Nitrogen regulator GlnR directly controls transcription of genes encoding lysine deacetylases in Actinobacteria

Ying Xu,1 Di You1 and Bang-Ce Ye1,2,*

Abstract

N-Lysine acetylation is a dynamic, reversible and regulatory post-translational modification (PTM) in prokaryotes as a means of modulating the activities of enzymes, transcription factors and molecular chaperones. Growing evidence suggests that reversible lysine acetylation (RLA) rapidly adjusts the activities of proteins that enable micro-organism growth plasticity to respond and adapt to fluctuating environments. The conventional mechanism for reversible acetylation of protein is enzyme-catalysed acetylation and deacetylation, relying on Gcn5-type lysine acetyltransferases (KAT) and deacetylases (histone deacetylases, HDACs). Expression of the genes encoding acetyltransferases and deacetylases is regulated in response to to intracellular nutritional signals to modulate the acetylation levels of specific proteins, which in turn affects the metabolic network. In Bacillus subtilis, regulation of expression of the acuA gene coding for acetyltransferase is under the control of CcpA, a global regulatory protein, which is affected by the quality of the carbon source that is available to the cell [1]. In Escherichia coli, the cAMP receptor protein CRP induces the expression of yfiQ, which encodes the acetyltransferase YfiQ and thus increases the acetylation level of proteins in response to the intracellular cAMP signal [2]. It was recently reported that the myo-inositol catabolism repressor, IolR, activates the expression of genes encoding components (pat and cobB) of the RLA system in Salmonella enterica [3]. More recently, we found that the nitrogen regulator GlnR of the Actinobacterium Saccharopolyspora erythraea directly regulates transcription of SACE_5148, which encodes a lysine acetyltransferase. Nevertheless, the molecular mechanism underlying the signalling pathway from nutrient sensing to protein acetylation remains poorly understood, especially HDAC deacetylases. The four HDAC protein family members (I, II, III and IV) can catalyse the N-lysine deacetylation reaction. Classes I, II and IV HDAC enzymes do not require cofactors [4] and can catalyse lysine deacylation via hydrolysis of the acyl group. Class III HDAC enzymes, commonly referred to as sirtuins, are a mechanistically distinct family of NAD+-dependent deacetylases.

Recent studies have found multiple nutrient-mediated signalling pathways that regulate the transcription of genes coding for sirtuin-type protein deacetylases in eukaryotic cells, and have established a connection between...
transcriptional regulation and protein deacetylation responding to environmental changes. The sirtuin-type deacetylase SIRT1 expression was conversely controlled at the transcriptional level by the activation of the CREB (cyclic AMP response element-binding)-acylated CRTC2 complex and the repression of ChREBP (carbohydrate response element-binding) protein in response to energy availability [5]. On the other hand, Forkhead transcription factor FoxO1 directly activates SIRT1 transcription through its binding to the IRS-1- and FKHD-like responsive elements within the rat sirt1 promoter region. SIRT1 increases FoxO1 DNA-binding ability by deacetylating FoxO1 and its co-activator PGC-1α, and enhances their transcription activity, indicating a possible forward auto-feedback loop mechanism of the FoxO1-SIRT1 circuit [6].

Although a role for nutrient-mediated signalling in deacetylase gene regulation has been implicated in S. enterica [3], it remains unclear how signals control deacetylase expression in micro-organisms. In this study, we found that global nitrogen regulator GlnR directly controls the transcription of genes encoding lysine deacetylases in Actinobacteria. Electrophoretic mobility shift assays and real-time PCR (RT-PCR) in three Actinobacteria species (Saccharopolyspora erythraea, Streptomyces coelicolor and Mycobacterium smegmatis) revealed that the GlnR regulator protein is able to interact with the promoter regions of these genes and activate their transcription. Furthermore, it was demonstrated that cellular acetylation status (acetylome) is modulated by extracellular nitrogen availability. Our results present an example of the novel complete signal transduction mechanism of regulating protein deacetylation through a nutrient-sensing pleiotropic regulator in response to nutrient availability.

