A novel GlnR target gene, \textit{nnaR}, is involved in nitrate/nitrite assimilation in \textit{Streptomyces coelicolor}

Rafat Amin, Jens Reuther, Agnieszka Bera, Wolfgang Wohlleben and Yvonne Mast

Microbiology/Biotechnology, Interfaculty Institute of Microbiology and Infection Medicine, Faculty of Science, University of Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany

GlnR is the global transcriptional regulator of nitrogen assimilation in \textit{Streptomyces coelicolor}. Under nitrogen starvation, GlnR controls the transcription of at least nine genes associated with nitrogen metabolism. In this study, we identified a new GlnR target gene, SCO2958, named \textit{nnaR} (nitrate/nitrite assimilation regulator). \textit{In silico} analysis of NnaR revealed the presence of two distinct domains: an N-terminal uroporphyrinogen-III synthase (HemD)-like enzymatic domain and a C-terminal DNA binding domain. Complementation experiments with a haemin auxotroph \textit{Escherichia coli} \textit{Dh}emD mutant strain revealed that NnaR has no HemD activity. Physiological studies of an \textit{S. coelicolor nnaR}::\textit{Tn}5062 mutant showed that NnaR is involved in regulating nitrite reduction. By electrophoretic mobility shift assays the functionality of the NnaR DNA binding domain was confirmed, and it was found that NnaR binds in front of the genes \textit{narK} (putative nitrate extrusion protein), \textit{nirB} (nitrite reductase), \textit{nirA} (putative nitrite/sulphite reductase) and \textit{nasA} (putative nitrate reductase), which are associated with nitrate/nitrite assimilation. Furthermore, a cooperative binding of NnaR together with GlnR to the \textit{nirB} promoter was observed, suggesting that NnaR may act as a GlnR co-activator.

INTRODUCTION

Streptomycetes are Gram-positive bacteria found predominantly in soil. They are characterized by a unique complex life cycle, which includes the formation of vegetative mycelium, differentiation of aerial hyphae, and spore formation. This morphological differentiation involves complex regulation of gene expression in space and time, and often coincides with the production of diverse secondary metabolites, including antibiotics and biologically active substances (Hopwood \textit{et al.}, 1995).

In their soil habitat, the non-motile streptomycetes compete with other bacteria for nutrients, especially for nitrogen (Reuther & Wohlleben, 2007), which is an important element required for the synthesis of almost all essential biomolecules, such as amino acids, nucleotides and aminosugars (Arcondéguy \textit{et al.}, 2001). Micro-organisms are able to assimilate nitrogen from various organic and inorganic sources by using a number of enzymatic systems (Merrick & Edwards, 1995). Generally, the preferred inorganic nitrogen source is ammonium, which can be taken up directly from the environment by ammonium transporters. Furthermore, ammonium can be made available through the reduction of nitrate to nitrite and subsequently to ammonium by the action of nitrate and nitrite reductases, respectively (Moir & Wood, 2001). Two main pathways, depending on the nitrogen status of the cell, are used for ammonium assimilation: the glutamate dehydrogenase (GDH) pathway and the glutamine synthetase/glutamate 2-oxoglutarate aminotransferase (GS/GOGAT) pathway. GDH is active under high-nitrogen concentrations and forms a molecule of glutamate from ammonium and 2-oxoglutarate in an NADPH-dependent reaction. The GS/GOGAT system catalyses the formation of L-glutamine and L-glutamate. The GS converts glutamate and ammonia to L-glutamine in an ATP-dependent step, whereas the GOGAT catalyses the synthesis of two molecules of L-glutamate from L-glutamine and 2-oxoglutarate (Magasanik, 1982).

To respond to the changes in nitrogen availability, streptomycetes have developed a complex regulation system for nitrogen assimilation, involving transcriptional and post-translational control.

In \textit{Streptomyces coelicolor} and many other actinomycetes the response regulator GlnR plays a central role in the transcriptional regulation of genes involved in nitrogen
metabolism (Pullan et al., 2011; Tiffert et al., 2008). GlnR belongs to the OmpR family of response regulators (Wray et al., 1991). These regulators usually act together with sensor kinases in so-called two-component systems. These signal transduction systems are involved in the regulation of cellular functions in response to changes in environmental conditions (Hutchings et al., 2004). Surprisingly, the *S. coelicolor* glnR gene is not physically linked to a sensor kinase gene and thus corresponds to an orphan response regulator (Wray et al., 1991). Up to now, the *S. coelicolor* GlnR regulon comprises 16 genes, of which nine are directly involved in nitrogen assimilation (Tiffert et al., 2008; Wang & Zhao, 2009). These GlnR target genes are responsible for nitrogen uptake and regulation (*amtb*-*glnK-gldH* operon), reduction (*nitB* and *nasA*) and cleavage (*ureA*) of nitrogen sources, and synthesis of the central metabolic nitrogen donors glutamine and glutamate (*glnA*, *glnII* and *gdhA*) (Tiffert et al., 2008). GlnR is able to act as an activator and a repressor for its target genes. Under nitrogen-limiting conditions it activates the transcription of *glnA* (encoding GS I), *glnII* (encoding GS II), *amtb* (encoding an ammonium transporter) and *nitB* (encoding a nitrite reductase), and represses the transcription of genes such as *gdhA* (encoding a GDH) and *ureA* (encoding a urease) (Tiffert et al., 2008). Specific GlnR binding sites have been identified in the upstream regions of 13 genes. These GlnR binding boxes are composed of two sites, an *a*-site (gTnAc), which is located 6 bp (n6) upstream of a *b*-site (GaAAc), with the consensus sequence gTnAc-n6-GaAAc. According to this model, two GlnR dimers bind to the whole GlnR binding site gTnAc-n6-GaAAc-GtnAC-n6-GaAAc-n6 (Tiffert et al., 2008).

