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GlnR positively regulates *nasA* transcription in *Streptomyces coelicolor*

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ABSTRACT

The model actinomycete, *Streptomyces coelicolor* is able to grow with nitrate as the sole nitrogen source. In this study, an assimilatory nitrate reductase encoding gene, *nasA* (SCO2473) was, for the first time, identified from the genome of *S. coelicolor* by genetic and physiological means. We also proved that GlnR, a previously characterized global nitrogen regulator in *S. coelicolor*, positively regulated the transcription of *nasA* via specific binding to a *cis*-element similar but different from the previously characterized consensus sequence. This finding will certainly facilitate the better understanding about both the functional scope and the mechanism of action of GlnR in regulating nitrogen metabolism in *S. coelicolor*.

Introduction

The soil-dwelling *Streptomyces coelicolor*, which produces many antibiotics and other secondary metabolites, can utilize diverse nitrogen sources, including nitrate as sole nitrogen sources under aerobic conditions [1]. The assimilation of nitrate begins with the reduction of nitrate to nitrite catalyzed by an assimilatory nitrate reductase and then followed by the assimilatory nitrite reductase catalyzed conversion of nitrite to ammonia. In *S. coelicolor*, the assimilatory nitrite reductase encoding genes *nirB1B2C* (SCO2486-SCO2488) were identified and proven to be positively regulated by the GlnR protein [2]. On the other hand, unlike the previously characterized nitrate assimilatory operons of *nasFEDCBA* in *Klebsiella pneumoniae* M5a1 [3] and *nasABCDEF* in *Bacillus subtilis* [4], both of which encode nitrate reductase and nitrite reductase in one operon, no putative nitrate reductase encoding genes were found near the *nirB1B2C* operon in *S. coelicolor*. To this date, three copies of *narGHJI* operons encoding respiratory nitrate reductase have been found in *S. coelicolor* [5,6], suggesting that the bacterium has the capacity to generate energy using nitrate as an electron receptor under anaerobic conditions [7–9]. However, no assimilatory nitrate reductase has ever been reported in *S. coelicolor*, neither for its encoding gene nor for the mechanism of its regulation.

GlnR has been characterized as a global regulator controlling most of the nitrogen metabolism processes in *S. coelicolor* [1,2] except for the nitrate reduction, which was the first step of nitrate assimilation. In this report, an assimilatory nitrate reductase encoding gene *nasA* (SCO2473) was identified and proven to be positively regulated by GlnR under nitrogen-limited condition in *S. coelicolor*. This finding was surely an important supplement for the previously proposed GlnR regulon [2].

Materials and methods

Bacterial strains, primers, and media. Escherichia coli DH5α was used for subcloning. *S. coelicolor* was grown at 30 °C in either the nitrogen-rich S medium [10] or nitrogen-limited N-Evans medium [1] with either 5 mM nitrate or 5 mM nitrite or 20 mM glutamine as the sole nitrogen sources. When needed, aparamycin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), thiostrepton (50 μg ml^{-1}), ampicillin (100 μg ml^{-1}) and nalidixic acid (25 μg ml^{-1}) were added into media. Primers used in this study were listed in Table 1.

Construction and complementation of the nasA mutant. The *nasA* null mutant of *S. coelicolor*, M145Δ*nasA* was constructed using the PCR-targeting method provided by the John Innes Centre [11], employing primers SCnasAPTF and SCnasAPTR. Mutants were confirmed via PCR with primers SCnasACF and SCnasACR, which located outside the homogenous recombination regions. To

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Table 1
Primers used in this study.