METHODS

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The wild-type Saccharopolyspora erythraea NRRL 23338, Streptomyces coelicolor A3(2) and their glnR deletion and complemented strains were grown in R2M agar plates. An agar section (1 cm²) was cut and inoculated into a 150 ml flask containing 30 ml TSB (tryptic soy broth) media for 48 h at 30 °C with shaking at 220 r.p.m. for preparation of seed stock. The wild-type Mycobacterium smegmatis MC2 155, its glnR deletion and complemented strains were grown in LB agar plates. The cells were inoculated into a 150 ml flask containing 30 ml of Luria–Bertani (LB) for 24 h at 37 °C and 220 r.p.m. for seed stock preparation. Escherichia coli strains were grown in LB medium at 37 °C. Modified Evans medium was used for S. erythraea and S. coelicolor. Basic Evans medium contains 25 mM TES [N- (Tris(hydroxymethyl)methyl)-2-aminoethanesulfonic acid sodium salt], 2 mM citric acid, 10 mM KCl, 0.25 mM CaCl₂, 1.25 mM MgCl₂, 2 mM Na₂SO₄, 1 mM Na₂MoO₄, 0.5% trace elements (20 M MnSO₄·4H₂O, 6 M ZnSO₄·7H₂O, 20 M H₃BO₃, 1 M KI, 2 M Na₂MoO₄·2H₂O, 50 M CuSO₄·5H₂O, 50 M CoCl₂·6H₂O), 2.5% (mass/vol) glucose and 2 mM NaH₂PO₄, at a final pH of 7.0. Nitrogen-limiting Evans contains 1 mM l-glutamine and C and P as in the basic, and nitrogen-rich Evans contains 30 mM l-glutamine. Sauton’s medium was used for M. smegmatis. Nitrogen-free Sauton’s medium contains 0.05% (w/v) KH₂PO₄, 0.05% (w/v) MgSO₄, 0.2% (w/v) citric acid, 0.005% (w/v) ferric citrate, 0.2% (v/v) glycerol, 0.001% (v/v) ZnSO₄ and 0.015% (v/v) Tyloxapol. Next, 1 mM (NH₄)₂SO₄ was added as nitrogen-limiting Sauton’s medium while 30 mM (NH₄)₂SO₄ was added as nitrogen-rich Sauton’s medium [7]. All media were sterilized by autoclaving at 121 °C for 20 min. Carbon sources (20% sterile) autoclaved at 115 °C for 15 min were also added to Evans medium.

Overexpression and purification of GlnR in E. coli

The glnR genes were amplified from S. erythraea, S. coelicolor, M. smegmatis and M. tuberculosis genomic DNA by PCR using the primers listed in Table S1 (available in the online Supplementary Material). The PCR products were cloned into pET-28a (+) (with His tag), generating the recombinant plasmid pET-glnR. After validating the correct gene sequence by sequencing analysis, the recombinant plasmid was introduced into E. coli BL21(DE3) for overexpression of GlnR proteins. The E. coli cells with kanamycin resistance were grown in 100 LB medium containing kanamycin, at 37 °C and 250 r.p.m. to an OD of 0.6. GlnR proteins were induced with IPTG at a final concentration of 0.5 mM, followed by incubation at 20 °C for 10–12 h. For protein purification, the cells were collected through centrifugation then washed twice in phosphate-buffered saline (PBS, pH 8.0), and lysed using an ultrasonic cell crusher. Cell debris and supernatant were separated by centrifugation (9000 r.p.m., 20 min, 4 °C). His-GlnR proteins were purified by Ni-NTA Superflow columns (Qiagen, Germany), and mixed with protein-loading buffer (6x protein loading buffer, TransGen Biotech) for the subsequent electrophoresis. The purified protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the protein concentration was determined using Bradford reagent.