In the present study, we report the characterization of a novel GlnR target gene, *nnaR* (*SCO2958*), which encodes a transcriptional regulator protein, consisting of a predicted N-terminal HemD-like enzymatic domain and a C-terminal DNA binding domain. NnaR is not enzymatically active but is involved in the transcriptional control of nitrate/nitrite assimilation genes as a GlnR co-activator.

**METHODS**

**Strains, media and growth conditions.** Strains and plasmids used in this study are described in Table 1 and primers are listed in Table S1 available with the online version of this paper. *Escherichia coli* strains were grown on solid or in liquid Luria–Bertani (LB) medium at 37 °C (Sambrook et al., 1989). *S. coelicolor* M145 was cultivated at 30 °C on R2YE medium, Mannitol Soy Flour medium (MS), Difco nutrient (DN) agar, in rich liquid YEME medium (Kieser et al., 2000), nitrogen-rich S-medium (Okanishi et al., 1974) or nitrogen-limited N-Evans minimalmedium (Fink et al., 2002). Media were supplemented with 150 μg ampicillin ml⁻¹, 50 μg kanamycin ml⁻¹, 25 μg chloramphenicol ml⁻¹ or 12.5 μg streptomycin ml⁻¹, when required. Manipulation of *S. coelicolor* M145 and *E. coli* was performed as described by Kieser et al. (2000) and Sambrook et al. (1989). For preparation of genomic DNA, *S. coelicolor* M145 was grown for 4 days in S-medium and DNA was isolated with the NucleoSpin Tissue Kit (Macherey-Nagel).

**Generation of an *S. coelicolor nnaR* mutant.** Cosmid SCE59.1H09 (kindly provided by Paul Dyson, University of Swansea), carrying a Tn5062 insertion in the *nnaR* (*SCO2958*) gene, was introduced into *S. coelicolor* M145 by intergeneric conjugation, using *E. coli* ET12567/pUZ8002 as donor (Kieser et al., 2000). The resulting replacement mutant, *nnaR*:Tn5062, was isolated as an apramycin-resistant and kanamycin-sensitive exconjugant and was confirmed by PCR and Southern blot experiments, as described by Southern (1975).

**Complementation of *nnaR*:Tn5062.** For complementation studies, the *nnaR* gene with its 250 bp upstream region was amplified by PCR using *S. coelicolor* genomic DNA and the primers compl-*nnaR* fw (with an EcoRI restriction site attached to the 3' end) and compl-*nnaR* rev (with an XbaI restriction site attached to the 3' end) (Table S1). The 1456 bp amplicon *PnaR* was cloned into pJE1T2 (Fermentas), resulting in construct pJE1T2PnaR. The *PnaR* fragment was excised from this plasmid as an EcoRI/XbaI fragment and cloned into the respective restriction sites of pTSKan (T. Schaberle, Eberhard-Karls-Universität Tübingen). The resulting plasmid pTSKan/PnaR was cloned into *E. coli* S17-1 and then transferred to the *nnaR*:Tn5062 mutant by conjugation, as described elsewhere (Kieser et al., 2000). Apramycin- and kanamycin-resistant transconjugants were confirmed by PCR, and the phenotype of the resulting complementation strain *nnaR*:Tn5062 pTSKan/PnaR was analysed on N-Evans medium with 50 mM NaNO₃.

**Generation of *Rhodococcus jostii*Δ*naR* mutant.** The *naR* homologous gene of *R. jostii* RHA1, R06368, was disrupted by targeted mutagenesis using a sacB counterselection system, as described elsewhere (Ahmad et al., 2011). The oligonucleotides used for the construction of the mutant are listed in Table S1. Plasmids generated in this process are listed in Table 1.

**Complementation of *E. coli* ΔhemD.** For complementation studies of the *E. coli* ΔhemD strain SASZ31 (Chartrand et al., 1979), the genes *nnaR* and SCO3317 were amplified from *S. coelicolor* M145 genomic DNA using Phusion DNA polymerase (Finnzyme) and Taq DNA polymerase (Qiagen), respectively. For the amplification of *nnaR* and SCO3317, primers *nnaR* fw/naR rev and 3317 fw/3317 rev were used (Table S1), respectively, which were designed in such a way that a sequence encoding an N-terminal His-tag, as well as a 3' Ndel and a 5' HindIII restriction site, were added to the *nnaR* gene sequence, and a sequence encoding a 3' Ndel and 5' HindIII restriction site was added to the SCO3317 gene sequence, which were cloned into pDrive (Qiagen), leading to the constructs pDrive/naR and pDrive/his-SCO3317, respectively (Table 1). *his-naR* and his-SCO3317 were excised from pDrive/naR and pDrive/his-SCO3317 as Ndel- and HindIII-fragments and were subcloned into Ndel- and HindIII-restricted plasmid pY9 under the control of the *P*<sub>hasB</sub> promoter (Tiffert et al., 2008), resulting in pY9/naR and pY9/his-SCO3317, respectively (Table 1). Both constructs as well as plasmid pYT-glnII were transferred to *E. coli* SASZ31, and ampicillin-resistant transformants were isolated. *E. coli* SASZ31 pYT-<i>nnaR</i>, *E. coli* SASZ31 pYT-SCO3317 and *E. coli* SASZ31 pYT-glnII (control) were grown in LB medium without haemin for 24 h at 37 °C. Protein synthesis was induced at OD<sub>578</sub> 0.3 by the addition of 0.2% rhamnose. After 24 h, cell densities were measured by spectrophotometer at OD<sub>578</sub>. As a control, 25 μg haemin ml⁻¹ (Fluka) was added to the cultures, when appropriate.

