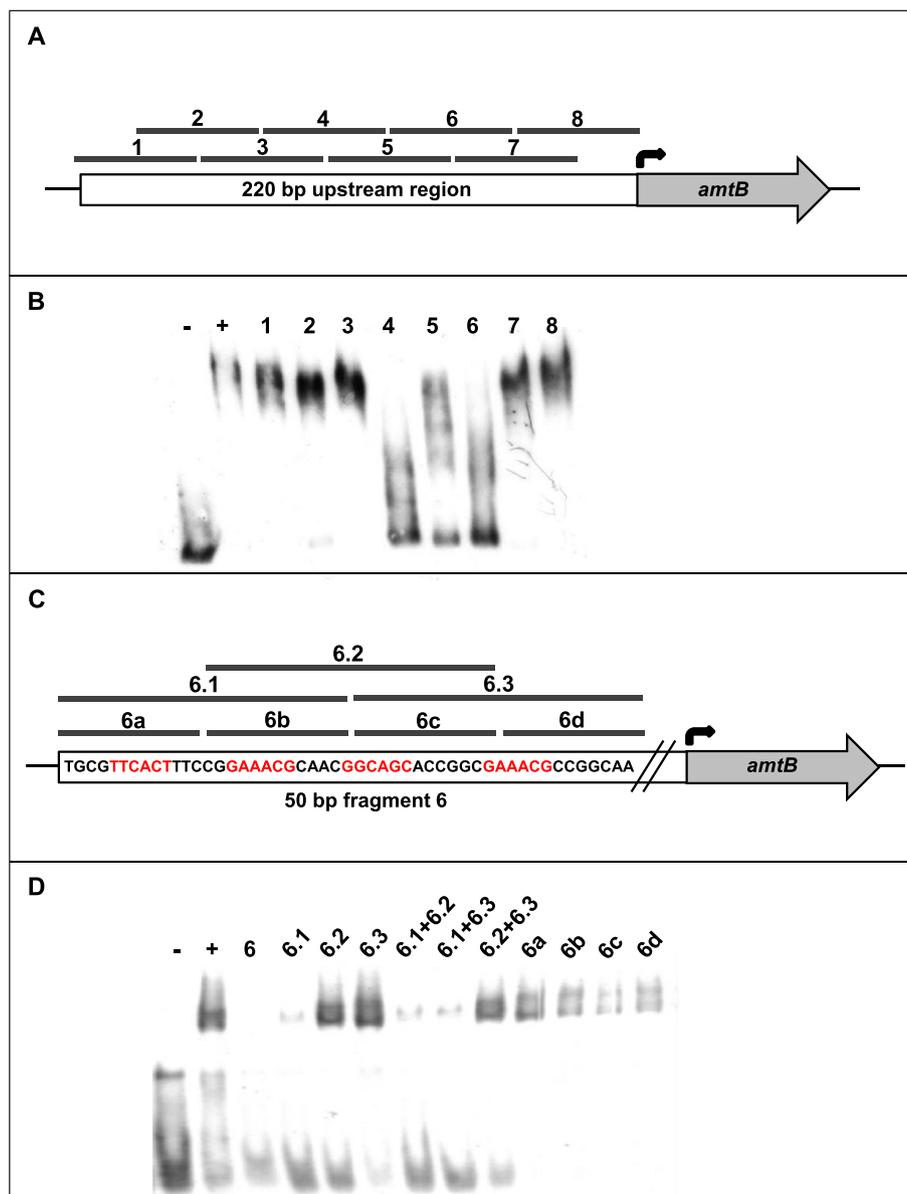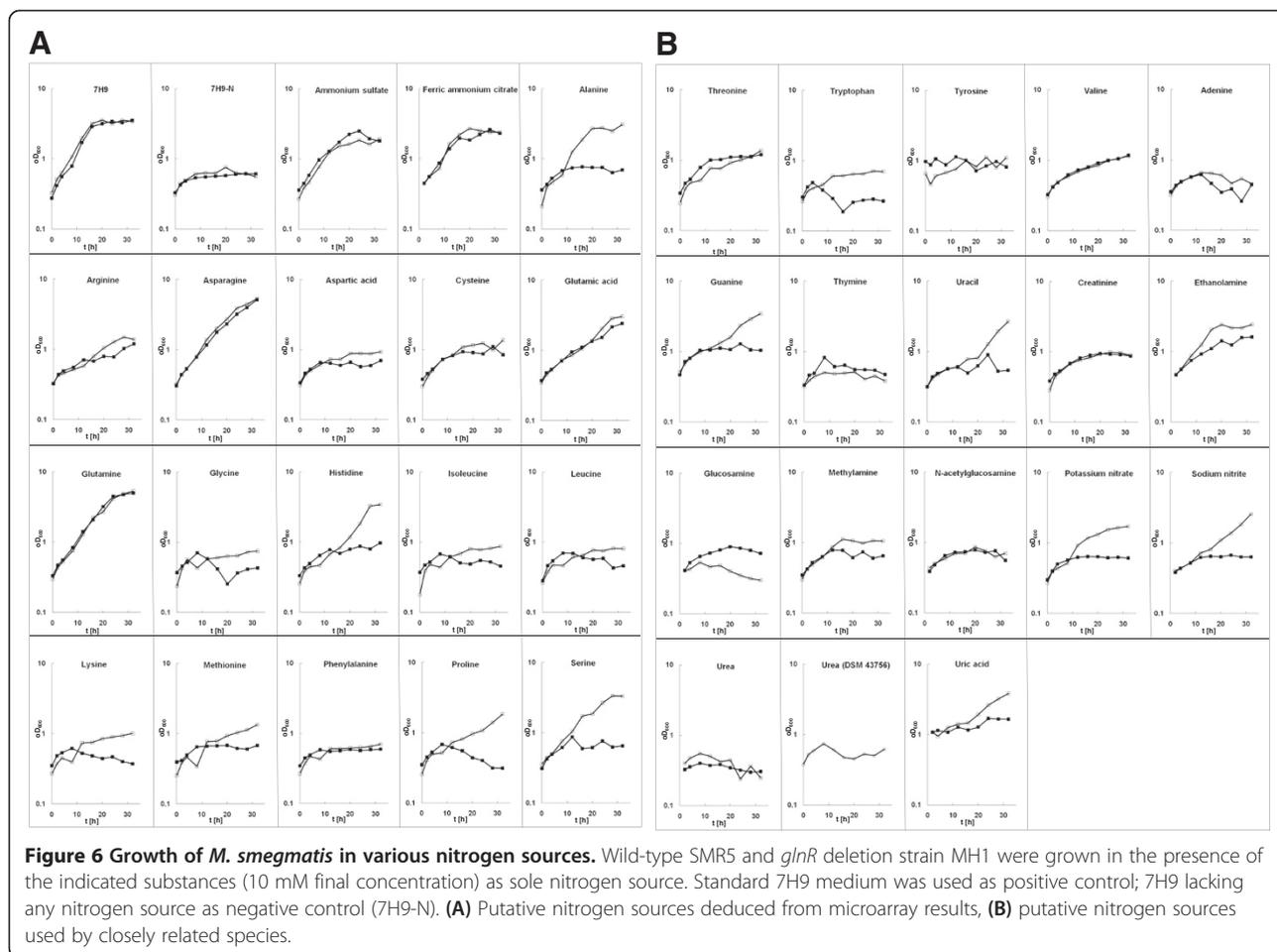
Figure 5 Competitive gel retardation experiment. (A) Model of eight overlapping 50 bp DNA fragments covering the 220 bp upstream region of *amtB*. **(B)**. When each of these fragments was added in 1000-fold surplus to gel retardation samples consisting of 220 bp digoxigenin-labeled *amtB* promoter fragment and purified MBP-GlnR, an inhibition of the shift was spotted for fragments 4, 5, 6 and weakly 2. **(C)** Model of three overlapping 25 bp DNA fragments (6.1-6.3) and four 15 bp fragments (6a-d) covering the 50 bp fragment 6 in the upstream region of *amtB*. **(D)** Addition of these fragments in 1,000-fold surplus to gel retardation samples containing 50 bp digoxigenin-labeled fragment 6 and 600 ng MBP-GlnR each. An inhibition of the shift was detected for fragment 6.1. -: free DNA as negative control. +: DNA plus 600 ng MBP-GlnR as positive control.