Electrophoretic mobility shift assay (EMSA)

The upstream regions (upstream 350 bp from about –300 to +50) of the deacetylase genes containing the putative GlnR binding sites in S. erythraea, S. coelicolor, M. smegmatis and M. tuberculosis were amplified by PCR using the primers listed in Table S2 (available in the online Supplementary Material). PCR products were labelled with biotin using a universal biotinylated primer (5’-AGCCGATGGCGA TAA-3’). The resulting products were analysed by agarose gel electrophoresis and purified using a PCR purification kit (Shanghai Generay Biotech) for EMSA assay as probes. The concentrations of the biotin-labelled probes were determined by a microplate reader (Biotek, USA). EMSAs were carried out using a Chemiluminescent EMSA Kit (Beyotime Biotechnology, China), according to the manufacturer’s instructions. The binding reaction mixture contained 10 mM
strains were inoculated in nitrogen-rich (N\textsuperscript{+}) media. Evans media with 30 mM L-glutamine (N\textsuperscript{-}) or 1 mM SO\textsubscript{4} as the sole nitrogen source were used for the growth of \textit{S. coelicolor} and \textit{S. erythraea}. Sauton’s media with 30 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} (N\textsuperscript{XS}) or 1 mM (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4} (N\textsuperscript{+}) as the sole nitrogen source were used for the growth of \textit{M. smegmatis}. The strains were grown to the mid-log phase, then cells were collected by centrifugation (12 000 r.p.m., 5 min, 4°C). Total RNA was prepared using an RNPrep Pure Cell/Bacteria Kit (Tiangen Biotech, Beijing, China), and validation of RNA was analysed by 1 % agarose gel electrophoresis. RNA concentrations were determined using a microplate reader (BioTek). Total RNA (1 µg) was reverse transcribed using a PrimeScript reverse transcription (RT) reagent kit with gDNA Eraser (TaKaRa, Japan). DNase digestion was performed to remove genomic DNA before reverse transcription for 5 min at 42°C. Real-time RT-PCR experiments for the deacetylase genes were conducted using a SYBR Premix Ex Taq GC kit (TaKaRa, Japan), and about 100 ng cDNA was added to yield a final PCR reaction volume of 20 µl. All procedures were carried out according to the manufacturer’s instructions. RT-PCR was performed using the primers listed in Table S3 (available in the online Supplementary Material). PCR assays were carried out using a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA), and PCR conditions were as follows: 95°C for 5 min, followed by 40 cycles of 95°C for 10 s, 30°C for 10 s and 72°C for 30 s, with a final extension cycle at 72°C for 10 min.

**Western blotting analysis**

Total proteins (10 µg) from \textit{S. erythraea}, \textit{S. coelicolor}, \textit{M. smegmatis} and their mutant strains inoculated in nitrogen-rich (N\textsuperscript{XS}) or nitrogen-limited (N\textsuperscript{-}) media were electrophoresed on SDS-polyacrylamide gels (12 % acrylamine, 1.2 % bis-acrylamide), and transferred to a nitrocellulose filter membrane for 90 min at 100 V. The membranes were blocked in 3 % BSA blocking buffer for 60 min, and were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1 : 10 000) in 1×TBST three times for 10 min, the membranes were washed three times for 10 min using 1×TBST (20 mM Tris [Tris( Hydroxymethyl)aminomethane], 137 mM NaCl, pH 7.6 and 0.05 Tween 20). Next, the membranes were incubated with anti-acetyllysine polyclonal antibody (1 : 1000, catalogue no. PTM-105; PTM Biolabs, Hangzhou, China) in 1×TBST with 3 % BSA overnight at 4°C. After washing with 1×TBST three times for 10 min, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1 : 10 000) in 1×TBST with 3 % BSA at room temperature with gentle shaking for 1 h. After washing with 1×TBST three times for 10 min again, an ECL kit was used for signal detection.