**Expression and purification of GlnR and NnaR.** GlnR was expressed in *E. coli* with an N-terminal StreptIgG superflow gravity-flow columns (IBA), as described previously (Tiffert et al., 2008).
Table 1. Strains, cosmid and plasmids used in this work

<table>
<thead>
<tr>
<th>Strain cosmid or plasmid</th>
<th>Genotype</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli XL1-Blue</td>
<td>recA1 hisD17 relA1 lac[F proAB lacZM15 Tn10(TetR)]</td>
<td>Bullock et al. (1987)</td>
</tr>
<tr>
<td>E. coli SASZ31</td>
<td>hisG(lacF), yCl, mfe, metD31, argH1, lacZM15, tetR-lacZ15</td>
<td>Chartrand et al. (1979)</td>
</tr>
<tr>
<td>E. coli DH5z</td>
<td>F′-λ- enda1 hisD81 hsdR17 hsdM′ supE44 thi-1 recA1 gyrA96 relA1 Δ(argF lacZ)Y169 f80d Δ(lacZ)15, used to clone and propagate pGM190</td>
<td>Bethesda Laboratories (1983)</td>
</tr>
<tr>
<td>E. coli S17-1</td>
<td>recA1 pro thi; has the tra genes from plasmid RP4 integrated in the chromosome; donor strain for conjugation</td>
<td>Simon et al. (1983)</td>
</tr>
<tr>
<td>S. coelicolor M145 parental strain</td>
<td>S. coelicolor A3(2), plasmid-free derivative</td>
<td>Kieser et al. (2000)</td>
</tr>
<tr>
<td>S. coelicolor nnaR::Ts1062</td>
<td>nnaR mutant strain of S. coelicolor M145, Tn5062 insertion mutant</td>
<td>This work</td>
</tr>
<tr>
<td>S. coelicolor mnaR::Ts1062 pTSKan/P</td>
<td>nnaR mutant strain of S. coelicolor M145, complemented with plasmid pTSKan/PnaR</td>
<td>This work</td>
</tr>
<tr>
<td>S. coelicolor ΔglnR</td>
<td>glnR mutant strain of S. coelicolor M145, glnR replaced by an aac(3)IV cassette, ApraR</td>
<td>Tiffert et al. (2011)</td>
</tr>
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<td>R. jostii RHA1</td>
<td>Wild-type, CmR</td>
<td>Lindsay Eltis</td>
</tr>
<tr>
<td>R. jostii Δr06368</td>
<td>Mutant derivative of RHA1 with Δr06368 disrupted</td>
<td>This work</td>
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<tr>
<td>Cosmid</td>
<td>S. coelicolor cosmid with egfp containing transposon</td>
<td>Paul Dyson</td>
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<td>Cosmid SCE59.17c</td>
<td>SCE59.1.00111303.1seq</td>
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<td>Plasmids</td>
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<td>pDRIVE</td>
<td>TA cloning vector, blu, kan</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pDRIVE/his-nnaR</td>
<td>pDRIVE derivative, carrying his-nnaR fragment</td>
<td>This work</td>
</tr>
<tr>
<td>pDRIVE/his-SCO3317</td>
<td>pDRIVE derivative, carrying his-SCO3317 fragment</td>
<td>This work</td>
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<tr>
<td>pYT9</td>
<td>pJOE2775 derivative with P_rham expression cassette and C-terminal (His)_6 tag, blu, with amplified strep-glnR cloned via NdeI/HindIII</td>
<td>Volf et al. (1996); Tiffert et al. (2008)</td>
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<td>pYT9/hs-nnaR</td>
<td>pYT9 derivative, carrying P_rham, expression cassette, blu, P_rha, his-glnR insertion</td>
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<td>pYT9/hs-SCO3317</td>
<td>pYT9 derivative, carrying his-SCO3317</td>
<td>This work</td>
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<td>pGM190/hs-nnaR</td>
<td>pGM190 derivative, carrying the his-nnaR fragment</td>
<td>Wolhleben et al. (2009)</td>
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<td>pJET1.2</td>
<td>Cloning vector, AmpR</td>
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<td>pTSKan</td>
<td>pSET152 derivative, carrying an aphII instead of an aac(3)IV resistance cassette</td>
<td>This work</td>
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<td>pTSKan/PnaR</td>
<td>pTSKan, carrying the nnaR gene and its 250 bp upstream region</td>
<td>T. Schäferle, unpublished results</td>
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<td>pK18/ro06368</td>
<td>5.7 kb mobilizable suicide vector used for triple ligation; sacB aphII</td>
<td>This work</td>
</tr>
<tr>
<td>pK18/ro06368</td>
<td>pK18/nobsacB containing 2.0 kb fusion PCR fragment flanking Δr06368; used to make R. jostii Δr06368</td>
<td></td>
</tr>
</tbody>
</table>

For the expression of NnaR in S. coelicolor M145, his-nnaR was excised from pYT/his-nnaR and inserted into NdeI- and HindIII-restricted plasmid pGM190, resulting in pGM190/his-nnaR, where the nnaR gene is under the control of the thistreptone-inducible promoter (P_{ipExpression}). pGM190/his-nnaR was introduced into S. coelicolor M145 by protoplast transformation (Hopwood et al., 1985) and kanamycin-resistant clones were selected. S. coelicolor pGM190/his-nnaR was grown in S-medium for 48 h. Protein expression was induced with 12.5 μg thiostrepton ml⁻¹ for a further 36 h. Cells were harvested by centrifugation (15 min, 13,000 r.p.m.), washed with a solution of 50 mM Tris, 100 mM NaCl, 20 mM imidazole and 10 mM β-mercaptoethanol, pH 8, and disrupted (6500 r.p.m., 1 x 20–30 s) with a Precellys homogenizer (Peqlab). To prevent proteolytic degradation, the Complete Protease Inhibitor Cocktail (Roche) was added. Cell debris and membrane fractions were separated from the soluble fraction by centrifugation (15 min, 13,000 r.p.m.). His–NnaR protein was detected by Western blot analysis, and protein concentration was determined by Bradford assay (Bradford, 1976).

