The NnaR orphan response regulator is essential for the utilization of nitrate and nitrite as sole nitrogen sources in mycobacteria

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Nitrogen is an essential component of biological molecules and an indispensable microelement required for the growth of cells. Nitrogen metabolism of Mycobacterium smegmatis is regulated by a number of transcription factors, with the glnR gene product playing a major role. Under nitrogen-depletion conditions, GlnR controls the expression of many genes involved in nitrogen assimilation, including the msmeG_0432 gene encoding NnaR, the homologue of a nitrite/nitrate transport regulator from Streptomyces coelicolor. In the present study, the role of NnaR in the nitrogen metabolism of M. smegmatis was evaluated. The ΔglnR and ΔnnaR mutant strains were generated and cultured under nitrogen-depletion conditions. Total RNA profiling was used to investigate the potential role of NnaR in the GlnR regulon under nitrogen-depletion and in nitrogen-rich media. We found that disruption of MSMEG_0432 affected the expression of genes involved in nitrite/nitrate uptake, and its removal rendered mycobacteria unable to assimilate nitrogen from those sources, leading to cell death. RNA-Seq results were validated using quantitative real-time polymerase chain reaction (qRT-PCR) and electrophoretic mobility shift assays (EMSAs). The ability of mutants to grow on various nitrogen sources was evaluated using the BIOLOG Phenotype screening platform and confirmed on minimal Sauton’s medium containing various sources of nitrogen. The ΔglnR mutant was not able to convert nitrates to nitrites. Interestingly, NnaR required active GlnR to prevent nitrogen starvation, and both proteins cooperated in the regulation of gene expression associated with nitrate/nitrite assimilation. The ΔnnaR mutant was able to convert nitrates to nitrites, but it could not assimilate the products of this conversion. Importantly, NnaR was the key regulator of the expression of the truncated haemoglobin trHbN, which is required to improve the survival of bacteria under nitrosative stress.

The genus Mycobacterium contains obligatory pathogens known to cause serious diseases in mammals, including tuberculosis (Mycobacterium tuberculosis, Mtb) and leprosy (M. leprae) in humans, as well as a large number of opportunistic pathogens and/or free-living saprophytes, such as Mycobacterium smegmatis (M. smegmatis)1. The growth of bacteria occupying various environmental niches depends on the growth conditions and availability of essential elements such as carbon, oxygen and nitrogen that are used for biosynthesis of proteins, nucleic acids and cell wall components2. The genome of M. smegmatis contains a number of genes believed to be involved in nitrogen metabolism, but the real functions of the majority of them are still unknown. For bacteria, an important source of nitrogen is ammonium entering cells by diffusion across the cytoplasmic membrane or via protein-dependent transport. Three ammonium transporters (Amt1, AmtA, AmtB) have been identified in the cell wall of M. smegmatis. However, the bacterium also possess genes allowing assimilation of the nitrogen

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from urea or nitrite. The accessibility of the nitrogen determines the mode of its utilization. Glutamate is the main nitrogen-storage molecule in bacteria. In nitrogen-rich environments, glutamate dehydrogenase (GDH) plays a main role in nitrogen accumulation, as it is able to convert ammonium to L-glutamate. Under nitrogen deficiency, the glutamine synthetase/glutamate synthase (GS/GOGAT) is activated. Switching between the pathways is accompanied by changes in gene expression of the amtB (msmeg_2425) operon, consisting of ammonium transporter AmtB, nitrogen regulatory protein P-II (msmeg_2426), and GlnD adenyl transferase (msmeg_2427), as well as two additional ammonium transporters, AmtA (msmeg_4635) and Amt1 (msmeg_6259), glutamine synthetase and glutamate synthase, encoded by glnA1 and gltBD, respectively. Under nitrogen depletion, GlnD adenylates the GlnK (PII) protein, allowing its dissociation from the AmtB porin channel, resulting in ammonium inflow, and the glutamine synthetase is de-adenylated by GlnE.

Two major transcriptional regulators of nitrogen metabolism have been identified in Actinobacteria, AmtR and GlnR. The AmtR of Corynebacterium glutamicum regulates more than 30 genes, whereas the GlnR of Streptomyces coelicolor (S. coelicolor) affects the expression of more than 50 genes. The homologs of both regulators, AmtR (msmeg_4300) and GlnR (msmeg_5784), were identified in M. smegmatis, but MtB only encodes GlnR. In both MtB and M. smegmatis, GlnR is considered a key transcriptional regulator of nitrogen metabolism. GlnR controls the expression of ammonium transporters (amt1, amtB), signal transduction components (glnK, glnD), glutamine synthetase (glnA) in M. smegmatis and nirBD expression in M. tuberculosis. Response of MtB to nitrogen starvation is fairly well characterized, including identification of GlnRs DNA binding consensus and regulon. Very recently Liu and colleagues demonstrated GlnR mediated regulation of short chain fatty acid synthesis in M. smegmatis. GlnR was found to bind to promoter regions of prepE (AMP-forming propionyl-CoA synthase) and 4 AMP-forming acs genes (acetyl-CoA) under nitrogen starvation. A separate study revealed GlnR’s involvement in the regulation of methylcitrate cycle by directly controlling the expression of prepDBC operon. Furthermore, the GlnR-dependent regulation of more than 100 genes under nitrogen depletion has been demonstrated using ChIP-seq technology in M. smegmatis. Among them are those involved in ammonium, nitrate/nitrite, amino acid/peptide and urea uptake, genes encoding the nitrite reductase NirBD, amine oxidase, urea amidolase, deaminase and hydrolases acting on carbon-nitrogen bonds, as well as regulatory genes, including msmeg_0432, the homologue of the nitrite/nitrate transport regulator (NnaR) of S. coelicolor also present in M. tuberculosis. GlnR and NnaR both belong to the OmpR-type family of two-component signal transduction system elements, acting as orphan response regulators (RRs). GlnR and NnaR conserved the aspartic acid residue that could potentially be phosphorylated by the cognate histidine kinase, but no kinase has been identified as a partner for those regulators. On the other hand, GlnR is phosphorylated by the serine/threonine kinases and is acetylated in Streptomyces, and those modifications supposedly regulate the protein’s activity as a response regulator. Here, we have engineered ∆nnaR and ∆glnR M. smegmatis mutants to evaluate systematically the role of NnaR as a regulator of nitrogen metabolism in M. smegmatis by using phenotypic microarray technology and RNA-Seq analysis and by monitoring the kinetics of growth and viability in the presence of various nitrogen sources.

Results

M. smegmatis ΔnnaR and ΔglnR mutant strains are defective in assimilation of various nitrogen sources according to phenotype microarray profiling.