**In-gel digestion and label-free quantification of acetylated peptides**

Gel bands were excised from the SDS-PAGE gels, then de-stained with 50 % ethanol overnight. After washing with H\textsubscript{2}O, the gels were sliced into small pieces and dehydrated with 100 % acetonitrile. The proteins in gels were reduced by 10 mM DTT at 56°C for 40 min followed by alkylation with 55 mM iodoacetamide in darkness for 40 min at room...
Peptide samples dissolved in solvent A (0.1% formic acid and 2% acetonitrile in water) were injected onto a manually packed reverse-phase C18 column (10 cm length × 75 µm inner diameter; C18 resin with 3 µm particle size; 90 Å pore diameter; Dikma Technologies Inc., Lake Forest, CA) coupled to a nano-HPLC system (Thermo Fisher Scientific, Waltham, MA), and eluted over 120 min gradient with 2–35% solvent B (0.1% formic acid and 10% water in acetonitrile) for 100 min, 35–80% solvent B for 2 min, 80% solvent B for 10 min, 80–2% solvent B for 2 min and 2% solvent B for 6 min at a flow rate of 300 nL min⁻¹. A Q Exactive mass spectrometer (Thermo Fisher Scientific) was used for sample analysis. For the MS1 scan, an m/z range of 350–1300 with mass resolution of 70000 at m/z 1000 was set, and the 16 most intense ions were sequentially isolated for MS/MS fragmentation using high-energy collision-induced dissociation (HCD) with normalized collision energy (NCE) of 35%. Ions with either a single charge or more than six charges were discarded. The dynamic exclusion duration was maintained at 30 s.

The S. erythraea database from UniProt (7166 sequences), M. smegmatis database from UniProt (12,683 sequences), and S. coelicolor database from UniProt (8033 sequences) with a reversed decoy database were used for mass spectrometric (MS) data analysis. MaxQuant software (version 1.4.1.2) was used for qualitative and quantitative analysis of proteins. The cleavage enzyme was specified as trypsin/P. The maximum missing cleavages was set at two. Precursor error tolerance was 20 ppm, and fragment ion was ±0.02 Da. Carbamidomethyl (C) was set as fixed modification, while oxidation (M), acetylation (Protein N-term) and acetylation (K) were set as variable modifications. False discovery rates (FDR) for protein, peptide and modification site identification were all set at 1%. Minimum peptide length was set to 7. The match between runs was used and the match time window was set to 2 min. For qualitative and quantitative analysis of acetylated peptides, raw data were converted to an mgf file by using a Thermo Proteome Discoverer v1.4.0.288 for mascot (v2.1; Matrix Science London, UK) search; 10 ppm for MS and 0.02 Da for MS/MS were used as mass error settings.

After database searching, the m/z of acetylated peptides from mascot search results was used to extract precursor ion peaks. Areas under the curve (AUC) were used to denote the intensity of acetylated peptides and were normalized by protein intensities from MaxQuant search results [10]. Two replicates were conducted.

**Computational analysis**

The MEME/MAST tools (http://meme.sdsc.edu) and the PREDetector software program were used to search GlnR binding sites within the upstream regions of the deacetylase genes in S. erythraea, S. coelicolor, M. smegmatis and M. tuberculosis. The training set of sequences used to generate weight matrices and the prediction method were described in our previous studies [11, 12].

**RESULTS AND DISCUSSION**

Two classes of lysine deacetylases were present in Actinobacteria: NAD⁺-dependent sirtuin deacetylase and zinc-dependent lysine deacetylase