Electrophoretic mobility shift assay (EMSA). DNA fragments containing the different upstream regions were amplified from S. coelicolor M145 genomic DNA by using Taq DNA polymerase (Qiagen) and primers listed in Table S1. Fragment labelling was performed as described by Tiffert et al. (2008). The Cy5-labelled DNA fragments were mixed with different amounts of purified tagged protein and reaction buffer (50 mM Tris, 100 mM NaCl, 5 mM MgCl₂, and 10 mM β-mercaptoethanol, pH 8.0) in a total volume of 20 μl, which was incubated for 10 min at room temperature. After incubation, loading buffer [0.25 × Tris-borate-EDTA (TBE) buffer, 60 % (v/v) glycerol] was added and the fragments were separated on a 2 % agarose gel. DNA bands were visualized by fluorescence imaging using a Typhoon Trio variable mode imager (GE Healthcare).

Gene expression analysis by RT-PCR. For RT-PCR experiments, the S. coelicolor M145 wild-type, the nnaR::Ts1062 strain and the S. coelicolor A3(2) nnaR mutant strain were grown in S-medium. After 4 days, cells were washed twice with either S-medium or minimal Evans medium and incubated for 5 h in S-medium or minimal Evans
medium with 5 mM NaNO₃, respectively. Prior to RNA isolation the cell pellet was treated with RNAprotect Bacteria reagent (Qiagen) for RNA stabilization. Cells were disrupted using glass beads (150–212 μm, Sigma) and a Precellys homogenizer (6500 r.p.m., 20–30 s; Peqlab). RNA isolation was performed with an RNaseasy kit (Qiagen). RNA preparations were treated twice with DNase (Fermentas). First, an on-column digestion was carried out for 30 min at 24 °C, and afterwards RNA samples were treated with DNase for 1.5 h at 37 °C. RNA concentrations and quality were checked using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). cDNA from 3 μg RNA was generated with random nonamer primers (Sigma), reverse transcriptase and cofactors (Fermentas). PCR reactions were performed with the primers listed in Table S1. The PCR conditions were 95 °C for 5 min; 35 cycles of 95 °C for 15 s, 55 °C for 30 s and 72 °C for 20 s; and 72 °C for 5 min. As a positive control, cDNA was amplified from the major vegetative sigma factor (hrcB) transcript, which is produced constitutively. To exclude DNA contamination, negative controls were carried out by using total RNA as a template for each RT-PCR reaction.

**Griess–Ilosvay assay.** *S. coelicolor* M145 and the *nnaR*::Tn5062 mutant were grown at 30 °C in S-medium for 48 h. Cells were washed twice with N-Evans medium containing 5 mM NaNO₃ and then transferred into fresh 5 mM NaNO₃ N-Evans medium. Samples were taken after 12 h and cells were centrifuged at 13 000 r.p.m. for 15 min. A 0.6 ml volume of Griess–Ilosvay reagent (Merck) was added to 2 ml of 1:10-diluted cell supernatant, and accumulation of nitrite was determined by detection of a red colour.

**RESULTS**

**Identification of nnaR as a novel GlnR target gene**

The gene *SCO2958*, named *nnaR* (nitrate/nitrite assimilation regulator), was identified as a putative novel GlnR target gene by using hidden Markov models (HMMER software; http://hmmer.janelia.org) for screening the *S. coelicolor* M145 genome against the GlnR binding motif, which escaped in previous analysis with the MEME/MAST tool done by Tiffert et al. (2008) (http://meme.sdsc.edu/meme/intro.html). The predicted GlnR binding site is located in between −60 and −110 nt upstream of the translational start site of *nnaR*. To investigate the ability of GlnR to bind to the *nnaR* promoter region, an EMSA was performed. For this purpose purified Strep-tagged GlnR protein (0.2 and 1.0 nM) was added to a 250 bp Cy5-labelled DNA fragment harbouring the *nnaR* upstream region, and the mixture was analysed in a 2 % agarose gel. To verify the specificity of the GlnR–DNA interaction, a 500-fold excess of unlabelled, specific and non-specific DNA was added to the protein–DNA mixture. In this assay, GlnR interacted specifically with the *nnaR* upstream region (Fig. 1). Furthermore, the effect of GlnR on *nnaR* transcription was analysed in semiquantitative RT-PCR. For this purpose, RNA was isolated from the *SCO2958* mutant as well as from the M145 wild-type strain as a control. Both strains were grown under different nitrogen conditions, in the nitrogen-rich S-medium and the nitrogen-limiting N-Evans medium, supplemented with 5 mM NaNO₃. The RT-PCR demonstrated that the *nnaR* transcript was absent in the ΔglnR mutant under the different nitrogen conditions tested, whereas it was present in the wild-type under these conditions (Fig. 2a), suggesting that GlnR is an activator of *nnaR* expression. Altogether, these data clearly show that *nnaR* is a target of GlnR.