about the GlnR-dependent or -independent regulation of assimilation pathways.

Growth experiments were carried out using the various substances tested as sole nitrogen source and comparing wild-type and *ΔglnR* strain MH1 (Figure 6). 7H9 medium was used as positive control and nitrogen-free 7H9 (7H9-N) as negative control. Standard 7H9 medium contains ammonium sulphate, ferric ammonium citrate

and glutamic acid as nitrogen sources. When ammonium sulphate and ferric ammonium citrate were tested alone, a reduced growth rate and final OD_{600} was observed, independently from the presence or absence of GlnR, reflecting that ammonium (NH_4^+) is membrane-permeable in its unprotonated form ammonia (NH_3) and that under the growth conditions tested, ammonium was not limiting.

Figure 6 Growth of *M. smegmatis* in various nitrogen sources. Wild-type SMR5 and *glnR* deletion strain MH1 were grown in the presence of the indicated substances (10 mM final concentration) as sole nitrogen source. Standard 7H9 medium was used as positive control; 7H9 lacking any nitrogen source as negative control (7H9-N). **(A)** Putative nitrogen sources deduced from microarray results, **(B)** putative nitrogen sources used by closely related species.

Further growth tests revealed that metabolism of alanine, histidine, proline and serine was significantly impaired in strain MH1 compared to the wild-type. Consequently, uptake and/or metabolism of these amino acids seem to be directly or indirectly regulated by GlnR. No significant influence of a *glnR* deletion was observed when arginine, asparagine, glutamate, glutamine and valine were used as sole nitrogen source, indicating a GlnR-independent regulation of the metabolism of these amino acids. Aspartate, cysteine, glycine isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and tyrosine are poor or not suitable nitrogen sources for *M. smegmatis* under the conditions tested here (Figure 6A).

The increased levels of various genes connected to purine and pyrimidine uptake and metabolism (Table 1) in response to nitrogen starvation prompted us to test these also as nitrogen sources. Guanine and uracil supported growth in a GlnR-dependent manner, which is in accordance with increased mRNA levels for (*msmeg_5730* 45 x, *msmeg_1293* 15 x, *msmeg_6660* 11 x, encoding putative uracil transport proteins; Table 1), while adenine and thymine were no suitable nitrogen sources.

Additionally other substrates, which are for example used by corynebacteria or streptomycetes, were tested (Figure 6B). GlnR-dependent growth was observed when ethanolamine, nitrate, nitrite or uric acid were added, which is in accordance with the transcriptome data. Growth was not supported by creatinine, glucosamine, methylamine, *N*-acetylglucosamine and urea. The latter finding was especially puzzling, since *M. smegmatis* is characterized as urease-positive and gene clusters for two ureases and one urea transporter were described based on bioinformatic analyses [12]. Furthermore, when transcript patterns of nitrogen-supplied and -starved wild-type cells were compared in this study, increased mRNA levels were observed at least for one gene of a putative *urt* (urea transporter) gene cluster (*msmeg_2982* 136 x) and for a putative urea ammonium lyase (*msmeg_2187* 3 x). To exclude strain-specific variations, type-strain DSM 43796 (equivalent to ATCC 19420 and NCTC 8159) was tested under the same growth conditions as SMR5 and MH1. Also in this case, supply of urea alone did not support growth in 7H9-N independent of the addition for example of different trace element solutions (data not shown). Subsequently carried out

enzyme activity measurements revealed a urease activity of 1 μmol of ammonium released min^{-1} (mg protein)$^{-1}$, independent of growth of strains SMR5 and DSM 43756 in nitrogen surplus or incubated for 30 minutes under nitrogen starvation. This correlates with data obtained for *C. glutamicum*, which shows a urease activity of 0.9 μmol min^{-1} (mg protein)$^{-1}$ in complex medium and a urease activity of 0.9-2.2 μmol min^{-1} (mg protein)$^{-1}$ when ammonium is used as single nitrogen source. In this bacterium, however, urease activity is increased to 7.8 μmol min^{-1} (mg protein)$^{-1}$, when cells are exposed to nitrogen starvation [19,20]. Despite the fact that urease activity is not upregulated in response to starvation, *M. smegmatis* shows at least low urease activity and the reason for the complete growth deficiency with urea as single nitrogen source remains unclear.

In summary, as a soil bacterium and opportunistic human pathogen, *M. smegmatis* is able to metabolize a wide variety of nitrogen sources. Transcriptome and growth data presented here hint to a major role of GlnR in ammonium, amino acid, nitrate/nitrite and purine/pyrimidine assimilation, while additionally other regulators seem to be present as well as indicated by the GlnR-independent metabolism of several amino acids as nitrogen sources.

Analysis of the AmtR regulon

The experiments described above supported the idea that GlnR is a major nitrogen regulator in *M. smegmatis*, which consequently leads to the question of AmtR function in this organism. In a first approach to characterize the AmtR regulon, a bioinformatic analysis was carried out to identify AmtR-controlled genes in *M. smegmatis.* For this purpose, a co-occurrence analysis was carried out using the genome information of ten actinobacterial genomes, i.e. *Arthrobacter aurescens*, *C. michiganensis*, *C. efficiens*, *Gordonia bronchialis*, *K. radiotolerans*, *M. smegmatis*, *Nocardia farcinica*, *Rhodococcus jostii*, *Streptomyces avermitilis* and *Tsukumurella paurometabola*. From these genomes, one conserved operon was extracted, which (i) shows a high degree of co-occurrence with the *amtR* gene, (ii) shows co-localization with *amtR* in *A. aurescens*, *Rhodococcus* sp. RHA1 and *S. avermitilis* and (iii) seems to be involved in nitrogen metabolism based on their annotation (Table 3). *K. radiotolerans* features the most condensed AmtR gene cluster (Figure 7); all open reading frames of this putative operon feature overlapping start and stop codons (GTGA or ATGA). While AmtR and the amino acid permease are highly conserved and well consistently annotated in the different genomes, the exact functions of unknown reading frames urf1 and urf2, the urea carboxylase and the amidase are highly speculative and deduced from annotations (automatic and manual), domain analyses and PFAM

searches. The location of these unknown reading frames in direct genomic neighborhood to the putative urea carboxylase-encoding gene is highly conserved in all investigated genomes, while the locations of the genes coding for amino acid permease and the amidase are more variable. Particularly the *amtR* gene can be found in close vicinity as well as completely elsewhere in the respective genome sequences (Table 3, Figure 7).