N-Lysine protein acetylation is reversible by protein deacetylases, which are historically referred to as histone deacetylases (HDACs) (Pfam 08295) [13]. It was found that two classes of lysine deacetylases were present in prokaryotes: NAD⁺-dependent sirtuin deacetylase (Pfam 02416) and zinc-dependent lysine deacetylase [13]. Some bacterial sirtuin deacetylases, such as SeCoB enzyme from S. enterica [14], EcCoB from E. coli and the YhdZ protein of B. subtilis, have been studied. The AcuC deacetylase in B. subtilis is a typical example of bacterial zinc-dependent lysine deacetylase [15]. The transcriptional regulation of the genes encoding these deacetylases in prokaryotes remains to be elucidated. No protein deacetylase in Actinobacteria has been investigated to date. In general, bioinformatics analyses reveal that 1–3 NAD⁺-dependent sirtuin deacetylases, or one Zn²⁺-dependent protein deacetylase, are encoded in Actinobacteria genomes. As shown in Table 2, the S. coelicolor genome encodes two sirtuin-type deacetylases (SCO0452 and SCO6464) and one Zn²⁺-dependent deacetylase (SCO3330). The M. smegmatis genome has two sirtuin-type deacetylases (MSMEG_4620 and MSMEG_5175) and one Zn²⁺-dependent deacetylase (MSMEG_0171). Saccharophythora erythraea contains one NAD⁺-dependent deacetylase (SACE_3798) and one Zn²⁺-dependent deacetylase (SACE_1779). Streptomyces avermitilis genome harbours one sirtuin-type deacetylase (SAV_537) and one AcuC-like deacetylase (SAV_4729). Amycolatopsis mediterranei possesses three sirtuin-type deacetylases (AMED_5937, AMED_4209 and AMED_8708) and one AcuC-like deacetylase (AMED_2418). Mycobacterium tuberculosis possesses only one deacetylase (sirtuin-type deacetylase, Rv1151c).

GlnR-binding sequences were identified in the upstream region of the genes encoding lysine deacetylases in S. coelicolor, S. erythraea and M. smegmatis

The DNA binding sites of GlnR of Actinobacteria were definitively identified in previous studies [16, 17]. The predicted GlnR-binding sites were identified in the upstream regions of the genes encoding lysine deacetylases.
in *S. coelicolor*, *S. erythraea* and *M. smegmatis* using MSAT/MEME tools and PREDetector software (Table 2). The absence of a putative GlnR-box was observed in only two deacetylase genes, MSMEG_0171 and MSMEG_5175, from *M. smegmatis*.

To determine whether the GlnR regulator binds directly to the upstream regions of these genes with the putative GlnR-box, electrophoretic mobility shift assays (EMSAs) were performed using purified His-GlnR protein with biotin-labelled DNA probes containing the predicted GlnR-box. To verify the specificity of the binding, an unlabelled specific probe (200-fold) or non-specific competitor DNA (200-fold, salmon sperm DNA) was used. As shown in Fig. 1, obvious shift bands were observed for all genes containing the putative GlnR-box when the purified regulator proteins were added. No shift band was found following the addition of the unlabelled specific probe (200-fold). The results revealed that His-tagged GlnR was able to interact with all upstream regions tested (Fig. 1).

### GlnR directly induces or represses the transcription of deacetylase genes

In order to investigate the regulatory effect of GlnR on the deacetylase genes, we constructed *glnR* deletion mutants (strains *S. erythraea* ΔglnR, *S. coelicolor* ΔglnR and *M. smegmatis* ΔglnR) and *glnR* complemented mutants (strains *S. erythraea* ΔglnR::glnR and *M. smegmatis* ΔglnR::glnR). Transcription analysis of the deacetylase genes was performed for the wild-type (wt), ΔglnR and ΔglnR::glnR strains in nitrogen-limited media using real-time PCR. As shown in Fig. 2, the deletion of *glnR* led to lower expression levels of all deacetylase genes in *S. erythraea* and *S. coelicolor*. Transcription of SCO0452, SCO6464, SCO3330, SACE_1779 and SACE_3798 was quantified to about 20-, 6-, 10-, 7- and 3-fold lower in the ΔglnR mutant compared to the wild-type strain, respectively. However, MSMEG_4620 showed about 4-fold increased expression in *M. smegmatis* as a consequence of *glnR* deletion. The Δ*glnR* strain complemented with the *glnR* gene (Δ*glnR*:glnR) showed similar transcription levels of these genes, as did the wild-type strains (Fig. 2). These results indicate that GlnR plays a regulatory role as a transcriptional activator of the deacetylase genes in *S. erythraea* and *S. coelicolor*, whereas it also represses the transcription of the deacetylase gene in *M. smegmatis*.