**In silico analysis of nnaR**

The predicted NnaR protein consists of two conserved domains: the N-terminal part of the protein resembles an enzymatic domain that is similar to uroporphyrinogen-III synthases (HemD). These enzymes catalyse the formation of uroporphyrinogen III from hydroxymethylbilane, which is an important step in sirohaem biosynthesis (Stamford et al., 1995). Sirohaem is a prosthetic group of sulphite/nitrite reductases, and its synthesis initiates from L-glutamate. The C-terminus of NnaR is similar to the DNA binding domains of OmpR-like response regulators. Protein sequence comparisons showed that homologues of the predicted NnaR protein are present not only in different streptomycetes, such as *Streptomyces scabies*, *Streptomyces avermitilis*, *Streptomyces griseus* and *Streptomyces venezuelae* (75–87 % sequence identity), but also in other actinomycetes, such as *Nocardia* spp., *Rhodococcus* spp., *Frankia* spp. and *Mycobacterium* spp. (55–65 % sequence identity). The existence of nnaR-like genes in most of the genomes of the actinomycetes as well as its highly conserved nucleotide and protein (data not shown) sequence identity in all strains indicates its evolutionary importance. DNA sequence comparisons of the up- and downstream regions of *nnaR* in different actinomycetes revealed that in *S. coelicolor*, *nnaR* is located next to the nitrate/nitrite transporter gene *narK*, whereas in other actinobacteria it is also often close to the nitrite reductase genes *nirB* and *nirD* (Fig. 3), which suggests the functional importance of this gene in nitrogen assimilation.
NnaR lacks enzymatic activity

To analyse whether the uroporphyrinogen-III synthase (HemD)-like domain of NnaR is active, complementation analyses with an *E. coli* ΔhemD haemin auxotroph strain, SASZ31 (Chartrand *et al.*, 1979), were performed. The *S. coelicolor* genome contains two genes encoding HemD-like proteins, SCO2958 (*nnaR*) and SCO3317. Both proteins were analysed for their catalytic functionality by complementation analyses with *E. coli* SASZ31, which lacks HemD and cannot grow without haemin. For this purpose the genes *nnaR* and SCO3317, located on plasmids pYT/his-nnaR and pYT/his-SCO3317, respectively, were transferred to *E. coli* SASZ31. As a negative control, the gene *glnII*, located on plasmid pYT/his-*glnII*, was used for complementing *E. coli* SASZ31. Only *E. coli* SASZ31 pYT/his-SCO3317 was able to grow in LB medium without the addition of haemin, whereas *E. coli* SASZ31 pYT/his-*nnaR* and the negative control *E. coli* SASZ31 pYT/his-*glnII* did not (see Fig. S1 available with the online version of this article).

![Fig. 2](image_url). Transcriptional analysis of *S. coelicolor* M145, *S. coelicolor* nnaR::Tn5062 and *S. coelicolor* ΔglnR. (a) RT-PCR analysis of the *nnaR* gene in M145 and ΔglnR. (b) RT-PCR analyses of genes putatively involved in nitrate/nitrite metabolism in M145 and nnaR::Tn5062. (c) RT-PCR analysis of the *nirB* gene in M145, nnaR::Tn5062 and ΔglnR. (d) RT-PCR analyses of putative haem biosynthetic genes in M145 and nnaR::Tn5062.

![Fig. 3](image_url). Genomic organization of *nnaR* in *S. coelicolor* and its homologues in other actinobacteria. Black arrows represent *nnaR*. Arrows indicate the positions, relative lengths and directions of the genes. *nirB* and *nirD*, genes for nitrite reductase large and small subunits, respectively; *narK* and *narK3*, nitrate/nitrite transporter genes; *nasA* and *nasC*, genes for nitrate reductase catalytic and electron transfer subunits, respectively.

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**Fig. 2.** Transcriptional analysis of *S. coelicolor* M145, *S. coelicolor* nnaR::Tn5062 and *S. coelicolor* ΔglnR. (a) RT-PCR analysis of the *nnaR* gene in M145 and ΔglnR. (b) RT-PCR analyses of genes putatively involved in nitrate/nitrite metabolism in M145 and nnaR::Tn5062. (c) RT-PCR analysis of the *nirB* gene in M145, nnaR::Tn5062 and ΔglnR. (d) RT-PCR analyses of putative haem biosynthetic genes in M145 and nnaR::Tn5062.

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**Fig. 3.** Genomic organization of *nnaR* in *S. coelicolor* and its homologues in other actinobacteria. Black arrows represent *nnaR*. Arrows indicate the positions, relative lengths and directions of the genes. *nirB* and *nirD*, genes for nitrite reductase large and small subunits, respectively; *narK* and *narK3*, nitrate/nitrite transporter genes; *nasA* and *nasC*, genes for nitrate reductase catalytic and electron transfer subunits, respectively.
Characterization of an S. coelicolor nnaR::Tn5062 mutant

To investigate the function of nnaR, we generated an nnaR::Tn5062 insertional mutant in the parental strain S. coelicolor M145 and analysed its phenotype. The nnaR::Tn5062 mutant showed no significant morphological changes compared with the M145 wild-type strain, and grew equally when surface-grown on complex media, such as MS, R5 and DN agar, and on N-Evans medium supplemented with L-aspartate. However, nnaR::Tn5062 showed a defect in aerial mycelium and spore formation, and antibiotic production, on solid agar with various nitrogen sources (NH₄Cl, urea, L-glutamate, L-glutamine, L-aspartic acid and L-arginine; data not shown). Furthermore, when plated on N-Evans agar with NaNO₃ and NaNO₂ as a sole nitrogen source, the nnaR::Tn5062 mutant showed greatly retarded growth compared with M145 (Fig. 4). This phenotype resembles the phenotype of the S. coelicolor glnR mutant, which shows nearly no growth on nitrate and a delayed growth on ammonium as the result of the inability to activate the transcription of the nitrate reductase gene nirB (Tiffert et al., 2008). This observation indicates that nnaR::Tn5062 is also impaired in its ability to reduce nitrite to ammonium. Finally, the complementation of the nnaR::Tn5062 mutant by a wild-type copy of the nnaR gene under its native promoter restored the wild-type phenotype (see Fig. S2 available with the online version of this paper).