The mutants defective in the synthesis of NnaR or GlnR were subjected to phenotypic analysis using the BIOLOG Phenotype Microarray screening platform, which allows for convenient testing of growth kinetics of bacteria under various conditions. We exploited 6 PM plates (PM3-8) representing 576 different growth conditions, including various nitrogen sources, phosphorus and sulphur sources, nutrient supplements, and peptide nitrogen sources for simultaneous testing.

When comparing the area under the curve (AUC) values of the wild-type M. smegmatis and the ΔmnaR and ΔglnR mutants, several changes were noted in the assimilation of various nitrogen sources (Table S2). The mutant defective in the synthesis of NnaR was significantly less metabolically active than wild-type in the media containing guanosine (AUC difference >9,500), nitrite (>8,500), γ-amino-N-butyrinic acid (>7,500) or nitrate (>6,500). The list of significant differences between the mutant and wild-type strain was much longer in the case of ΔglnR. The mutant defective in the synthesis of GlnR was significantly less metabolically active than wild-type in the media containing various peptides and amino acids as nitrogen sources (approximately 100 hits with AUC differences between 7,000 and 30,000). The kinetics of ΔglnR metabolism was also affected in the presence of, e.g.,...
Figure 1. The survival of *M. smegmatis* strains propagated on nitrogen-limiting Sauton’s medium containing various nitrogen sources. Wild-type, Δ*nraR*, Δ*glnR*, Δ*nraR–attB::phsp*nraR*, Δ*glnR–attB::phsp*glnR* and Δ*nraR, glnR* were grown in the presence of the (A) urea (pH = 4.5), (B) uric acid, (C) histidine (pH = 9.5), (D) leucine, (E) ammonium sulphate, (F) allantoin, (G) hydantoin, (H) proline, (I) methionine, (J) L-glutamic acid potassium salt monohydrate (each at 10 mM final concentration) and (M) acetamide (5 mM). Standard (A) 7H9/OADC medium was used as a positive control, and (B) nitrogen-free Sauton’s medium was the negative control. The numbers of viable cells were determined by counting of the bacterial colony-forming units (CFU) on 7H10/OADC plates at 24 hours. Colony formation values are means ± standard deviation from three independent experiments. The statistical significance was determined using Student's t-test (*p < 0.03): (D) for Δ*glnR*, Δ*glnR–attB::phsp*glnR* p < 0.001, (E) for Δ*glnR* p < 0.001, (F) for Δ*glnR*, Δ*glnR–attB::phsp*glnR* p < 0.001, (G) for Δ*glnR* p < 0.001, (H) for Δ*glnR* p < 0.001, (I) for Δ*glnR* p = 0.014, (J) for Δ*glnR* p < 0.001, (L) for Δ*glnR* p < 0.001, (M) for Δ*glnR* p < 0.001.
Utilization of various nitrogen sources via the ΔnnaR and ΔglnR strains. The evidence from the global BIOLOG analysis prompted us to determine the growth and viability of the studied M. smegmatis strains in nitrogen-limiting medium containing various substances as the sole nitrogen sources. Most of the studied nitrogen compounds, such as urea (pH = 4.5), uric acid, histidine (pH = 9.5), leucine (pH = 9.5), methionine (pH = 9.5), allantoin and potassium L-glutamate, were selected based on BIOLOG Phenotype Microarray analysis. We additionally tested the ability of the ΔnnaR strain to assimilate nitrogen from ammonium sulphate, hydantoin and acetamide. The kinetics of the growth and number of viable cells were evaluated in the presence of the tested nitrogen sources, following the induction of the initial nitrogen starvation to reduce background interference. The CFU analysis did not indicate significant differences in viability of ΔnnaR mutant cells in comparison to wild-type in the presence of the selected nitrogen-containing compounds. The ΔnnaR strain efficiently incorporated nitrogen derived from a majority of tested compounds, except for urea, hydantoin and methionine. In contrast, ΔglnR had significantly reduced survival in the presence of uric acid (96.6%), histidine (40.2%), leucine (83.5%), ammonium sulphate (71.3%), allantoin (99.0%), hydantoin (42.9%), proline (88.4%), potassium L-glutamate (95.1%) and acetamide (88.5%) in comparison to wild-type. Urea, methionine and hydantoin were the worst at supporting the growth of all studied strains when used as sole nitrogen sources (Supplementary Table S2).

Transcriptome profiling reveals NnaR functions in the regulation of nitrate/nitrite transport and utilization systems. Total RNA sequencing was employed to establish the NnaR regulon in M. smegmatis. The RNA profiles were compared between the wild-type, ΔnnaR and ΔglnR strains cultured in the defined Sauton’s minimal medium containing high levels of nitrogen (30 mM ammonium sulphate) or under nitrogen-limiting conditions (1 mM ammonium sulphate). When comparing ΔnnaR to the wild-type strain, there were no significant differences between their transcriptomes when strains were cultured in nitrogen-rich media. Upon nitrogen depletion, a total of seven transcripts (corresponding to four single ORFs and three operons) were down-regulated in the mutant strain. In the wild-type strain, all these transcripts were induced specifically during nitrogen starvation, and their relative expression was low in nitrogen-rich growth medium. The proteins encoded by the down-regulated transcripts were involved in nitrogen utilization pathways. They included nitrate, nitrite and nucleotide-derived nitrogen cycle elements, as well as proteins implicated in nitrogen storage. One of the identified targets was the gene encoding bacterial truncated haemoglobin (MSMEG_5765, trHBn). MEME analysis of the promoter regions for the abovementioned transcripts revealed the presence of a partially degenerated palindromic motif (c-t-C-A-C-a/c- (16N)-t/g-G-T-G-a-g) located within the vicinity of the GlnR-binding motif (Supplementary Fig. S3). The two regulatory DNA motifs were spaced similarly in the majority of the transcripts. In the case of msmeg_4008, the spacing between the palindromic sequences was uneven, lacking a single nucleobase. Thus, it was only possible to assign two half-motifs, instead of the abovementioned motif. Consistently, the expression levels of all the seven transcripts were even more profoundly depleted in the ΔglnR strain. In line with the previously published microarray data, the ΔglnR strain showed a high number of transcriptional changes upon nitrogen depletion, as well as when grown on nitrogen-rich media (Supplementary Table S3). When cultured under nitrogen-limiting conditions, 537 individual genes (approximately 296 transcripts, counting operons as single transcripts) were up- or down-regulated by more than a log2 fold-change value of ±1.5. A total of 42 previously annotated GlnR motifs (out of 53) were associated with significant changes in gene expression under the conditions tested. The removal of glnR also led to a profound down-regulation of nnaR expression, making this strain a natural ΔnnaR mutant. The removal of glnR led to a transcriptional change of 633 genes when the mutant strain was grown on the nitrogen-rich medium. However, a different set of genes changed on the nitrogen-rich medium, and only 12 GlnR motifs were identifiable in front of the affected transcripts. Importantly, the differences observed between nitrogen-rich and nitrogen starvation growth conditions were much less sharp for the elements encoding nitrogen utilization enzymes, e.g., the log2 fold change of −2.33 versus −9.37 for the nnaR transcript. In fact, multiple nitrogen metabolism genes remained relatively unaffected, e.g., nitrate reductase (msmeg_0427, msmeg_0428) and nitrite extrusion protein (msmeg_0433), as they were not up-regulated under nitrogen-rich growth conditions and were rather associated with nitrogen starvation.