### Expression of deacetylase genes is repressed or induced by nitrogen starvation

The transcription level for these deacetylase genes was also investigated in three different Actinobacteria (*S. erythraea*, *S. coelicolor* and *M. smegmatis*) inoculated in nitrogen-rich (N\(^{XS}\)) or nitrogen-limited (N\(^{L}\)) media. Evans media with 30 mM l-glutamine (N\(^{XS}\)) or 1 mM l-glutamine (N\(^{L}\)) as the sole nitrogen source were used for the growth of *S. coelicolor* and *S. erythraea*. Sauton’s media with 30 mM (NH\(_4\))\(_2\)SO\(_4\) (N\(^{XS}\)) or 1 mM (NH\(_4\))\(_2\)SO\(_4\) (N\(^{L}\)) as the sole nitrogen source were used for the growth of *M. smegmatis*. Quantitative real-time RT-PCR showed that, compared to those in nitrogen-rich media, the levels of deacetylase gene transcripts in *S. erythraea* and *S. coelicolor* were significantly increased in the nitrogen-limited media, by a factor of 5 for SCO3330, 2.5 for SCO0452, 18 for SCO6464, 5.8 for SACE_1779 and 5 for SACE_3798 (Fig. 3a, b). As expected, the transcription level of MSMEG_4620 revealed a 10-fold repression when *M. smegmatis* was cultivated in nitrogen-limited Sauton’s medium (Fig. 3c). No obvious changes in the transcription levels of MSMEG_5175 and MSMEG_0171 were observed. Transcription of the *glnR* gene was induced by nitrogen starvation in three strains (Fig. 3d). These results further suggest that GlnR has a regulatory effect on transcription of the deacetylase genes in response to nitrogen availability in Actinobacteria. Previous research has demonstrated that GlnR displays the dual repressor/activator function, and

### Table 2. Genes in selected Actinobacteria encoding lysine deacetylases

<table>
<thead>
<tr>
<th>Actinobacterium</th>
<th>No. of deacetylases</th>
<th>Lysine deacetylase</th>
<th>Putative GlnR-box</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Saccharopolyspora erythraea</em></td>
<td>2</td>
<td>SACE_1779</td>
<td>CGGCCCGGGCCGTAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SACE_3798</td>
<td>TTTGCACTCGGTAAC</td>
</tr>
<tr>
<td><em>Streptomyces coelicolor</em></td>
<td>3</td>
<td>SCO3330</td>
<td>GTCGGGTTCTGAAAC</td>
</tr>
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<td></td>
<td></td>
<td>SCO0452</td>
<td>GTGCAGTGCGGGAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SCO6464</td>
<td>ACCAGCCCCGCGAGAC</td>
</tr>
<tr>
<td><em>Mycobacterium smegmatis</em></td>
<td>3</td>
<td>MSMEG_0171</td>
<td>ND</td>
</tr>
<tr>
<td></td>
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<td>MSMEG_4620</td>
<td>GCGAGCGGCGGGAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MSMEG_5175</td>
<td>ND</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>1</td>
<td>Rv1151c</td>
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</tr>
<tr>
<td><em>Amycolatopsis mediterranei</em></td>
<td>4</td>
<td>AMED_2418</td>
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</tr>
<tr>
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<td></td>
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<td>AMED_8708</td>
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<tr>
<td><em>Streptomyces avermitilis</em></td>
<td>2</td>
<td>SAV_4729</td>
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<td></td>
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<td>SAV_537</td>
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Fig. 1. DNA-binding properties of GlnR from *S. erythraea*, *S. coelicolor* and *M. smegmatis*. EMSAs of the biotin-labelled probes (350 bp, from −300 to +50 in the upstream regions of the deacetylase genes) with purified His-tagged GlnR from *S. erythraea*, *S. coelicolor* and *M. smegmatis* were performed. EMSAs with a 200-fold excess of unlabelled specific probes (S) or non-specific competitor DNA (sperm DNA) (N) were conducted as controls. The free probes did not bind with proteins.