nnaR::Tn5062 is impaired in nitrite reduction

To analyse whether the growth retardation of the nnaR::Tn5062 mutant on N-Evans medium with NaNO₃ is caused by the inability to reduce nitrite to ammonium, a Griess–Ilosvay assay was performed. The Griess–Ilosvay reaction is used to test for the presence of nitrite, which is visualized by the production of a red azo dye proportional to the amount of nitrite ions in solution. After addition of the Griess–Ilosvay reagent to the supernatant of a 12 h-old nnaR::Tn5062 culture, a deep red colour was observed, in contrast to the clear supernatant of the wild-type culture, demonstrating nitrite accumulation in the nnaR::Tn5062 culture (see Fig. S3 available with the online version of this paper). Clearly, the nnaR::Tn5062 mutant is able to reduce nitrate to nitrite, whereas it fails to further reduce the accumulating nitrite to ammonium.

NnaR is a transcriptional regulator for nitrate/nitrite assimilation genes

To investigate whether the DNA binding domain of NnaR is active, EMSAs were performed with the purified His-tagged NnaR protein isolated from S. coelicolor. Either 2.5 or 5 nM His–NnaR was mixed with 2 ng ~250 bp Cy5-labelled DNA fragments containing the upstream regions of various putative nitrate/nitrite assimilation genes [nirA (SCO6102), encoding a putative nitrite/sulphite reductase; nirB (SCO2486), encoding the large subunit of a nitrite reductase; nirC (SCO2959), encoding a putative nitrate extrusion protein; and nasA (SCO2473), encoding a putative nitrate reductase] and sirohaem biosynthetic genes [hemC (SCO7343), encoding a putative porphobilinogen deaminase; hemD (SCO3317), encoding a putative uroporphyrinogen-III synthase; hemE (SCO6031), encoding a putative uroporphyrinogen decarboxylase; cbiF (SCO1855), encoding a putative precorrin-4 C₁₁-methyltransferase; cysG (SCO1553), encoding a putative uroporphyrin-III methyltransferase]. Additionally, a DNA fragment harbouring the nnaR (SCO2958) upstream region was tested in EMSA. All samples were separated on a 2% agarose gel. Specific binding was verified by the addition of 500 x unlabelled specific DNA. In these assays, His–NnaR bound specifically to the upstream regions of the nitrogen assimilatory genes nirA, nirB, nirC and nasA (Fig. 5), but not to its own promoter region or to the upstream regions of the genes involved in sirohaem biosynthesis. In addition, all Glr targets were tested in EMSAs with His–NnaR, but except for the nirB and nasA upstream region, none of these targets was bound by His–NnaR (data not shown), showing that GlnR and NnaR do not have the same targets.

Furthermore, RT-PCR experiments were performed to elucidate the effect of NnaR on its own transcription as well
as on the transcription of genes putatively involved in nitrate/nitrite assimilation (nirA, nirB, narK and nasA) and sirohaem biosynthesis (hemC, hemE, cbiF and cysG) (Fig. 2). Total RNA samples were obtained from the M145 wild-type strain and the nnaR::Tn5062 mutant under nitrogen-rich S-medium or minimal N-Evans medium containing 5 mM NaNO₃. The isolated RNA was used as template in RT-PCR with primers annealing to internal parts of the various genes. The transcriptional analysis showed that under nitrogen-rich conditions there is no difference in expression of the various nitrogen genes (nirA, nirB, narK and nasA) between the nnaR::Tn5062 mutant and the M145 wild-type strain. However, under nitrogen-limiting conditions, the narK transcript was only present in the parental strain, whereas it could not be detected in the nnaR::Tn5062 mutant. The nirB and nasA genes showed strongly reduced transcription in the nnaR::Tn5062 mutant under nitrogen-limiting conditions compared with the wild-type, while the nirA transcription level was the same as that of the wild-type strain (Fig. 2b, c). As NnaR showed poor binding efficiency to the nirA upstream region in the EMSA studies (Fig. 5), this may be an explanation of why NnaR does not have such a strong influence on nirA expression. These data demonstrate that under nitrogen-limiting conditions, NnaR is an activator of nirB, narK and nasA transcription. In contrast, no difference in expression of the genes involved in haem biosynthesis could be observed in either the complex medium or the nitrogen minimal medium, which shows that NnaR has no influence on the regulation of haem biosynthesis (Fig. 2d). Together with the data from the EMSA analyses it can be concluded that NnaR has a regulatory function in nitrate/nitrite assimilation.