At least four of the identified six putatively AmtR-regulated *M. smegmatis* genes showed increased transcript levels in response to starvation in the wild-type and seem to be under control of GlnR (*msmeg_2184* 48 x, *msmeg_2185* 13 x, *msmeg_2186* 32 x, *msmeg_2187* 3 x, *msmg_2189* 4 x; Table 2). To investigate transcription control of these genes, a strain, designated YL1, with in frame deletion of gene fragment (*amtR$_{DBD}$*) encoding the DNA binding domain of AmtR and an *amtR$_{DBD}$*/*glnR* double deletion strain, designated YL2, were constructed, respectively. These strains were analyzed in respect to nitrogen-starvation-dependent transcription.

RNA hybridization experiments with RNA isolated from the wild-type and *amtR* deletion strain YL1, isolated from nitrogen-supplied cells and after different intervals of nitrogen starvation showed significantly stronger signals for the proposed AmtR-regulated genes *msmeg_2184* (Figure 8A), *msmeg_2187* and *msmeg_2189* (data not shown) in the mutant; however, nitrogen control was still intact. Wild-type regulation for all genes was restored by a plasmid carrying *amtR*, while the vector alone did not complement the deletion (see Figure 8B for *msmeg_2184*, others: data not shown). Nitrogen-dependent regulation of AmtR-regulated genes was abolished in *glnR* deletion strain MH1 (see Figure 8C *msmeg_2184*, others: data not shown), indicating that AmtR is a secondary regulator of the master regulator GlnR. This model is in accordance with the transcriptome analyses presented above (Table 1).
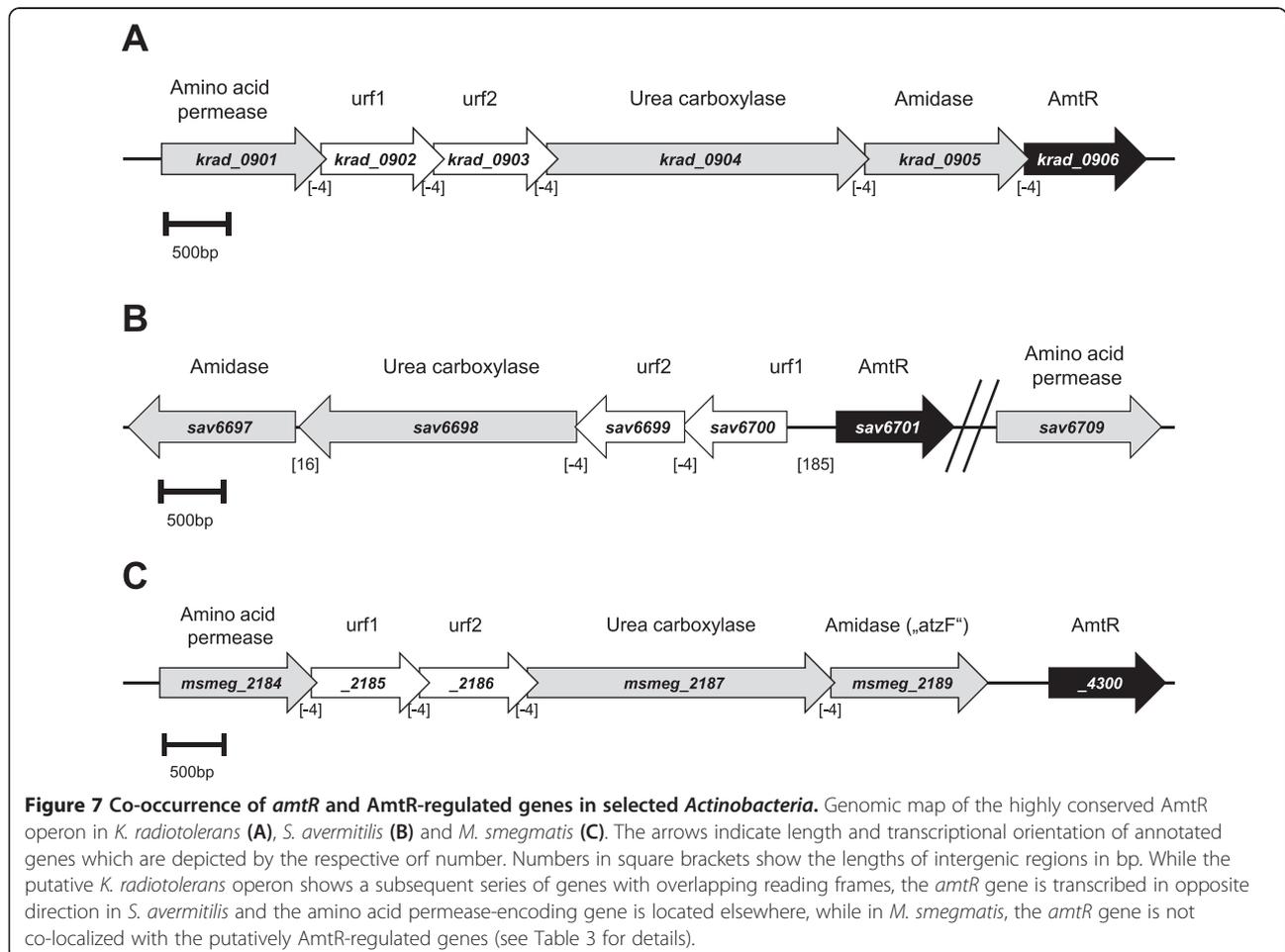
To verify direct AmtR-DNA interaction, *M. smegmatis* AmtR was overexpressed in *Eschericha coli*, purified and applied in gel retardation experiments. Binding of AmtR was tested for upstream DNA of *msmeg_2184* and binding to the *msmeg_2184* upstream region was demonstrated (Figure 9). Since TetR-type regulators often bind small effector molecules that induce release of the repressor from its binding site, addition of putative AmtR effectors deduced from the putative function of target genes, i.e. urea and different amino acids, were tested in various concentrations (0.5 to 5 mM) but had no influence on binding to the *msmeg_2184* upstream region (data not shown).