Validation of RNA-Seq results by quantitative qRT-PCR and electrophoretic mobility shift assay (EMSA). To validate the expression profiles obtained by RNA-Seq, qRT-PCR was performed on eight chosen transcripts that enabled us to differentiate between the wild-type and the investigated mutant strains cultured under nitrogen starvation or nitrogen-rich conditions. We selected four genes that had a reduced level of expression in the ΔnnaR and ΔglnR mutant strains: msmeg_0427 (nirB, nitrate reductase), msmeg_0433...
(narK, nitrate extrusion protein), msme_5360 (formate/nitrate transporter), msme_5765 (truncated bacterial haemoglobin, trHbN); we also selected two genes that had very reduced expression in ΔglnR compared to ΔnnaR and that are involved in ammonia and urea transport, respectively: msme_2425 or msme_2982. As a control, we selected two genes unrelated to nitrogen metabolism: the gene encoding AccD5 carboxyltransferase (msme_1813), involved in cell envelope lipid biosynthesis, and rpoB (msme_1367), encoding the β-subunit of the RNA polymerase. The same RNA samples as extracted for library preparation were used for qRT-PCR validation. qRT-PCR analyses confirmed the results obtained from RNA-Seq. Under nitrogen depletion, the transcript levels of msme_0427, msme_0433, msme_5360, and msme_5765 from M. smegmatis ΔnnaR and ΔglnR mutants were down-regulated compared to wild-type. The levels of tested here transcripts remained low in ΔglnR strain complemented with nnaR (ΔglnR-attB::phspNnaRnnaR). On the other hand, the same set of transcripts returned to wild-type levels for ΔnnaR complemented mnaR (ΔnnaR-attB::phspMnaR) (Fig. 2A). The collected data revealed no significant changes in the expression levels in the above genes for ΔnnaR, ΔglnR, ΔglnR-attB::phspMnaR and the wild-type strain, under nitrogen-rich environment (Fig. 2B). The qRT-PCR analyses confirmed lower expression of msme_2425 (1.32 log10 fold change (FC)) and msme_2982 (1.48 log10 FC) in ΔglnR in comparison to ΔnnaR under nitrogen depletion (Fig. 2C) and in rich environment: msme_2425 (2.50 log10 FC) and msme_2982 (2.99 log10 FC) (Fig. 2D). As expected, no significant changes in accD5 or rpoB were observed in either studied condition (Fig. 2C,D).

The EMSA assay was applied to further facilitate the validation of RNA-Seq results and to cross-confirm the NnaR-specific DNA-binding motif identified via MEME search. To show a direct regulation of identified genes by NnaR and GlnR, we purified the recombinant versions of both response regulators to assess their DNA-binding properties. The NnaR protein was purified as a full-length polypeptide possessing both the uroporphyrinogen-III cosynthetase and the NnaR-specific DNA-binding motif identified via MEME search. The NnaR protein bound avidly to the putative promoters of msme_0427, msme_0433, msme_5360, and msme_5765 but did not recognize the sequence upstream of msme_4008 gene (Fig. 3A). On the other hand, modest interaction was observed for GlnR with msme_0427, msme_0433 and msme_5765 promoter sequences but not with msme_4008 and msme_5360 promoters. Interestingly, the mobility of oligos representing msme_4008 and msme_5360 promoters was affected in the presence of both investigated proteins applied simultaneously, indicating possible cooperation of NnaR and GlnR (Fig. 3A). To test if the proteins may act as a stable protein complex we have performed a pull-down experiment with recombinant response regulators used for EMSA. We could only observe weak association between the two proteins (Supplementary Fig. S4) and they failed to interact with each other when tested in the bacterial two hybrid method (data not shown).
To further confirm the specificity of the NnaR protein towards the tested sequences, we also carried out a competition experiment. The NnaR protein caused a shift of the entire labelled \( msme_{g\_0427} \) DNA fragment when it was incubated with the sole labelled probe. The binding was nearly abolished in the presence of a competitor DNA fragment used at 100-fold excess relative to the labelled probe (Fig. 3B).

The \( \Delta nnaR \) and \( \Delta glnR \) mutant strains show severe growth defects on nitrate and nitrite. The collective evidence from the global RNA-Seq and high-throughput BIOLOG Phenotype screening platforms indicated that NnaR functions as a nitrogen assimilation regulator in mycobacteria. To evaluate the importance of NnaR protein in nitrogen metabolism/assimilation in \( M. \) \( smegmatis \) cells, we examined the growth of \( \Delta nnaR \) mutant and \( \Delta nnaR-attB_{\text{phage}} nnaR \) complementation strain on nitrogen-limiting agar, YNB, containing sodium nitrate as sole nitrogen source (later referred to as “YNB-nitrate”). We used \( \Delta glnR, \Delta glnR-attB_{\text{phage}} glnR \) complementation strain and wild-type strain as controls. Full growth for all tested strains was observed on nitrogen-rich agar plates (7H10/OADC). The \( \Delta nnaR \) and \( \Delta glnR \) cells did not exhibit any growth on YNB-nitrate plates, while

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**Figure 3.** Interactions between NnaR, GlnR or NnaR/GlnR mixture and putative promoter regions of \( msme_{g\_0427}, msme_{g\_0433}, msme_{g\_4008}, msme_{g\_5360} \) and \( msme_{g\_5765} \) analysed by EMSA. For EMSA reactions (A), approximately 30 nM hexachlorofluorescein-labelled DNA was incubated with 0 or 2 \( \mu \)M NnaR, 4 \( \mu \)M GlnR or 2 \( \mu \)M NnaR combined with 4 \( \mu \)M GlnR for 10 min in EMSA reaction buffer containing 10 mM Tris, 100 mM KCl, 1 mM DTT, 2.5% glycerol, 20 mM MgCl\(_2\), 500 ng poly(dI·dC), and 0.05% NP-40 (pH 7.5). (B) NnaR binding specificity to the promoter region of \( msme_{g\_0427} \) was analysed using the competition test. Approximately 30 nM hexachlorofluorescein-labelled DNA or a combination of both 30 nM labelled and 100-fold excess unlabelled probe was incubated with 2 \( \mu \)M NnaR for 10 min in EMSA reaction buffer. The samples were resolved in 2% agarose gel and visualized on a GE Typhoon 8600 Imager.
the ΔnnaR-attB::pBSM M. smegmatis strains and wild-type cells showed full growth on nitrogen-limiting medium (Fig. 4A).