**NnaR and GlnR bind cooperatively to the nirB promoter**

One of the GlnR target genes is nirB, which encodes a nitrite reductase that reduces nitrite to ammonium. nirB expression is under tight GlnR control (Tiffert et al., 2008). Transcriptional analysis showed that nirB transcription is completely abolished in the S. coelicolor ΔglnR mutant, whereas it is strongly reduced in the nnaR::Tn5062 mutant (Fig. 2c). Despite the strong effect on nirB expression, GlnR displayed only weak binding to the nirB promoter (Tiffert et al., 2008). Since NnaR exhibits a binding pattern to the nirB promoter region similar to that of GlnR, the binding efficiency of both proteins together to the nirB upstream region was analysed. EMSAs were performed with purified Strep–GlnR and His–NnaR and the Cy5-labelled nirB upstream region. Specific binding was verified by the addition of 100-, 200- and 500-fold unlabelled specific DNA. In these EMSAs the application of Strep–GlnR and His–NnaR led to a supershift of the nirB upstream region (Fig. 6), which suggests a cooperative binding of these proteins to the nirB promoter.

In *silico* analysis revealed an inverted repeat sequence upstream of the genes involved in nitrate/nitrite assimilation along with the GlnR binding boxes (Fig. 7a). This palindromic consensus sequence is not found upstream of any other GlnR target gene and may constitute the binding sites for NnaR. To prove the functionality and specificity of the nnaR and glnR binding sites, EMSAs were performed with the respective predicted binding motifs. For these assays a 48 bp (nnaRbox) and a 51 bp (glnRbox) Cy5-labelled DNA fragment, which contained only the predicted nnaR and glnR binding motifs, respectively, were amplified from *S. coelicolor* genomic DNA with the primers listed in Table S1. Two nanograms of each DNA fragment was tested in EMSAs together with purified His–NnaR (0.1, 0.15 and 0.2 nM) and Strep–GlnR proteins (0.05, 0.1, 0.15 and 0.2 nM). In these assays His–NnaR specifically bound to the nnaRbox fragment but not to the glnRbox, whereas Strep–GlnR only bound to the glnRbox but not to the nnaRbox fragment (Fig. 7b), which proves the specificity of NnaR for the nnaR binding motif and of GlnR for the glnR binding box.

**DISCUSSION**

Nitrogen assimilation in actinomycetes is controlled by a complex system of transcriptional and post-translational regulation. On the transcriptional level, GlnR represents the central regulator, and controls a number of target genes (Tiffert et al., 2008). In *S. coelicolor*, a novel GlnR target gene, nnaR, was identified via *in silico* screening of the genome for further GlnR binding sites. The promoter region of nnaR contains two semi-conserved GlnR boxes,
and their functionality was demonstrated in EMSAs. Furthermore, RT-PCR experiments with the *S. coelicolor* Δ*glnR* mutant showed that *nnaR* expression is dependent on GlnR, which demonstrated that *nnaR* is a new member of the GlnR regulon. Transcriptomic analyses in *S. venezuelae* have demonstrated that the expression of the gene corresponding to *nnaR*, Sven_2720, is strongly dependent on nitrogen availability. ChIP-chip analyses show that Sven_2720 is regulated by GlnR, though no direct binding of GlnR to the Sven_2720 promoter region is observed. Thus, Sven_2720 has been suggested to be directly involved in primary nitrogen metabolism (Pullan et al., 2011).

The NnaR protein is predicted to be composed of an N-terminal enzymatic domain, which resembles a uroporphyrinogen-III synthase (HemD), and a C-terminal regulatory domain. Amino acid sequence alignments showed that NnaR has only weak similarity (18% sequence identity) with the functional HemD of *Methanothermobacterthermoautotrophicum* and lacks all important residues normally present at the active site of HemD enzymes. In contrast, another HemD homologue, SCO3317, shares 54% identity and possesses all the important residues at its active site. Complementation analyses with an *E. coli* hemD mutant finally revealed that SCO3317, but not *nnaR*, encodes a functional HemD enzyme in *S. coelicolor*. The non-functional HemD-like

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**Fig. 6.** EMSA of Strep–GlnR and His–NnaR with the *nirB* upstream region. Two nanograms of Cy5-labelled *nirB* upstream fragment was incubated with different amounts of Strep–GlnR and His–NnaR. Lane 1: no protein; lanes 2, 5, 6, 7 and 8: 0.5 nM His–NnaR; lanes 3, 5, 6, 7 and 8: 0.5 nM Strep–GlnR; lane 4: 1.0 nM Strep–GlnR. Unlabelled specific DNA at 100-, 200- and 500-fold excess was added in lanes 6, 7 and 8, respectively, to confirm the specificity of the shifts.

**Fig. 7.** (a) Comparison of upstream regions of different nitrate/nitrite genes from *S. coelicolor* (SCO) and *M. tuberculosis* (MTU). Conserved GlnR binding boxes are underlined. Grey boxes indicate the predicted NnaR recognition sites. *N* shows the distance to the translational start point. (b) The *nirB* upstream region, showing the localization of the *nnaRbox* and *glnRbox* fragments (top). EMSAs using His–NnaR and Strep–GlnR and the *nnaRbox* and *glnRbox* fragments, respectively (bottom). Lane 1: no protein (-); lanes 2, 3, 4 and 5: 0.1, 0.15, 0.2 and 0.25 nM His–NnaR, respectively (left); lanes 2, 3, 4 and 5, 0.05, 0.1, 0.15 and 0.2 nM Strep–GlnR, respectively (right).
enzymatic domain may have a function as signal receiver domain, like trigger enzymes (Commichau & Stülke, 2008), and could modulate gene expression in response to an as-yet-unknown ligand, which could be nitrate, nitrite, or one of the pyrrole nitrogens of hydroxymethylbilane, which is a natural substrate of HemD enzymes. To test the ability of nitrate and nitrite to act as NnaR ligands, EMSAs were performed with purified His–NnaR together with NaN₃ and NaN₅O₂, respectively. However, none of the molecules influenced the DNA binding activity of NnaR (data not shown), which suggests that neither nitrate nor nitrite is a ligand for NnaR. In E. coli and other enterobacteria, the multifunctional protein PutA, which is involved in proline degradation, has two enzymatic domains (proline dehydrogenase and Δpyrroline-5-carboxylate dehydrogenase) and a DNA binding domain, by which transcription of the genes putA and putP, which are involved in proline utilization and transport, respectively, is controlled (Ling et al., 1994). These two disparate functions of PutA are both proline-dependent: in the presence of proline, PutA in its reduced form associates with the membrane and performs its enzymatic functions, while in the absence of proline, PutA in its oxidized form binds to the promoter regions of the respective genes. Thereby, the substrate binding site for proline plays a central role in determining the respective functions of PutA (Zhu & Becker, 2003). NnaR may act in a similar way. Although the enzymatic domain is inactive, the binding pocket could bind an as-yet-unknown effector molecule, which would lead to a conformational change of the protein structure and subsequently to the modulation of the DNA binding affinity of NnaR for its target genes.