Primer name	DNA sequences (5′–3′)
For construction of S. coelicolor GlnR expression plasmid in E. coli BL21 (DE3)	
EXSCR_f	ACGGCTAGCTCTCTGCTGCTCCTGA
EXSCR_r	TATGGATCCAGTCGCGCCACATCAT
For construction and verification of S. coelicolor nasA null mutant	
SCnasAPTF	CCGGTCGCGTAACACGGCAGCCGCAGGCTGGGCGGCATGATTCCGGGGATCCGTCGACC
SCnasAPTR	CTCACGTGGAGATCATGGGAGGAGAGGATGACGCCGCTATGTAGGCTGGAGCTGCTTC
SCnasACF	CCGTCCCCGCCGTGTAACAG
SCnasACR	GGCGCTGGTGCTGACTCGGA
For construction of nasA and glnR complementary plasmids	
SCnasACOM1	CACGACGTGACCTGGTACTA
SCnasACOM2	GGCGCTGGTGCTGACTCGGA
SCglnRCOM1	ATTGGATCCTGCGGACGATTGGCTG
SCglnRCOM2	TATGGATCCAGTCGCGCCACATCAT
For GMSA	
nasAbs1	The same as SCnasACOM1;
nasAbs2	GCCGCCCAGCCTGCGGCT
For DNase I footprinting	
nasAFP1	CGCCGACGAGCACGACGTGA
nasAFP2	GGGCAGTGCGTGGGGGTGGT
For RT-PCR	
hrdB_f	GAGTCCGTCTCTGTCATGGCG
hrdB_r	TCGTCCTCGTCGGACAGCACG
nasA_f	GCTGCGCTACTTCACCGAAC
nasA_r	GGTGATCCGCTCCACGTACT

complement the *nasA* mutation, an intact *S. coelicolor nasA* gene with its proper promoter region was firstly amplified with primers SCnasACOM1 and SCnasACOM2 using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) to produce blunt ends, which was then directly introduced into the BamHI site of the integrative pSET1521 [12] blunted with Klenow (NEB, MA, USA), resulting in the complementation plasmid pSETSC*nasA*. The inserted DNA was further verified by DNA sequencing before the plasmid was subsequently conjugated with M145Δ*nasA* from the donor *E. coli* ET12567/pUZ8002 [13]. Exconjugants were selected on MS media flooded with nalidixic acid and thiostrepton to obtain the *nasA* complementation strain, SC*nasA*+.

Complementation of the glnR null mutant. The *glnR* null mutant M145Δ*glnR* was obtained in the same way using the PCR-targeting strategy (unpublished data). For the complementation of *glnR* null mutation, an intact *glnR* gene together with its proper promoter region was amplified with primers SCglnRCOM1 and SCglnRCOM2 using KOD-plus (Toyobo), which was further digested with BamHI before inserted into the same site of pSET1521 [12], resulting in the complementation plasmid pSCglnR2. After the inserted *glnR* gene being verified via DNA sequencing, pSCglnR2 was conjugated into M145Δ*glnR* the same as we described above, obtaining the complemented strain SC*glnR*+.

Expression and purification of the recombinant GlnR. The *S. coelicolor glnR* gene was amplified with primer EXSCR_f and EXSCR_r using the chromosome as a template, which was digested with EcoRI and HindIII and then inserted into pET28a (Novagen, Darmstadt, Germany) to form the expression plasmid pEXSCR. Expression and purification of the recombinant GlnR were performed according to the methods recommended by the manufacturer. GlnR protein was stored in buffer (20 mM Tris–HCl [pH 8.0], 25 mM KCl) with its concentration determined using Bradford method [14].

Gel mobility shift assay (GMSA) and DNase I footprinting assay. For GMSA, the *S. coelicolor nasA* promoter region was PCR amplified with primers nasAbs1 and nasAbs2, which was further purified from agarose gel. The probe was labeled with γ-^{32}P-ATP using T4 polynucleotide kinase (T4 PNK) (NEB). In a total volume of 20 μl, 0.04 pmol γ-^{32}P labeled probe was incubated with different amounts of purified SCO_GlnR in 1× binding buffer containing 10 mM Tris–HCl (pH 8.0), 25 mM KCl, 2.5 mM $MgCl_2$, 1.0 mM DTT and 2 μg salmon sperm DNA.

The DNase I footprinting experiment was carried out according to Leblanc and Moss [15]. Primer nasAFP2 was firstly end-labeled with γ-^{32}P-ATP using T4 PNK, and then a 249-bp DNA fragment was amplified by primer pair nasAFP1/^{32}P-nasAFP2, followed by purification with the Wizard® SV Gel and PCR Clean-Up System (Promega, Wisconsin, USA). Proper probe (about 20,000 cpm) was incubated at 37 °C for proper time with different amounts of purified His_6-GlnR, 2.5 μg sheared salmon sperm DNA and 0.025 Unit DNase I (TaKaRa, Shiga, Japan) in a total volume of 50 μl in the same buffer as GMSA described above. The sequencing ladders were prepared with the same primer nasAFP2 using the fmol DNA Cycle Sequencing System (Promega). The digestion products together with the ladders were analyzed on 6% polyacrylamide, 7 M urea sequencing gels. Gels were scanned with a FLA-7000 phosphoimager (FujiFilm Corporation, Japan).