For a more global analysis of the AmtR regulon, DNA microarray experiments were carried out. RNA samples isolated from the wild-type and *amtR* deletion strain YL1 grown under nitrogen limitation were

Table 3 Distribution of AmtR and screening for AmtR-regulated genes

Organism (Reference)	Putative function and gene identifier			
	Amino acid permease	**Urea carboxylase**	**Amidase**	**AmtR**
Arthrobacter aurescens TC1 [21]	AAur_0190	AAur_0187	AAur_0186	AAur0192
Corynebacterium efficiens YS-314 [22]	ce0711	ce0713	ce0710	ce0939
Clavibacter michi-ganensis NCPPB 382 [23]	CMM_0123	CMM_0120	CMM_2824	CMM_0119
Gordonia bronchialis DSM 43247 (NCBI GenBank, acc. no. CP001802.1)	Gbro_3891	Gbro_3888	Gbro_3887	Gbro_0881
Kineococcus radiotolerans SRS30216 [24]	Krad_0901	Krad_0904	Krad_0905	Krad_0906
Mycobacterium smegmatis mc^2 155 (NCBI GenBank, acc. no. CP000480)	msmeg_2184	msmeg_2187	msmeg_2189	msmeg_4300
Nocardia farcinica IFM10152 [25]	nfa22220	nfa22190	nfa22180	nfa22230
Rhodococcus jostii RHA1 (NCBI GenBank, acc. no. CP000431.1)	RHA1_ro06919	RHA1_ro06922	RHA1_ro02136	RHA1_ro06918
Streptomyces avermitilis MA-4680 [26]	sav6709	sav6698	sav6697	sav6701
Tsukamurella paurometabola DSM20162 (NCBI GenBank, acc. no. CP001966)	Tpau_1591	Tpau_1594	Tpau_1595	Tpau_1590

The genome sequences of various *Actinobacteria* were screened for an *amtR* gene and co-occurrence of putatively AmtR-regulated genes (hypothetical urea carboxylase-associated unknown reading frames not shown, for details see text).
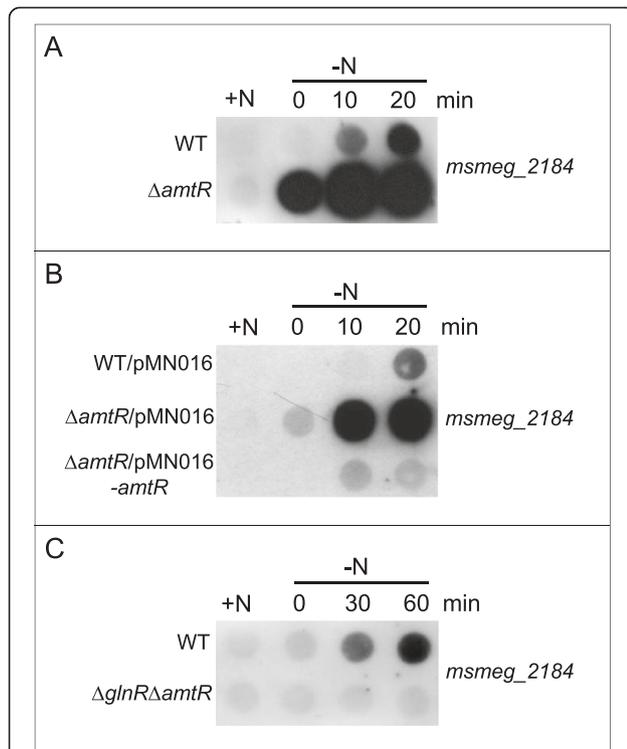

Figure 7 Co-occurrence of *amtR* and AmtR-regulated genes in selected *Actinobacteria*. Genomic map of the highly conserved AmtR operon in *K. radiotolerans* **(A)**, *S. avermitilis* **(B)** and *M. smegmatis* **(C)**. The arrows indicate length and transcriptional orientation of annotated genes which are depicted by the respective orf number. Numbers in square brackets show the lengths of intergenic regions in bp. While the putative *K. radiotolerans* operon shows a subsequent series of genes with overlapping reading frames, the *amtR* gene is transcribed in opposite direction in *S. avermitilis* and the amino acid permease-encoding gene is located elsewhere, while in *M. smegmatis*, the *amtR* gene is not co-localized with the putatively AmtR-regulated genes (see Table 3 for details).

Figure 8 Influence of AmtR on target gene expression.
Hybridization experiments were carried out with RNA isolated from
nitrogen-supplied cells and after different intervals of nitrogen
starvation and a probe specific for *msmeg_2184* mRNA. **(A)** Comparison
of wild-type SMR5 (WT) and *amtR* deletion strain YL1 (Δ*amtR*).
(B) Complementation of *amtR* deletion strain YL1 with plasmid
pMN016-*amtR* (Δ*amtR*/pMN016-*amtR*); wild-type and strain YL1
transformed with vector pMN016 (WT/pMN016 and Δ*amtR*/pMN016)
were used as control. **(C)** Influence of a *glnR/amtR* double
deletion (Δ*glnR*Δ*amtR*).

Figure 9 Test of AmtR binding by gel retardation assays. The
indicated amounts of purified AmtR proteins were added to a
^{32}P-labeled *msmeg_2184* promoter fragment (437 bp) in the presence
of 2ug sperm DNA (non-specific).

hybridized, and also RNA samples from *amtR* deletion
mutant YL1 grown with and without nitrogen source
were analyzed. No further AmtR targets were observed
(data not shown), indicating that AmtR controls only a
very small regulon, which is part of the GlnR modulon
in *M. smegmatis*.

In an additional approach, autoregulation of AmtR was
analyzed. This regulatory mechanism typical for TetR-type
regulators [27] was in fact observed (Figure 10). Since the
amtR deletion only comprised the part of the gene encod-
ing the DNA binding domain, an *amtR* target was detect-
able, which was stronger in the deletion mutant YL1
compared to the wild-type (Figure 10A). Induction of
nitrogen starvation or an additional deletion of *glnR*
(Figure 10B) had no effect, indication a nitrogen-independent
regulation of AmtR expression.

Conclusions

Deprivation of nitrogen leads to growth arrest in
M. smegmatis and induces not only specific transcrip-
tional alterations in respect to genes encoding nitrogen-
related proteins, but a general starvation response at the
transcriptional level. This includes changes in the mRNA
levels of several hundred genes encoding transporters,
proteins involved in nitrogen metabolism and regulation,
energy generation and protein turnover etc. and was for
example also observed in other *Actinobacteria* such as
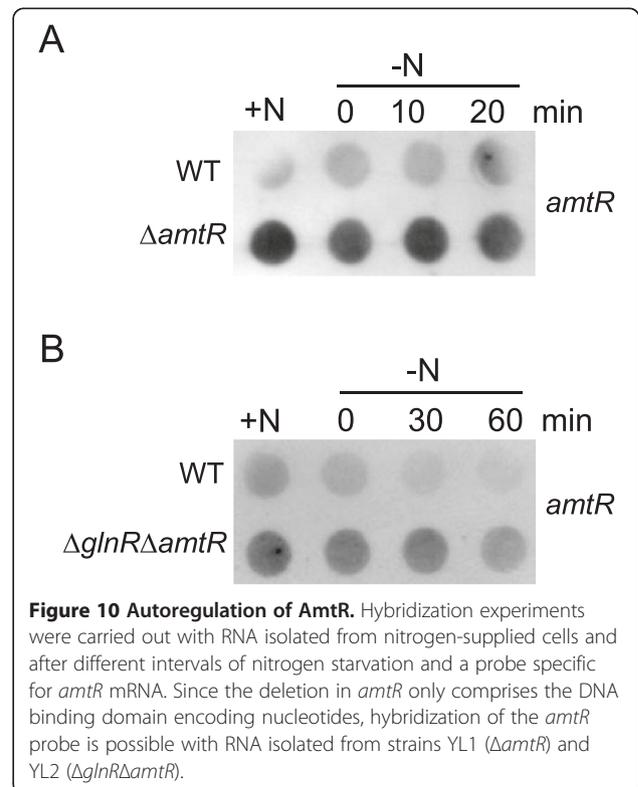*C. glutamicum* [14,28].