Fig. 2. Transcriptional analysis of the genes encoding lysine deacetylases in wild-type strain (WT), *glnR*-deleted mutant (*ΔglnR*) and *glnR*-complemented strains (*ΔglnR::glnR*) of *S. coelicolor*, *S. erythraea* and *M. smegmatis* grown in nitrogen-limited medium (N\(^{-}\)). Fold changes represent the levels of expression compared to that in WT.
regulates the transcription of the genes encoding proteins related to nitrogen uptake, metabolism and regulation, as well as proteins with unknown functions in Actinobacteria [11]. In the present work, we found that GlnR also plays a dual regulatory role as a transcriptional activator of the deacetylase genes in *S. erythraea* and *S. coelicolor*, but as a transcriptional repressor of these genes in *M. smegmatis*. One potential reason for the presence of either positive or negative control of GlnR of deacetylase genes in *S. erythraea*, *S. coelicolor* and *M. smegmatis* is that different Actinobacteria species have evolved various GlnR-mediated RLA mechanisms to adapt to changes in the availability of nitrogen in their various ecological niches. Why GlnR exerts such variable regulation on deacetylase genes remains to be elucidated in future studies.

**Effects of GlnR on the acetylome in Actinobacteria in response to nitrogen availability**

Next, we examined the effect of GlnR on the global levels of acetylation in Actinobacteria under different nitrogen conditions. To investigate the acetylation status (acetylome) of cells in response to nitrogen availability, two strains (wild type and ΔglnR) of *S. erythraea*, *S. coelicolor* and *M. smegmatis* were cultured at middle exponential phase in nitrogen-rich (N^XS^) or nitrogen-limited (N^L^) media as described above. Total proteins (10 µg) were extracted from bacterial samples under different nitrogen conditions and used for Western blotting analyses. The protein acetylation levels of these samples were detected with anti-acetylated-lysine (anti-AcK) antibody. As shown in Fig. 4, we found that the global acetylation levels of proteins in the wild-type strains under nitrogen-limiting conditions were lower than those under nitrogen-rich conditions. No obvious difference in acetylation level under different nitrogen conditions was observed in ΔglnR strains (Fig. 4).

To confirm the effects of GlnR on acetylation levels, quantitative analysis was also performed using in-gel digestion coupled with LC-MS/MS analysis. Protein samples from wild-type (WT) or ΔglnR strains of *S. erythraea*, *S. coelicolor* and *M. smegmatis* cultured in nitrogen-limited media were separated by SDS-PAGE. Gel bands showing the different acetylation levels (red frame in Fig. 4a–c) were cut and trypsin digested. The digested peptides were loaded onto LC-MS/MS. About 1500, 550 and 830 proteins in these bands were identified in *S. erythraea*, *S. coelicolor* and *M. smegmatis*, respectively. In these proteins, 36 and 38 acetylated peptides in *S. erythraea*, 34 and 28 acetylated peptides in *S. coelicolor* and 14 and 17 acetylated peptides in *M. smegmatis* were identified in the WT and ΔglnR strains, respectively. Compared to WT strains, the acetylation levels of certain peptides were increased in ΔglnR strains by a