To further confirm the above results, we examined the kinetics of growth and viability of the ΔnnaR mutant and the ΔnnaR-attB::pBSM M. smegmatis strains in the presence of nitrate (sodium nitrate) and nitrite (sodium nitrite) as the sole source of nitrogen. The ΔnnaR mutant and the ΔglnR strain complemented with an additional copy of nnaR, controlled by a heat shock promoter, as well as wild-type, were the additional controls in this experiment. Growth experiments for all tested strains were carried out following a 16 hours of nitrogen starvation to deplete the intracellular pool of nitrogen stored inside the cells. A significant reduction in the growth kinetics and viability of ΔnnaR and ΔglnR cells was observed compared to wild-type. The mutants had completely lost their ability to assimilate both nitrate and nitrite, which could be explained by the RNA-Seq results (Fig. 4B).

After 24 hours of growth on the sodium nitrate-containing media, the viability of ΔnnaR and ΔglnR cells was observed compared to wild-type. The mutants had completely lost their ability to assimilate both nitrate and nitrite, which could be explained by the RNA-Seq results (Fig. 4B). Interestingly,
the survival of ΔglnR mutant cells was not rescued when a complementing copy of nnaR was introduced to this mutant. Hence, NnaR protein was not able to compensate for the lack of GlnR. The observed results suggest that both response regulators are required for nitrate/nitrite assimilation.

The ability of ΔnnaR and ΔglnR of M. smegmatis to convert nitrate to nitrite and nitrite to ammonia. To determine whether the observed reduction in survival of ΔnnaR cells was due to the inability of the mutant cells to convert nitrate to nitrite, the Griess Reagent System was applied following the manufacturer's instructions. Measurement of nitrite in the supernatants obtained after centrifugation of bacterial cells from the medium containing sodium nitrate as sole nitrogen source revealed that ΔnnaR reduced nitrate to nitrite, but it could not assimilate the product of this conversion (Fig. 5A). Compared to the wild-type strain, the growth of the mutant was not observed, but the increase in the amount of nitrite (as a nitrate conversion product) in growth medium was noticeable. The restoration of the functional NnaR in the cell, as in the NnaR complementation strain, restored the ability of the bacterium to incorporate nitrite into the intracellular biomolecules. This observation suggests that the nitrate reduction pathway is at least partially active in ΔnnaR and other factors are responsible for the observed loss of viability. From previous work in Streptomyces and based on the RNA-Seq data, it seemed that nirBD expression may be the essential factor providing bacterial survival when nitrate/nitrite is the only available source of nitrogen. Thus, we tested the presence of ammonium ions in the supernatants from cultures of the wild-type and the ΔnnaR-attB::pHspnnaR strains at 72 hours (Fig. 5B). The concentration of ammonium remained at the background level in the culture media from the NnaR mutant strain. In contrast, ΔglnR could not reduce nitrate to nitrite, which is consistent with the complete lack of growth of this strain in the presence of sodium nitrate. The introduction of an intact copy of the nnaR gene under the highly active hsp60 promoter into ΔglnR mutant did not restore the ability of that strain to convert nitrate to nitrite, suggesting both transcriptional regulators need to be active for the effective expression of nitrogen-converting enzymes (Fig. 5A).

Biofilm formation of M. smegmatis ΔnnaR mutant strain. It has been observed that glnR response regulator is upregulated during biofilm formation of M. smegmatis. In a further study, Yang et al. has observed delayed biofilm development of ΔglnR as well as GlnR-dependent resistance to hydrogen peroxide under
nitrogen limiting conditions\textsuperscript{23}. To test whether the removal of NnaR affects biofilm development of \textit{M. smegmatis} we have examined the growth of \textit{\textDelta nnaR} strain on Sauton's\textsubscript{N0}, Sauton's\textsubscript{N1/2}, and Sauton's\textsubscript{N0} media\textsuperscript{23}, in the absence of any detergents. We compared the growth of \textit{\textDelta nnaR} strain to wild-type as well as \textit{\textDelta glnR} strains as controls for our experiment. While we have observed a delay in biofilm formation for \textit{\textDelta glnR}, as reported by Yang and colleagues\textsuperscript{23}, we were unable to note any significant changes in the kinetics of biofilm formation of \textit{\textDelta nnaR} strain, which behaved similarly to the wild-type strain (Supplementary Fig. S5). None of the strains was able to produce biofilm in Sauton's nitrogen-free medium (data not shown).

**Discussion**

The GlnR protein is currently considered the key response regulator contributing to the survival of mycobacterial cells during nitrogen starvation\textsuperscript{2-5,7,10}. The gene encoding GlnR is not essential when mycobacteria reside within nitrogen-rich niches, but it becomes indispensable under nutrient-limiting conditions\textsuperscript{5}. The regulatory DNA motifs are fairly well characterized for the GlnR protein from \textit{M. smegmatis} and \textit{M. tuberculosis}, as well as some other actinomycetes, showing good conservation among different species\textsuperscript{3,24,25}. During our analysis, we discovered that there was also a great level of similarity between the regulatory motifs recognized by the GlnR factor and another "global" response regulator, MtrA\textsuperscript{26,27}. The motif consensus and the internal spacing between the motif residues are very well conserved, with nearly identical sequences. Corroborating this, we discovered that the DNA binding domain of MtrA and GlnR were nearly identical, and both transcriptional regulators were thus likely to bind highly similar motifs (Supplementary Fig. S3C). On the other hand, MtrA was previously shown to bind the promoter sequence of the \textit{nirB} gene in mycobacteria\textsuperscript{28}. Another study found GlnR competed with PhoP to bind promoter regions of the \textit{glnA} and \textit{amtB} genes in \textit{S. coelicolor}\textsuperscript{29}. The regulatory networks require an interplay between all the response regulators acting in a highly orchestrated way to maximize the adaptive responses of the bacteria to changing environments. On the other hand, one gene's expression may be regulated by several factors, and they may play either synergistic or antagonistic roles in the regulation of gene expression. The complicated dependencies between factors contributing to the transcriptional regulation of a given gene are not very well understood.