The S. coelicolor nnaR::Tn5062 mutant showed greatly retarded growth on medium containing nitrate as the sole nitrogen source. This is in good accordance with the observation that the corresponding Δpro6368 deletion mutant of the closely related actinobacterium R. jostii RHA1 also does not grow on minimal medium with nitrate as a single nitrogen source (see Fig. S4 available with the online version of this paper). Furthermore, the deletion of the nnaR homologous gene nasE of Amycolatopsis mediterranei U32, which is part of an operon responsible for nitrate/nitrite assimilation, leads to an inability to grow on minimal medium with either nitrate or nitrite (Shao et al., 2011). These phenotypes resemble the phenotype of the S. coelicolor glnR mutant, which is not able to grow on nitrate, because it fails to activate the transcription of the nitrate reductase gene nirB. Also, the S. coelicolor nirB deletion mutant is not able to grow on nitrate (Shao et al., 2011). Thus, nitrite is accumulated, and is probably toxic to cells, but can no longer be converted to ammonium, which is subsequently required to synthesize glutamine and glutamate. This was demonstrated by a Griess–Ilosvay assay, in which the accumulation of nitrite was detected in the nnaR::Tn5062 and ΔglnR mutants but not in the wild-type strain. The reason why the nnaR::Tn5062 mutant only shows retarded growth, whereas the ΔglnR mutant cannot grow at all on nitrate minimal medium, may be because loss of nnaR only attenuates nirB transcription, while deletion of glnR may prevent nnaR and nirB transcription. Thus, in the nnaR::Tn5062 mutant, weak nirB expression may be possible, because GlnR still can activate transcription to some extent, whereas nirB expression is completely blocked in the ΔglnR mutant because of the additional failure of nnaR expression. This is also supported by the results obtained from transcriptional analysis, which showed that the amount of nirB transcript is strongly reduced in the nnaR::Tn5062 mutant, whereas nirB mRNA is completely absent in the ΔglnR mutant. To summarize, this would mean that both GlnR and NnaR are needed for full nirB expression.

The function of NnaR as a transcriptional regulator was verified by performing EMSAs and RT-PCR, in which NnaR was discovered to be an activator of nitrate/nitrite metabolism genes, although without any influence on the expression of sirohaem biosynthetic genes. His–NnaR and Strep–GlnR together with the nirB promoter region led to a supershift in the EMSA, which suggests that NnaR may act as a co-activator at certain GlnR-dependent promoters. This also answers the question raised by Tiffert et al. (2008) about the weak DNA shift of the nirB promoter region with GlnR. We also tested whether the NnaR/GlnR mix leads to a supershift with other DNA fragments containing the nnaR and glnR binding motif. However, only an indistinct supershift was found with the nasA upstream region (data not shown). Based on these findings, we propose a model for the NnaR-based nirB gene expression in S. coelicolor: under nitrogen-limiting conditions, GlnR binds to its specific binding boxes in the nirB promoter and activates its expression. Simultaneously, GlnR binds to its binding boxes in the nirB promoter, leading to a weak induction of gene expression. The binding of GlnR to the nirB promoter probably leads to a conformational change of the DNA structure in such a way that NnaR binding is facilitated. Subsequently, NnaR in the presence of a specific signal also binds to its proposed binding sites in the nirB promoter and supports the GlnR binding for full expression of nirB.

Such a dual pattern of regulation has also been reported for cyd (encoding a cytochrome d oxidase) and hemA (encoding a glutamyl-tRNA dehydrogenase) gene expression in E. coli (Darie & Gunsalus, 1994; Fu et al., 1991). Here, multiple transcription factors are required to accomplish the maximal control of the target genes. Moreover, a number of E. coli genes involved in nitrate/nitrite metabolism (e.g. narG, fdhG, narK, nirB and nrfA) are dependent on the FNR and NarP and/or NarL proteins for full expression. E. coli Fnr is an oxygen-responsive global transcriptional factor (Kiley & Beinert, 2003), whereas the two paralogues NarP and NarL are response regulators, which constitute two-component systems together with their cognate sensor kinases, NarQ and NarX, respectively (Darwin et al., 1997). The arrangement of their respective control regions permits the organization of multiple protein–protein interactions between RNA polymerase, FNR, NarL and NarP (Browning & Busby, 2004; Eiglemeier et al., 1989), and is similar to that...
of the *S. coelicolor* nirB upstream region. In the light of this example, we propose an analogous model of multi-transcriptional factor control of nitrate/nitrite metabolic genes in *S. coelicolor*. Furthermore, the synergistic binding of NnaR and GlnR proteins to the promoter region of nirB suggests that NnaR may act as a GlnR co-activator in the regulation of nitrogen metabolism.

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