Figure 10 Autoregulation of AmtR. Hybridization experiments
were carried out with RNA isolated from nitrogen-supplied cells and
after different intervals of nitrogen starvation and a probe specific
for *amtR* mRNA. Since the deletion in *amtR* only comprises the DNA
binding domain encoding nucleotides, hybridization of the *amtR*
probe is possible with RNA isolated from strains YL1 (Δ*amtR*) and
YL2 (Δ*glnR*Δ*amtR*).

Based on the DNA microarray data obtained for M. smegmatis wild-type SMR5 and glnR mutant MH1 and growth experiments, GlnR seems to play a crucial role in nitrogen metabolism in M. smegmatis., including the uptake and assimilation of ammonium, different amino acids (alanine, histidine, proline, serine), nitrate, nitrite, uric acid, ethanolamine, guanine and uracil, while, AmtR, the master regulator of nitrogen control in corynebacteria plays a minor role. All in all, nitrogen control in this species resembles carbon catabolite repression (CCR) in enterobacteria (for recent reviews on CCR in bacteria, see [29,30]. Like CcpA in CCR, GlnR seems to be the global regulator and functions as an activator in response to a starvation stimulus, while AmtR is a specific repressor of a small regulon, corresponding to LacI in CCR.

In addition to the GlnR-dependent regulation of nitrogen metabolism, genes not associated with nitrogen were indicated as putative GlnR targets by the DNA microarray analyses carried out. While these genes were not in the focus of this study, it can be assumed that the regulatory function of GlnR might not be restricted to nitrogen metabolism in M. smegmatis. This has also been suggested in similar studies focusing on S. coelicolor GlnR [3], where genes involved in carbon metabolism, stress response and antibiotic biosynthesis were also found to be GlnR-controlled confirming the regulatory function of GlnR beyond nitrogen metabolism. Future work will concentrate on the question, whether the observed transcription changes of non-nitrogen-related genes are directly GlnR-regulated or the result of perturbations caused by the glnR deletion, which indirectly affect expression.

Methods

Bioinformatics analyses

For bioinformatics analysis of the AmtR regulon, a screening and co-occurrence approach was carried out. Genome sequences were obtained from the NCBI Genbank (ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/) and manually investigated with the software program Artemis (Sanger Institute). Homology analyses were performed with the microbial genomes BLAST databases at the NCBI and JCVI CMR servers. Advanced protein sequence predictions were done at the EMBL SMART domain analysis server and co-occurrence screenings were performed with the EMBL STRING interaction networks database.

Growth conditions

Bacteria were routinely grown at 37°C in baffled flasks under agitation. Mycobacterial strains were grown in Middlebrook 7H9 liquid medium (Difco Laboratories; per 900 ml approx. 0.5 g ammonium sulfate, 0.5 g L-glutamic acid, 0.1 g sodium citrate, 1.0 mg pyridoxine, 0.5 mg biotin,

2.5 g disodium phosphate, 1.0 g mono potassium phosphate, 0.04 g ferric ammonium citrate, 0.05 g magnesium sulfate, 0.5 mg calcium chloride, 1.0 mg zinc sulfate, 1.0 mg copper sulfate), supplemented with 0.2% glycerol and 0.05% Tween 80 or on Middlebrook 7H9 medium (Difco Laboratories) with 1.5% agar supplemented with 0.2% glycerol and 0.05% Tween 80. If appropriate, antibiotics were added in the following concentrations: hygromycin (200 μg ml^{-1} for E. coli; 50 μg ml^{-1} for M. smegmatis), kanamycin (30 μg ml^{-1} for E. coli; 10 μg ml^{-1} for M. smegmatis) and streptomycin (400 μg ml^{-1} for M. smegmatis). In order to study the effects of nitrogen starvation, a fresh M. smegmatis culture was used to inoculate 7H9 medium for overnight growth. This culture, with an overnight OD_{600} of approximately three to four, was used to inoculate fresh 7H9 medium to an OD_{600} of approximately 0.2, and cells were grown for 10 to 11 hours until the exponential growth phase was reached (OD_{600} approximately 0.6 to 0.8). To induce nitrogen starvation, cells were harvested by centrifugation, washed and resuspended in pre-warmed 7H9 medium without nitrogen source (7H9 lacking ammonium sulfate and glutamic acid and containing iron citrate instead of iron ammonium citrate). For control, the unstarved cells were harvested, washed, and transferred using 7H9 medium.

General molecular biology techniques

For plasmid isolation, transformation, and cloning standard techniques were used [31]. E. coli strain DH5αMCR [32] was used as cloning host. Plasmids were subsequently brought into competent M. smegmatis cells by electroporation. Chromosomal DNA was extracted from 100 ml cultures grown to stationary phase as described [33]. DNA sequence analyses were carried out using Big Dye® Terminator V3.1 Cycle Sequencing kit (Perkin Elmer).

Construction of an amtR deletion strain

To inactivate the AmtR function, a mutant with in-frame deletion of the DNA fragment ($amtR_{DBD}$) encoding AmtR DNA-binding domain (DBD) (amino acid residue 26 to 72) was generated as described earlier [11]. Briefly, two DNA fragments (1 kb up- and downstream of $amtR_{DBD}$) were amplified via PCR using chromosomal DNA from M. smegmatis strain SMR5 as a template. SwaI and PacI restriction sites were introduced into the primer sequences used for amplification of the upstream arm and SpeI and PmeI sites into the primer sequences used for amplification of the downstream arm (Additional file 1: Table S3). The PCR products were then cloned in vector pML814 (ColE1 origin, FRT-hyg-FRT, rpsL, Amp^R, Hyg^R, 6220 bp, general deletion vector), resulting in the recombinant plasmid pML814ΔamtR$_{DBD}$, which carried a FRT-hyg-FRT expression cassette [34] flanked by the upstream

and downstream arms. *M. smegmatis* SMR5 was transformed with pML814Δ*amtR*$_{DBD}$ and the transformants were first selected on hygromycin-containing plates to obtain single crossovers [34]. After verification of the single crossover event *via* colony PCR, cells were further selected on hygromycin/streptomycin containing plates. Clones on these plates should have lost the vector and have the integrated *FRT-hyg-FRT* cassette in the chromosome. After verification of the double crossover event *via* PCR, the FLP recombinase was used to specifically remove the *hyg* gene from the chromosome, thus generating a marker-free deletion mutant. Selection of clones was performed using hygromycin/streptomycin-containing plates. Deletion of *amtR*$_{DBD}$ in the resulting strain MH2 was verified by PCR and Southern blotting (data not shown).