The GlnR regulon is fairly well characterized for some actinomycetes, with the response regulator acting mainly as a transcriptional activator during nitrogen starvation and less often playing a role of transcription repressor\textsuperscript{29,30}. Using the CHIP-Seq approach, a previous study identified approximately 53 GlnR boxes on the \textit{M. smegmatis} genome, most of them potentially being regulatory motifs\textsuperscript{3}. The same study identified a much wider set of genes with altered expression in the absence of GlnR using the microarray approach. Another study used the same model organism to discover that some additional transcripts changed significantly in the response to nitrogen depletion on the \textit{\textDelta glnR} background, also using microarray technology\textsuperscript{31}.

Among the multiple genes regulated directly by the activity of GlnR are transcription factors, adding to the overall complexity of the GlnR dependent response. One such transcription factors is NnaR, belonging to the same class of response regulators of the two-component systems family\textsuperscript{32}. Both GlnR and NnaR are considered orphan response elements, lacking any identifiable, genetically linked histidine kinase. A report from \textit{Streptomyces} revealed a potential role of serine/threonine phosphorylation and some lysine acetylation among the mechanisms by which the activity of GlnR protein is regulated\textsuperscript{33}. On the other hand, GlnR controls genes encoding lysine deacetylases in Actinobacteria\textsuperscript{34}. Such mechanisms may further alter the ability of the protein to bind DNA, modulating its DNA-binding strength and/or substrate specificity, making GlnR an intriguing model system to study. Another \textit{Streptomyces} study focused on elucidating the activity of NnaR protein, a regulator activating expression of a small set of genes critical for nitrate/nitrite assimilation. Based on \textit{in silico} analyses, the study identified a potential NnaR-binding motif in the vicinity of the GlnR binding motifs, present in front of a nitrite reductase, NirB, a putative nitrate reductase, NasA, and the NarK nitrate/nitrite transporter\textsuperscript{34,35}. To investigate the activity of NnaR in mycobacterial species, we used the \textit{M. smegmatis} as the model. Since NnaR expression is directly affected by the activity of GlnR, we also intended to study their interconnection in more detail.

We initially generated unmarked mutant strains lacking the NnaR or the GlnR orphan response regulator via the standard two-step recombination protocol\textsuperscript{19}. We next subjected such mutant strains to global analyses using BIOLOG Phenotype Microarray screen\textsuperscript{33} and transcriptomic profiling using total RNA sequencing. Using the two independent platforms, we detected nitrate/nitrite metabolism as the key pathway regulated by the activity of NnaR. We performed the transcriptional profiling under the conditions previously reported for the GlnR profiling microarray experiments\textsuperscript{5}, to be able to cross-validate our results. The removal of NnaR caused only subtle transcriptomic rearrangements, significantly changing the expression level of twenty genes (three operons and eight single open reading frames), but only when the strains were cultured under nitrogen-limiting conditions. Similar to what was predicted based on the \textit{in silico} analysis from \textit{Streptomyces}\textsuperscript{34}, the affected transcripts included the nitrite reductase NirBD (\textit{msmeg\_0427, msmeg\_0428}), the NarK transporter (\textit{msmeg\_0433}) and the pseudogene for NasA (\textit{msmeg\_4206}). We additionally identified \textit{msmeg\_5360}, a formate/nitrate transporter, as well as \textit{msmeg\_5765} as two more transcripts requiring the activity of NnaR for their expression under nitrogen depletion in \textit{M. smegmatis}. Two additional operons, encoding genes mainly required for nucleotide transport and recycling, were affected by the removal of NnaR. Both operons encoded phenylhydantoinase genes, needed for the recycling of nitrogen from hydantoin. Thus, we tested the ability of the \textit{\textDelta nnaR} mutant and wild-type strains to utilize hydantoin and allantoin as the sole source of nitrogen to support the growth of \textit{M. smegmatis}. However, the laboratory strain of \textit{M. smegmatis} failed to utilize hydantoin efficiently, showing only residual growth. It might have lost the full pathway necessary for the efficient utilization of such molecules as nitrogen sources. It is possible, however, that the environmental strains of mycobacteria use NnaR to control the expression of hydantoin-recycling factors, just as they express a full copy of the NasA nitrate reductase, which is only a pseudogene in the \textit{M. smegmatis} \textit{MC}2 155 (MSMG) laboratory strain. We noted virtually no significant differences between the transcriptomes of the wild-type strain and the NnaR mutant grown in the presence of excess...
ammonium. The transcriptome profiling was followed by a wide screening of any potential phenotypic changes that may have resulted from the observed changes at the RNA level. Only nitrite and nitrate were selected as critical target molecules for the regulation of NnaR in the laboratory MSSMEG strain. In our study, the ΔnnaR strain was completely unable to grow on nitrate or nitrite as sole nitrogen source, making the NnaR protein a conditionally essential gene for mycobacteria. The mechanism of bacterial killing relied most likely on the inability of the ΔnnaR strain to reduce nitrite to ammonia in the absence of sufficient expression of NirBD nitrite reductase. The ΔnnaR mutant showed no changes in growth, survival or transcriptomic profile when cultured on ammonium salts as nitrogen sources. Additional phenotypic alterations in response to NnaR inactivation cannot be ruled out and may be more pronounced in the environmental strains, with nucleotide scavenging and recycling being the most promising targets. This hypothesis was also confirmed by the BIOLOG screen, showing reduced growth of the ΔnnaR mutant on guanosine and γ-amino-N-butryc acid as nitrogen sources.

Interestingly, the NnaR protein contains an N-terminal HemD domain and was originally annotated as a bifunctional protein that was a putative uroporphyrinogen-III synthase and a response regulator. The above-mentioned study on Streptomyces showed that NnaR does not possess HemD activity. M. smegmatis possesses another HemD paralog, MSMEG_0954, which is likely the actual uroporphyrinogen-III synthase. Interestingly, NnaR is required to support the expression of the bacterial truncated haemoglobin, trHBn (msmeg_5765), which is one of the highest-affinity targets for NnaR-dependent expression. Studies on M. tuberculosis, M. bovis and M. smegmatis have shown that trHBn is involved in the adaptation to nitrite, acting as a nitrosative stress inducer. TrHBn together with another truncated haemoglobin, trHBo, contributes significantly to the survival of M. tuberculosis during macrophage infection, reducing the nitrosative and oxidative stress levels. TrHBn protects aerobic respiration from being inhibited by nitric oxide radical with its potent nitric oxide dioxygenase activity. Studies on the trHBn promoters from M. tuberculosis M. smegmatis suggest the presence of an iron/haeme-containing oxygen sensor that is involved in the modulation of the expression of truncated haemoglobins. Taking into account our transcriptomic data and the presence of a HemD-like domain (possibly interacting with haeme or haeme-like molecules) in the N-termius of NnaR, we are confident that NnaR is the regulator required for trHBn expression. This makes NnaR an important factor modulating the bacterial response to nitrosative stress, which is one of the key elements of the interplay between macrophages and M. tuberculosis, showing NnaR contributes to bacterial fitness and intracellular survival. The trHBn gene is listed among the elements of the GlnR regulon in M. tuberculosis, likely requiring co-regulation of NnaR-GlnR to fully activate its transcription, similarly to what we can see in M. smegmatis.