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**Fig. 3.** Transcriptional analysis of deacetylase genes and glnR in *S. erythraea*, *S. coelicolor* and *M. smegmatis* wild strain grown in nitrogen-rich (N^XS^) or nitrogen-limited (N^L^) medium. Fold changes represent the level of expression compared to that of these genes in N^XS^ medium. (a) Expression of genes encoding lysine deacetylase in *S. coelicolor*; (b) expression of genes encoding lysine deacetylase in *S. erythraea*; (c) expression of genes encoding lysine deacetylase in *M. smegmatis*; (d) expression of glnR in all three strains.
factor of 4.5 for A4F815 (EchA11, putative enoyl-CoA hydratase/isomerase family protein, K100Ac), 2 for A4F761 (SACE_0539, carbon monoxide dehydrogenase subunit G, K248Ac), 1.5 for A4F7x2 (SACE_0806, lipoprotein, K241Ac), 9.5 for A4F8G2 (TypA, GTP-binding elongation factor family protein, K356Ac), 2 for A4F5V9 (SACE_0080, possible glycine betaine/carnitine/choline-binding protein, K132Ac) in S. erythraea (Fig. 4d), 480 for O31212 (RpsB, 30S ribosomal protein S2, K325Ac) in S. coelicolor (Fig. 4e), 2.5 for A0QQU5 (GroL1, 60 kDa chaperonin 1, K325Ac), 2.5 for A0QU53 (MSMEG_2080, putative acyl-CoA dehydrogenase, K371Ac) and 2.5 for A0QVM0 (ProS, proline-tRNA ligase, K54Ac) in M. smegmatis (Fig. 4f). These results are consistent with those of Western blot (Fig. 4a–c), and demonstrated that GlnR exerted effects on acetylation levels in Actinobacteria in response to nitrogen availability.

GlnR-box was also observed in deacetylase genes in other Actinomycetes

Finally, we investigated the GlnR-binding motifs in upstream regions of the genes in other Actinobacterial species encoding putative deacetylases, by bioinformatics analysis, including A. mediterranei and S. avermitilis. According to the annotation from the KEGG database (http://www.genome.jp/kegg/), the A. mediterranei genome contains three sirtuin-type deacetylases (AMED_5937, AMED_4209, AMED_8708) and an AcuC-like deacetylase (AMED_2418), while S. avermitilis possesses one sirtuin-type deacetylase (SAV_537) and one AcuC-like deacetylase (SAV_4729). Using MSAT/MEME tools and PRDeeector software, putative GlnR-box sequences were found in the upstream regions of these deacetylase genes (Table 2), suggesting the investigation of potential GlnR-mediated regulation of deacetylation in other Actinomycetes in future research. However, no GlnR-box was observed in the gene Rv1151c, which encodes only one sirtuin-type deacetylase in M. tuberculosis.

In summary, we found that nutrient-sensing regulator GlnR directly regulates the transcription of genes encoding lysine deacetylases in response to nitrogen starvation in S. erythraea, S. coelicolor and M. smegmatis. GlnR plays a crucial role as a master regulator of nitrogen metabolism, integrating intracellular nitrogen signals to modulate (induce or repress) transcription of its target genes involved in nitrogen metabolism in response to nitrogen availability in Actinobacteria [17, 18]. Our previous research has revealed that GlnR directly regulates the transcription of acuA encoding Gcn5-type protein acetyltransferase (the catalytic enzyme of acetylation) in S. erythraea, indicating that GlnR plays an important role in acetylation of proteins [8]. Herein, we further demonstrated that GlnR also directly controls transcription of the genes encoding lysine deacetylases in Actinobacteria, suggesting that GlnR has an effect on
deacetylation of proteins. These results show that nitrogen-responsive GlnR, as a bifunctional regulatory protein (transcriptional activator or repressor), modulates the acetylation levels of proteins by adjusting the expression of two enzymes of the reversible lysine acetylation system. These findings suggest a novel molecular mechanism in Actinobacteria underlying the integration of nitrogen metabolism and protein lysine acetylation in response to environmental changes.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
No human or animal experiments were conducted in this work.

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