On basis of the mutant strain YL1, *glnR* was further deleted as described earlier [11], generating the mutant strain YL2, with the inactivation of both *amtR*$_{DBD}$ and *glnR*, which was confirmed by PCR and Southern blotting (data not shown).

Construction of *amtR* carrying plasmids
For complementation assays, the *amtR* gene was amplified by PCR using chromosomal DNA of *M. smegmatis* as template and the primers amtRcom-fw/amtRcom-rev (see Additional file 1: Table S3). The PCR products were ligated to plasmid pMN016 (p$_{smyc}$-*mspA*, ColE1 origin, pAL5000 origin, Hyg^R, 6164 bp, [35]) between the *Swa*I and *Pac*I sites. For the overexpression of AmtR protein in *E. coli*, the *amtR* gene was amplified by PCR (for primers, see Additional file 1: Table S3) and ligated to plasmid pQE70 (Qiagen, Hilden) to add a C-terminal His$_6$-tag to the expressed protein, leading to plasmid pQE-*amtR-His*$_6$. The resulting plasmids pMN016-*amtR* and pQE-*amtR-His*$_6$ were sequenced for verification (data not shown).

Construction of antisense probes
For the generation of antisense probes, internal DNA fragments of the corresponding genes were amplified by PCR (primers for the different probes are listed in supplementary materials, Additional file 1: Table S3). The reverse primers encoded the promoter region for T7 polymerase, which allowed *in vitro* transcription of probes using T7 polymerase.

RNA preparation, hybridization analyses
M. smegmatis RNA was prepared from 6 ml culture samples using the NucleoSpin RNA II Kit (Macherey Nagel, Düren). When necessary, a second DNase digestion was performed with Turbo-DNase (Ambion) to completely remove the chromosomal DNA. RNA samples were finally stored at −80°C.

Antisense probes with a size between 0.2 and 0.5 kb for the analysis of gene transcription were generated by

PCR and subsequent labelling with DIG RNA-labelling mix (Roche, Mannheim) and T7 polymerase (NEB, Frankfurt). RNA (1 μg per time point) was spotted onto nylon membranes using a Schleicher & Schuell (Dassel) Minifold I Dot Blotter. Hybridization of digoxigenin-labelled RNA probes was detected with X-ray films (Amersham Hyperfilm MP; GE Healthcare) using alkaline phosphatase-conjugated anti-digoxigenin Fab fragments and CSPD as light-emitting substrate as recommended by the supplier (Roche, Mannheim). All experiments were carried out at least twice with independent cultures (biological replicates).

Real-time reverse transcriptase PCR
For real-time RT-PCR, a MyiQ Single-Color Real-Time PCR Detection System (BioRad, Munich), the iScript One-Step RT-PCR Kit with SYBR Green (Biorad, Munich), 1 μM primers, and 100 ng of template RNA were used. Reverse transcription was carried out at 50°C for 10 min, the reverse transcriptase was inactivated and the polymerase activated by 5 min incubation at 95°C, PCR was carried out by 45 cycles of the following program: DNA denaturation for 10 sec at 95°C, primer annealing for 15 sec at 57°C, and DNA polymerization for 10 sec at 72°C. The PCR reaction was followed by a melting curve program (81 x 55–100°C with a heating rate of 1°C per 10 sec) and then a cooling program at 25°C. No-template controls were run with all reactions. Data were analyzed using the MyiQ Single-Color Real-Time PCR Detection System software.

Design of DNA microarrays, RNA quality control, labelling, hybridization and transcriptome analysis
Whole genome expression analyses were performed using custom *M. smegmatis* strain mc^2 155 8x15K microarrays (Agilent, Santa Clara). Based on annotated genes downloaded from the NCBI genome browser (http://www.ncbi. nlm.nih.gov/genomes), two specific probes per gene were designed using the eArray software (https://earray.chem. agilent.com).

Total RNA from two biological replicates of each bacterial strain or condition was isolated as described above and purified using RNeasy Mini Spin Columns (Qiagen, Hilden, Germany). RNA quantity was measured with the ND-100 Spectrophotometer v3.3.0 (NanoDrop Technologies). RNA integrity was verified using an Agilent RNA 6000 Nano Chip on an Agilent 2100 BioAnalyzer as recommended by manufacturer's protocol (Agilent RNA 6000 Nano Assay Protocol2). Reverse transcription of 300 ng total RNA was carried out, using a random T7N9 primer [36]. Further sample labeling and hybridization was essentially performed as described in the two-color microarray-based gene expression analysis protocol provided by Agilent including the two-color RNA spike-in kit (v5.7, 2008; Agilent

Technologies, Santa Clara). For each replicate the cy3/cy5 dye combination was flipped between control and experimental sample. Slides were scanned on the Agilent Microarray Scanner with extended dynamic range (XDR) at high resolution (5 μm). Data sets were extracted by the feature extraction software package (v9.5.3.1/ Agilent Technologies) using a standard protocol. Data were analyzed using the GeneSpring XI software (Agilent Technologies) with standard settings. Statistically significantly deregulated genes (> 2-fold change in comparison to respective control and p-value < 0.05) were identified using the *t-test against zero* function of GeneSpring XI including the Benjamini-Hochberg multiple test correction. The microarray datasets were deposited in GEO database (http://www.ncbi.nlm.nih.gov/geo/) under record number GSE30236.

Purification of GlnR and gel retardation experiments

For gel shift assays, protein extracts from *E. coli* Rosetta2 carrying plasmid pMal-c2-*glnR* were prepared. Cells were cultivated in LB medium containing antibiotics as described, harvested by centrifugation (4,000 x g, 15 min, 4°C) and suspended in 20 mM Tris, 200 mM NaCl, 1 mM EDTA, pH (HCl) 7.4 (2 ml g^{-1} cell weight) containing lysozyme (2 mg ml^{-1}) and Complete protease inhibitor as recommended by the supplier (Roche, Mannheim). Cells were subsequently disrupted by ultrasonic treatment. Cell debris was removed by centrifugation (14,000 x g, 30 min, 4°C) and the protein extract was loaded on a 10 ml amylose resin affinity column (GE Healthcare, Munich) in a chromatography device (Äkta prime, GE Healthcare, Munich). Washing and subsequent elution with 20 mM Tris, 200 mM NaCl, 1 mM EDTA, 20 mM maltose, pH (HCl) 7.4 were carried out as recommended by the supplier of the amylose resin matrix (GE Healthcare, Munich); the purified protein was stored at 4°C.

Target DNA for the gel shift assays was synthesized by PCR (primers listed in Additional file 1: Table S1) and purified by agarose gel electrophoresis. For labelling of the DNA and the setup of the reaction mixture for the gel shift assay, the DIG Gel Shift Kit (Roche, Mannheim) was used following the supplier's protocol. Separation by gel electrophoresis was performed in native 6% polyacrylamide gels (Anamed Electrophorese GmbH, Darmstadt) using 0.5 x TBE buffer as running buffer. Subsequently, the labelled DNA was blotted on a nylon membrane (Roche, Mannheim) by electro-blotting as described in the protocol of the DIG Gel Shift Kit (Roche, Mannheim). For detection of the labelled DNA, X-ray films were used.