All the transcriptomic changes caused by NnaR acting as a transcriptional activator were further deepened by the removal of GlnR. In other words, the NnaR regulon was even less efficiently expressed in the cells lacking GlnR. Adding another copy of NnaR under a strong promoter did not rescue the survival of the GlnR-deficient strain, suggesting that co-regulation rather than separate effects or the lack of NnaR expression on the ΔglnR mutant background were responsible for the observed phenomena. This notion was previously suggested by the above-mentioned work on Streptomyces’ NnaR protein. We have collected additional pieces of evidence to support that finding. Importantly, we have noticed some more unique characteristics of the transcripts most profoundly affected by the removal of GlnR and NnaR. The main observation was that the spacing between the two motifs was fairly conserved for most of the studied transcripts, which carry features of leaderless transcripts. The transcription start site was located downstream of the GlnR-binding motif. These characteristics also applied to the promoter region of the trHBn gene, containing a putative GlnR box, downstream of the NnaR-binding motif. It is thus likely that the two response factors co-regulate the expression of the entire NnaR regulon. In our case, it is likely that NnaR acts as a sensing molecule, using an iron/haeme antenna to detect oxygen and nitrogen levels and serving as a proxy for GlnR-dependent transcription activation. One cannot rule out a NnaR-GlnR-like mode of co-regulation between GlnR, as a regulatory hub, and other types of response regulators in mycobacteria.

### Materials and Methods

#### Strains and bacterial growth conditions.

_Escherichia coli_ strains were grown in Luria-Bertani broth (LB) or agar plates supplemented with ampicillin (50 μg/mL), kanamycin (50 μg/mL) or hygromycin (200 μg/mL). _M. smegmatis_ strains were propagated in Middlebrook 7H9 (Difco Laboratories) liquid medium supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase), 0.05% Tween 80 and kanamycin (25 μg/mL) or 7H10 medium supplemented with 10% OADC and 0.2% glycerol. For some experiments, such as culture preparation for RNA-Seq analysis and growth assays under nitrogen limitation, a defined nitrogen-free Sauton’s medium was applied (0.05% KH₂PO₄, 0.05% MgSO₄, 0.2% citric acid, 0.005% ferric citrate, 0.2% glycerol, 0.0001% ZnSO₄, 0.015% tyloxapol). To examine the growth of the studied strains on sodium nitrate as the sole source of nitrogen, 1.5% agar plates containing yeast nitrogen base medium lacking any amino acids and ammonium sulphate (YNB) supplemented with 10% AD were applied. All strains used in the study are listed in Table S1.

#### Gene cloning strategies.

All plasmid isolation, transformation and cloning techniques were performed essentially according to the protocols by Sambrook and Russell (2001). All PCR products were generated using thermostable AccuPrime Pfx DNA polymerase (Invitrogen) and cloned initially into a blunt vector (pJET 1.2/blunt; Thermo Fisher Scientific). Next, genes of interest were sequenced, released by digestion with appropriate restriction enzymes and cloned into the final vectors. The primers and plasmids used in this work are listed in Table S1.

#### Construction of gene replacement vectors and complementation plasmids.

Suicidal delivery vectors carrying deletion of _msmeg_ 0432 (nnaR) or _msmeg_ 5784 (glnR) were prepared in three steps. First, the 5’ upstream region of _nnaR_ or _glnR_ (1560 bp or 1484 bp, respectively) was cloned into a suicidal recombination delivery vector, p2NIL (Table S1). Next, a 1396 or 1660 bp fragment of _nnaR’s_ or _glnR’s_ downstream regions were
ligated with plasmids from step 1 to create truncated, out-of-frame copies of the respective genes. Finally, a 6 kb PacI cassette from pGOAL17 was added, resulting in suicidal delivery vectors pMA3 and pRD152, used to engineer the directed *M. smegmatis* mutant strains.

To prepare the complementation plasmid, the *nnaR* gene was subcloned under the control of a heat shock promoter Hsp60 (Table S1) in the pMV261 vector to create pMA4. Next, a 1576 bp fragment consisting of *nnaR* and the Hsp60 promoter was released using XbaI/HindIII and cloned into pMV306K to create the final pMA5 construct. The obtained plasmid was integrated into the *attB* attachment site of the directed Δ*glnR* and Δ*nnaR* *M. smegmatis* mutants.

In order to generate a *glnR* complementation plasmid, the *glnR* gene (786 bp) and its putative promoter (500 bp) was PCR amplified, verified by sequencing and cloned in the integration vector pMV306 (pRD154), allowing for integration of the whole plasmid into a single –*attB* site on *M. smegmatis* chromosome. The resulting pRD154 plasmid was electroporated into Δ*glnR* *M. smegmatis* competent cells and the integration was confirmed by PCR.

**Disruption of *M. smegmatis nnaR* and *glnR* genes at their native chromosomal loci.** A two-step protocol for homologous recombination19 was applied to generate defined mutant strains lacking functional NnaR or GlnR proteins as described previously13,19. The suicidal recombination plasmids pMA3 and pRD152 were treated with 0.2 mM NaOH and integrated into the *M. smegmatis* chromosome by homologous recombination. The obtained single-crossover (SCO) recombinants were blue, KanR and sensitive to sucrose. The site of recombination was confirmed by PCR and Southern hybridization (data not shown). The SCO strains were further processed to select for double-crossover (DCO) mutants that were white, KanR and resistant to sucrose (2%). The genotypes of the obtained mutant DCO strains were confirmed by Southern blot hybridization using the Amersham ECL Direct Nucleic Acid Labelling And Detection System (GE Healthcare) following the manufacturer’s instructions. Probes used were generated by PCR using primers listed in Table S1. In order to construct a double mutant strain, lacking functional copies of both *nnaR* and *glnR* genes, the suicidal delivery vector pRD152 carrying a Δ*glnR* gene was electroporated into Δ*nnaR* *M. smegmatis* competent cells. The SCO and further DCO screening was performed as described above. The genotype of the obtained double mutant strain Δ(*nnaR, glnR*) was confirmed by PCR and Southern hybridization using probe homologous to the 3′ of *glnR* gene (Supplementary Fig. S1).

**Cloning, expression and purification of NnaR and GlnR proteins.** The *nnaR* (*msmeg_0432*) and *glnR* (*msmeg_5784*) coding regions were PCR-amplified using primers listed in Table S1. The *nnaR* gene was cloned into the pMALC4e expression vector (pMA6), and *glnR* was cloned into the pET28a expression vector (pRD153). The resulting pMA6 plasmid was transformed into *E. coli* Arctic Express cells, whereas pRD153 was introduced into *E. coli* BL21 pLyS cells.

The biomas of *E. coli* Arctic Express cells overexpressing MBP-NnaR was cultured in LB broth supplemented with 0.2% glucose, 100 µg/mL ampicillin and 0.01% gentamycin with the shaking at 37 °C until the OD600 reached 0.6, and the cells were cooled down to 6 °C. Expression of protein was induced by addition of 0.4 mM IPTG followed by overnight incubation at 6 °C. Cells were harvested and suspended in column buffer (20 mM Tris-HCl, 1 M EDTA and 1 M DTT) with 100 µg/mL lysozyme and 1 M phenylmethylsulphonyl fluoride. For cell disruption, the suspension was sonicated, and the cell lysate was pre-cleared by centrifugation. Recombinant NnaR was obtained using affinity chromatography by passing the cell lysate through amylose resin (Amylose Resin High Flow), where the recombinant protein was washed with the column buffer and eluted with eluting buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT and 10 mM maltose). Next, NnaR was purified on a HiTrap SP FF column using AKTA start (GE Healthcare) and was eluted in a NaCl gradient with buffers containing 50 mM Tris-HCl and 10% glycerol.

The *E. coli* BL21 pLyS cells overexpressing His-GlnR were cultured in LB broth supplemented with 0.005% kanamycin and 34 µg/mL chloramphenicol with shaking at 37 °C until the OD600 reached 0.6, and the cells were cooled down to 20 °C. Expression of protein was induced by adding 1 mM IPTG followed by incubation overnight at 20 °C. Cells were harvested, centrifuged and suspended in column buffer (50 mM Tris-HCl, 1 M NaCl, 0.1% Triton X-100, 10 mM imidazole and 10% glycerol; pH = 7.8) with 1 mM phenylmethylsulphonyl fluoride. Cell lysate was prepared as described above. His-tagged GlnR was obtained by applying affinity chromatography purification using HisPur Ni-NTA Resin (Thermo Fisher Scientific), where the recombinant protein was washed with the column buffer and eluted with eluting buffer (50 mM Tris-HCl, 500 mM NaCl, 0.5 M imidazole and 10% glycerol; pH = 7.8). Next, to remove imidazole, GlnR was passed through a Sephadex G-25 M column (GE Healthcare), after which it was eluted with the buffer containing 50 mM Tris-HCl, 500 mM NaCl and 10% glycerol.

**Pull-down assay.** A pull-down assay was performed to investigate the interaction between NnaR and GlnR. The recombinant MBP-NnaR and His-GlnR were used to perform the experiment. BSA (albumin from bovine serum, SIGMA) was the control. Equimolar amounts of proteins (5 nM) were tested for interactions. In the first step, His-GlnR in the washing buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, pH = 8) was bound to HisPur Ni-NTA magnetic beads (rotating for 1 hour at 4 °C). The protein–coated beads were collected on a magnetic separator, washed six times. The protein complexes were eluted in buffer containing 500 mM imidazole (50 mM Tris, 500 mM NaCl, 10% glycerol, pH = 8). Finally, the load fraction (5 µg of each protein), the last wash after incubation with...
measuring the OD_{600}. To confirm nitrogen depletion in liquid cultures of 1 mM (nitrogen-limiting) or 30 mM (nitrogen-excess) ammonium sulphate. Growth kinetics were monitored by absence of ammonium ions was determined. After the ammonium ions were depleted, cultures (50 mL) were centrifuged, and the culture supernatant was used for testing the presence of ammonium ions in the growth media.

Growth assays under variable nitrogen sources. M. smegmatis strains ΔnnaR, ΔglnR, ΔnnaR-attB_\text{p}_\text{hsp60}nnaR, ΔglnR-attB_\text{p}_\text{hsp60}glnR, and wild-type were grown in 7H9 medium supplemented with Tween 80 and OADC up to the logarithmic stage of growth. To induce nitrogen starvation, cells were washed twice in nitrogen-free Sauton medium. The OD_{600} was adjusted to 0.2 in the same medium, and the cells were grown for 16 hours at 37 °C. Next, cells were harvested by centrifugation and diluted to OD_{600} 0.1 in Sauton’s medium supplemented with various substances that were tested as sole nitrogen sources: sodium nitrite (5 mM), sodium nitrate, acetamide (5 mM), ammonium sulphate, urea (pH 4.5), uric acid, histidine (pH 9.5), leucine (pH 9.5), allantoin, hydantoin, proline (pH 9.5), methionine (pH 9.5), L-glutamic acid (all at 10 mM concentration). 7H9 medium containing OADC and Tween 80 was used as a positive control, and nitrogen-free Sauton’s medium was the negative control. The kinetics of the growth was monitored by measuring absorbance at 600 nm at 3, 6, 9, 24, 36, 48 and 60 hours of growth. Cell viability was determined by measuring the colony-forming units (CFU) on 7H10 agar plates at the 24 hours timepoint. Plates were incubated at 37 °C for 3–5 days, colonies were counted, and data were plotted in Excel.

Griess-Llosvay assay. The ability to reduce nitrate to nitrates by ΔnnaR, ΔglnR, ΔnnaR-attB_\text{p}_\text{hsp60}nnaR, ΔglnR-attB_\text{p}_\text{hsp60}glnR mutant strains and wild-type was evaluated using the Griess Reagent System (Promega). The samples were collected at 0, 6, 12, 24, 36, 48, 60 and 72 hours of growth on Sauton’s medium containing sodium nitrate as a sole nitrogen source. The level of nitrite in supernatants was measured in 96-well plates. The nitrite standard reference curve (100–1.56 µM) was prepared by two-fold serial dilutions of the 100 µM nitrite standard in nitrogen-free Sauton's medium. Sulphanilamide solution (1% sulphanilamide in 5% phosphoric acid) was dispensed to experimental samples and nitrite standards, and plates were incubated in the dark for 10 minutes at room temperature. Next, the 0.1% N-1-naphthylethlenediamine dihydrochloride (NED) solution (in water) was added to all wells, and the plates were incubated as above. The absorbance was measured at 550 nm by Benchmark Plus Microplate Spectrophotometer (BioRad). The nitrite standard reference curve was generated using Excel, and concentrations of nitrates were determined by comparing the average absorbance to the nitrite standard reference curve.