Purification of AmtR and electrophoretic mobility shift assays

For purification of AmtR protein, *E. coli* BL21 (DE3) carrying plasmid pQE-*amtR*-His$_6$ was used to inoculate 200 ml LB and the cultures were induced with 0.5% α-lactose at an OD$_{600}$ of approximately 0.6 and grown for 12 h at 16°C. The cells were harvested by centrifugation, washed twice with lysis buffer (20 mM Tris–HCl, 500 mM KCl, 10% glycerol, pH 7.9), and resuspended in binding buffer (20 mM Tris–HCl, 500 mM KCl, 10% glycerol, 10 mM imidazole, pH 7.9). The cell suspension was stored on ice for 30 min and then lysed by sonication. The lysate were centrifuged at 15,000 x g for 60 min at 4°C and applied to a 1-mL Ni Sepharose column (GE Healthcare) following 1 h incubation on ice. After washing with 20 ml of binding buffer, the column was washed with 20 ml washing buffer A (20 mM Tris–HCl, 500 mM KCl, 10% glycerol, 20 mM imidazole, pH 7.9) and B (20 mM Tris–HCl, 500 mM KCl, 10% glycerol, 50 mM imidazole, pH7.9), respectively. The bound protein was eluted with elution buffer (20 mM Tris–HCl, 500 mM KCl, 10% glycerol, 500 mM imidazole, pH 7.9) and the purity of His-tagged protein was checked by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified protein was stored at –80°C.

Electrophoretic mobility shift assays (EMSAs) were performed according to the method described before with some modifications [37]. The promoter region of *msmeg_2184* (437 bp) was amplified using *M. smegmatis* genomic DNA as template and the primer pair, msmeg_2184pfw/msmeg_2184prev (see Additional file 1: Table S3). PCR product was labeled at the 5' ends with [γ-^{32}P]ATP using T4 polynucleotide kinase (Promega, China), and purified using illustra ProbeQuant G-50 Micro Columns (GE Healthcare, UK). For EMSA, the ^{32}P-labelled DNA probe (1,000 cpm) was incubated individually with various amounts of His$_6$-AmtR protein in 20 μl binding assays, containing 2 μg sperm DNA, 20 mM Tris–HCl (pH 7.9), 25 mM KCl, 1 mM dithiothreitol (DTT), 5 mM MgCl$_2$, 0.5 mg ml^{-1} calf BSA and 5% glycerol. After incubation at 25°C for 30 min, protein-bound and -free probe DNA were separated by electrophoresis on non-denaturing 6% polyacrylamide gels at 10 V cm^{-1} with a running buffer 0.5 x TBE (40 mM Tris–HCl, pH 8.0, 20 mM boric acid and 1 mM EDTA). After electrophoresis, gels were dried and scanned with a FLA-7000 phosphoimager (FujiFilm Corporation, Japan).

Availability of supporting data

The datasets supporting the results of this article are available in the GEO repository (http://www.ncbi.nlm.nih.gov/geo/) under record number GSE30236.

Additional file

Additional file 1: Table S1. List of all 231 genes with decreased transcript levels in the *M. smegmatis* wild type strain SMR5 under nitrogen starvation. **Table S2.** List of all 284 genes with increased

transcript levels in the *M. smegmatis* wild type strain SMR5 under
nitrogen starvation. **Table S3.** Primers used in this study. Restriction sites
are highlighted bold.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NJ carried out RNA experiments and growth tests; YL was responsible for
AmtR experimental work, while bioinformatic analyses of AmtR were carried
out by JA. SR supported the DNA microarray experiments, FT, SS and AB
were responsible for general experimental design and supervision of
experiments. The manuscript was mainly written by NJ and AB. All authors
read and approved the final manuscript.

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References

1. Amon J, Titgemeyer F, Burkovski A: **Common patterns - unique features:
   nitrogen metabolism and regulation in Gram-positive bacteria.**
   FEMS Microbiol Rev 2010, **34**:588–605.
2. Fink D, Weissschuh N, Reuther J, Wohlleben W, Engels A: **Two
   transcriptional regulators GlnR and GlnRII are involved in regulation of
   nitrogen metabolism in *Streptomyces coelicolor* A3(2).** *Mol Microbiol* 2002,
   46:331–347.
3. Tiffert Y, Supra P, Wurm R, Wohlleben W, Wagner R, Reuther J: **The
   Streptomyces coelicolor GlnR regulon: identification of new GlnR targets
   and evidence for a central role of GlnR in nitrogen metabolism in
   actinomycetes.** *Mol Microbiol* 2008, **67**:861–880.
4. Tiffert Y, Franz-Wachtel M, Fladerer C, Nordheim A, Reuther J, Wohlleben W,
   Mast Y: **Proteomic analysis of the GlnR-mediated response to nitrogen
   limitation in *Streptomyces coelicolor* M145.** *Appl Microbiol Biotechnol* 2011,
   89:1149–1159.
5. Reuther J, Wohlleben W: **Nitrogen metabolism in Streptomyces coelicolor:
   transcriptional and post-translational regulation.** *J Mol Microbiol
   Biotechnol* 2007, **12**:139–146.
6. Jakoby M, Nolden L, Meier-Wagner J, Krämer R, Burkovski A: **AmtR, a global
   repressor in the nitrogen regulation system of *Corynebacterium glutamicum*.**
   Mol Microbiol 2000, **37**:964–977.
7. Nolden L, Beckers G, Burkovski A: **Nitrogen assimilation in *Corynebacterium
   diphtheriae*: pathways and regulatory cascades.** *FEMS Microbiol Lett* 2002,
   208:287–293.
8. Walter B, Hänßler E, Kalinowski J, Burkovski A: **Nitrogen metabolism and
   nitrogen control in corynebacteria: variations of a common theme.**
   J Mol Microbiol Biotechnol 2007, **12**:131–138.
9. Beckers G, Strösser J, Hildebrandt U, Kalinowski J, Farwick M, Krämer R,
   Burkovski A: **Regulation of AmtR-controlled gene expression in
   Corynebacterium glutamicum: mechanism and characterization of the
   AmtR regulon.** *Mol Microbiol* 2005, **58**:580–595.
10. Buchinger S, Strösser J, Rehm N, Hänßler E, Hans S, Bathe B, Schomburg D,
    Krämer R, Burkovski A: **A combination of metabolome and transcriptome
    analyses reveals new targets of the *Corynebacterium glutamicum*
    nitrogen regulator AmtR.** *J Biotechnol* 2009, **140**:68–74.
11. Amon J, Bräu T, Grimrath A, Hänßler E, Hasselt K, Höller M, Jeßberger N, Ott
    L, Szököl J, Titgemeyer F, Burkovski A: **Nitrogen control in *Mycobacterium
    smegmatis*: nitrogen-dependent expression of ammonium transport
    and assimilation proteins depends on the OmpR-type regulator GlnR.**
    J Bacteriol 2008, **190**:7108–7106.
12. Amon J, Titgemeyer F, Burkovski A: **A genomic view on nitrogen
    metabolism and nitrogen control in mycobacteria.** *J Mol Microbiol
    Biotechnol* 2009, **17**:20–29.
13. Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, Koonin EV, Krylov
    DM, Mazumder R, Mekhedov SL, Nikolskaya AN, *et al*: **The COG database: an
    updated version includes eukaryotes.** *BMC Bioinformatics* 2003, **4**:41.
14. Silberbach M, Hüser A, Kalinowski J, Pühler A, Walter B, Krämer R, Burkovski
    A: **DNA microarray analysis of the nitrogen starvation response of
    Corynebacterium glutamicum.** *J Biotechnol* 2005, **119**:357–367.
15. Pullan ST, Chandra G, Bibb MJ, Merrick M: **Genome-wide analysis of the
    role of GlnR in *Streptomyces venezuelae* provides new insights into
    global nitrogen regulation in actinomycetes.** *BMC Genomics* 2011, **12**:175.
16. Ahmad S, Bhatnagar RK, Venkitasubramanian TA: **Changes in the enzyme
    activities involved in nitrogen assimilation in *Mycobacterium smegmatis*
    under various growth conditions.** *Ann Inst Pasteur Microbiol* 1986,
    137B:231–237.
17. Iwainsky H, Sehrt I: **The nitrogen metabolism of mycobacteria.** *Z Tuberk
    Erkr Thoraxorg* 1968, **128**:145–150.
18. Sritharan V, Ratledge C, Wheeler PR: **Effect of homoserine on growth of
    Mycobacterium smegmatis: inhibition of glutamate transport by
    homoserine.** *J Gen Microbiol* 1987, **133**:2781–2785.
19. Nolden L, Beckers G, Möckel B, Pfefferle W, Nampoothiri KM, Krämer R,
    Burkovski A: **Urease of *Corynebacterium glutamicum*: organization of
    corresponding genes and investigation of activity.** *FEMS Microbiol Lett*
    2000, **189**:305–310.
20. Puskás LG, Inui M, Yukawa H: **Structure of the urease operon of
    Corynebacterium glutamicum.** *DNA Seq* 2000, **11**:383–394. 467.
21. Mongodin EF, Shapir N, Daugherty SC, DeBoy RT, Emerson JB, Shvartzbeyn
    A, Radune D, Vamathevan J, Riggs F, Grinberg V, *et al*: **Secrets of soil
    survival revealed by the genome sequence of *Arthrobacter aurescens*
    TC1.** *PLoS Genet* 2006, **2**:e214.
22. Nishio Y, Nakamura Y, Kawarabayasi Y, Usuda Y, Kimura E, Sugimoto S,
    Matsui K, Yamagishi A, Kikuchi H, Ikeo K, Gojobori T: **Comparative complete
    genome sequence analysis of the amino acid replacements responsible
    for the thermostability of *Corynebacterium efficiens*.** *Genome Res* 2003,
    13:1572–1579.
23. Gartemann KH, Abt B, Bekel T, Burger A, Engemann J, Flügel M, Gaigalat L,
    Goesmann A, Gräfen I, Kalinowski J, *et al*: **The genome sequence of the
    tomato-pathogenic actinomycete *Clavibacter michiganensis* subsp.
    michiganensis NCPPB382 reveals a large island involved in pathogenicity.**
    J Bacteriol 2008, **190**:2138–2149.
24. Bagwell CE, Bhat S, Hawkins GM, Smith BW, Biswas T, Hoover TR, Saunders
    E, Han CS, Tsodikov OV, Shimkets LJ: **Survival in nuclear waste, extreme
    resistance, and potential applications gleaned from the genome
    sequence of *Kineococcus radiotolerans* SRS30216.** *PLoS One* 2008, **3**:e3878.
25. Ishikawa J, Yamashita A, Mikami Y, Hoshino Y, Kurita H, Hotta K, Shiba T,
    Hattori M: **The complete genomic sequence of *Nocardia farcinica* IFM
    10152.** *Proc Natl Acad Sci USA* 2004, **101**:14925–14930.
26. Omura S, Ikeda H, Ishikawa J, Hanamoto A, Takahashi C, Shinose M, Takahashi Y,
    Horikawa H, Nakazawa H, Osonoe T, *et al*: **Genome sequence of an industrial
    microorganism *Streptomyces avermitilis*: deducing the ability of producing
    secondary metabolites.** *Proc Natl Acad Sci USA* 2001, **98**:12215–12220.
27. Ramos JL, Martínez-Bueno M, Molina-Henares AJ, Terán W, Watanabe K,
    Zhang X, Gallegos MT, Brennan R, Tobes R: **The TetR family of transcrip-
    tional repressors.** *Microbiol Mol Biol Rev* 2005, **69**:326–356.
28. Silberbach M, Schäfer M, Hüser AT, Kalinowski J, Pühler A, Krämer R,
    Burkovski A: **Adaptation of *Corynebacterium glutamicum* to ammonium
    limitation: a global analysis using transcriptome and proteome
    techniques.** *Appl Environ Microbiol* 2005, **71**:2391–23402.
29. Deutscher J: **The mechanisms of carbon catabolite repression in bacteria.**
    Curr Opin Microbiol 2008, **11**:87–93.
30. Görke B, Stülke J: **Carbon catabolite repression in bacteria: many ways to
    make the most out of nutrients.** *Nat Rev Microbiol* 2008, **6**:613–624.
31. Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning. A Laboratory
    Handbook.* 2nd edition. Cold Spring Harbor, N.Y: Cold Spring Harbor
    Laboratory Press; 1989.

32. Grant SG, Jessee J, Bloom FR, Hanahan D: **Differential plasmid rescue from transgenic mouse DNAs into** *Escherichia coli* **methylation-restriction mutants.** *Proc Natl Acad Sci USA* 1990, **87**:4645–4649.

33. Belisle JT, Sonnenberg MG: **Isolation of genomic DNA from mycobacteria.** *Methods Mol Biol* 1998, **101**:31–44.

34. Stephan J, Stemmer V, Niederweis M: **Consecutive gene deletions in** *Mycobacterium smegmatis* **using the yeast FLP recombinase.** *Gene* 2004, **343**:181–190.

35. Stephan J, Bender J, Wolschendorf F, Hoffmann C, Roth E, Mailänder C, Engelhardt H, Niederweis M: **The growth rate of** *Mycobacterium smegmatis* **depends on sufficient porin-mediated influx of nutrients.** *Mol Microbiol* 2005, **58**:714–730.

36. Moreno-Paz M, Parro V: **Amplification of low quantity bacterial RNA for microarray studies: time-course analysis of** *Leptospirillum ferrooxidans* **under nitrogen-fixing conditions.** *Environ Microbiol* 2006, **8**:1064–1073.

37. Li R, Xie Z, Tian Y, Yang H, Chen W, You D, Liu G, Deng Z, Tan H: **polR, a pathway-specific transcriptional regulatory gene, positively controls polyoxin biosynthesis in** *Streptomyces cacaoi* subsp. *asoensis.* *Microbiology* 2009, **155**:1819–1831.