RNA isolation, removal of rRNA and library preparation. M. smegmatis strains lacking mnaR or glnR, grown overnight in 7H9 liquid broth supplemented with OADC and Tween 80, were washed three times in nitrogen-free Sauton’s medium. Bacteria were diluted to an OD_{600} of 0.1 in the same medium supplemented with 1 mM (nitrogen-limiting) or 30 mM (nitrogen-excess) ammonium sulphate. Growth kinetics were monitored by measuring the OD_{600}. To confirm nitrogen depletion in liquid cultures of ΔnnaR, ΔglnR and wild-type strains, the presence of ammonium ions was monitored during bacterial growth by the ammonium test (Merck) following the manufacturer’s protocol. Six milliliters of each culture was collected at the indicated time points and centrifuged, and the culture supernatant was used for testing the presence of ammonium ions in the growth media. Based on each sample’s colour compared to the blank on the colour card attached to the kit, the presence or absence of ammonium ions was determined. After the ammonium ions were depleted, cultures (50 mL) were centrifuged (4500 rpm for 10 min at room temperature), and the total RNA was isolated as described previously. Briefly, Trizol LS reagent (Invitrogen) was used to extract RNA. Cells were disrupted twice using the MP disruptor system with the Quick prep adapter (MP Biomedicals) and 0.1 mm silica spheres (45 seconds, 6.0 m/s with 5 min intervals). DNase I turbo (Invitrogen by Thermo Fisher Scientific) was used to remove DNA contamination according to the manufacturer’s instructions. The RNA quantity was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific), and RNA integrity was verified using an Agilent 2100 BioAnalyzer following the manufacturer’s protocol (Agilent RNA 6000 Nano Kit). Before rRNA removal, the RNA samples were purified using AMPure magnetic beads (Becton Dickinson). To remove rRNA from the samples, the Ribo-Zero rRNA Removal Kit (Illumina) was applied, and the libraries were prepared using the KAPA Stranded RNA-Seq Kit (KAPA Biosystems) following the detailed method provided by the manufacturer. The resulting libraries were examined on an Agilent 2100 BioAnalyzer on a DNA 1000 chip and then quantified and then quantified by qPCR with the NEBNext Library Quant Kit for Illumina (New England Biolabs). The libraries were sequenced using the NextSeq500 System from Illumina with the NextSeq 500/550 Mid Output v2 sequencing kit (150 cycles, Illumina) ensuring around 5 mln pair-end reads per sample. Experiments were performed in triplicate, averaged results are shown.

Bioinformatics. The bioinformatic analyses of the total RNA sequencing results were performed in-house using an array of scripts and programmes. The sequencing indexes were initially removed using Cutadapt software, and quality trimming of sequencing reads was performed with help of the Sickle programme. Trimmed
reads were next mapped to the *M. smegmatis* genome (NC_008596.1, from NCBI; source: [https://www.ncbi.nlm.nih.gov/nuccore/NC_008596](https://www.ncbi.nlm.nih.gov/nuccore/NC_008596)) using the Bowtie2 short sequence aligner [1]. Mapped reads derived from the bacterial RNA were counted to the corresponding genes using HTSeq-count script [41]. The differences between the tested conditions were examined using the online Degust RNA-Seq analysis platform with default parameters ([http://degust.erc.monash.edu/](http://degust.erc.monash.edu/), originally designed by D. R. Powell). Statistical analysis of differential gene expression (DGE) was performed using the empirical Bayes quasi-likelihood F-test. For all DGE analysis carried out in this study, genes having a false discovery rate (FDR) of <0.05 and a log2 fold change of >1.5 were considered significantly differential.

**Quantitative real-time (qRT) PCR.** The qRT-PCR technique was applied as a validation experiment of RNA-Seq data. The transcript levels for selected genes were studied using the Maxima SYBR green qPCR master mix (Thermo Fisher Scientific) and 7900HT real time PCR system (Applied Biosystems). The reverse transcription reaction and qRT-PCR were carried out as described previously [20,33]. Briefly, the SuperScript III First-Strand Synthesis Super Mix kit with random hexamers (Invitrogen) was used for reverse transcription of 1 μg of total RNA according to the manufacturer's protocol. Each qPCR reaction (total volume of 25 μl) contained: 1x Maxima SYBR green qPCR master mix, 0.3 μM each primer, 50 ng of cDNA (primers sequences used for qRT-PCR are listed in Table S1). Real-time PCR conditions were as follows: initial activation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec (denaturation), 62 °C for 30 sec (annealing), 72 °C for 30 sec (extension). The melting curve analysis was performed at the end of each qPCR reaction to verify a single, specific product was generated. The threshold cycle (Ct) value for each studied gene was normalized to the expression of *sigA* (∆Ct) and converted to linear form (2-ΔCt). The RNA samples for each strain were isolated from three independently grown cultures.

**Electrophoretic mobility shift assay (EMSA).** Interactions of MBP-NnaR and His-GlnR with the hexachlorofluorescein-labelled promoter regions of *msmeg_0427, msmeg_0433, msmeg_4008, msmeg_5360, msmeg_5765* were assessed using EMSA. First, 0 μM NnaR, 2 μM NnaR, 4 μM GlnR or 2 μM NnaR combined with 4 μM GlnR was incubated with 30 nM of labelled DNA in buffer containing 10 mM Tris, 1 mM DTT, 2.5% glycerol, 20 mM MgCl2, 500 ng poly(dI-dC), and 0.05% NP-40 (pH 7.5). Samples were incubated for 10 min at room temperature and resolved on 2% agarose gels. The protein-DNA complexes were visualized by a GE Typhoon 8600 Imager Scanner.

To confirm the specificity of the shifts, competitive EMSA for the promoter region of *msmeg_0427* was performed. To do this, 30 nM of the labelled region of *msmeg_0427* and the mixture of labelled (30 nM) with 100-fold excess of unlabelled specific DNA was incubated with 2 μM of MBP-NnaR for 10 min at room temperature. The complexes were resolved and visualized as above.

**Biofilm formation.** *M. smegmatis* strains were cultured in Middlebrook 7H9 (Difco Laboratories) liquid media supplemented with 10% OADC, 0.05% Tween 80 and kanamycin (25 μg/mL) when necessary. Pellicle biofilms of tested here strains were grown as described earlier [22,23]. 10 μl of a saturated planktonic culture of each strain was inoculated into 10 mL of detergent-free Sauton’s, Sauton’s and Sauton’s media and transferred onto 6-well polystyrene plates. The biofilms were incubated at 30 °C for up to 7 days.

**Statistical analysis.** Statistical analyses in this study were carried out with GraphPad Software (La Jolla, CA, USA) for Windows.

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Author Contributions

Designed the study J.D., R.P., P.P. Performed the experiments M.A., R.P., P.P., A.Z., D.S., A.R.-G. Analyzed the data M.A., R.P., P.P., A.Z., J.D. Wrote the manuscript M.A., R.P., P.P., J.D.

Additional Information

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