RNA extraction, reverse transcription-PCR (RT-PCR). S. coelicolor M145, M145Δ*glnR* and SC*glnR*+ were grown in nitrogen-rich liquid S medium for 3 days and 0.4% glutamine was added in the culture for M145Δ*glnR*. Cells were then harvested and washed twice with nitrogen-limited N-Evans medium with 5 mM nitrate at 4 °C before inoculating individually into the fresh S medium and the fresh N-Evans medium with 5 mM nitrate simultaneously for 5 h' further culturing [2]. Total RNA was extracted using Trizol Reagent (Invitrogen, California, USA) following the instructions recommended by the manufacture. DNase I (Promega, Wisconsin, USA) treatment was performed at 37 °C for 1 h. Two micrograms of RNA was used for cDNA synthesis with 6-bp random primers employing the Superscript III Reverse Transcriptase (Invitrogen) in 20 μl volume.

For analyzing gene transcription in *S. coelicolor*, 20 ng cDNA reaction mixture was used as the template for the following PCR reaction, employing the *hrdB* gene as an internal control. Equal amount of RNA without reverse transcription was used as a negative control. The PCR procedures were: 95 °C heating 5 min; then 25 cycles were conducted with 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s extension; finally with 5 min incubation at 72 °C.

RNA extracted from M145 cultured under nitrogen-limited condition was used for determining co-transcription of SCO2472 and *nasA* gene (SCO2473), and 30 cycles were conducted employing primer pair SCnasACOM1/nasA_r under the same PCR procedures shown above.

PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. Two independent samples were used for RT-PCR analysis and the results were found to be consistent.

Results and discussion

Gene SCO2473 (nasA) of S. coelicolor encodes an assimilatory nitrate reductase

An open reading frame (ORF) SCO2473 was annotated as a putative nitrate reductase encoding gene [5] and designated *nasA* (nitrate assimilatory gene A) hereafter. This ORF was completely deleted from the chromosome via the PCR-targeting strategy and verified by specific PCR against the targeted gene (*Materials and methods*). The resulting *nasA* null mutant (M145Δ*nasA*) grew poorly on N-Evans plate with 5 mM nitrate as the sole nitrogen source but grew well when 5 mM nitrite was supplemented (Fig. 1). When an intact *nasA* gene with its proper upstream region was genetically complemented via integrative plasmid pSET1521 [12], the resulting strain SC*nasA*+ was able to grow on both nitrogen sources, with the same growth phenotype as that of the wild-type M145 (Fig. 1). It is thus evident that the *nasA* gene does encode an assimilatory nitrate reductase in *S. coelicolor*.

We also noticed that during the prolonged incubation, M145Δ*nasA* did grow slowly on N-Evans plate with nitrate supplied as the sole nitrogen sources (data not shown). In *S. coelicolor*, besides *nasA* and three copies of respiratory nitrate reductase encoding clusters [6,9], blast search (http://blast.ncbi.nlm.nih.gov/) identified another ORF SCO7374 bearing 54% amino acid sequence identity with a putative periplasmic nitrate reductase from *Penicillium marneffei* ATCC 18224 (PMAA_083320). It has been known that the assimilatory, respiratory, and dissimilatory processes were interconnected and nitrite formed via the catalysis of dissimilatory periplasmic nitrate reductase could be assimilated for cellular metabolism [16], which probably led to the poor/slow growth of M145Δ*nasA* on N-Evans medium with nitrate supplied. However, based on the present data, we cannot exclude the possibility of existence of other uncharacterized assimilatory nitrate reductases in *S. coelicolor* although they are unlikely to be functional, particularly under the tested physiological conditions.

The S. coelicolor nasA is positively regulated by GlnR under nitrogen-limited condition

Semi-quantitative RT-PCR analysis showed (Fig. 2) that in the wild-type M145, *nasA* transcription was remarkably induced under the nitrogen-limited condition; while in the *glnR* null mutant M145Δ*glnR*, no transcription of *nasA* could be detected under the same condition. In the *glnR* complementation strain SC*glnR*+, the *nasA* transcript was detectable and the transcription was inducible under nitrogen starvation conditions, similar to that in M145, although the expression level of *nasA* was apparently lower. Therefore, the *S. coelicolor nasA* ought to be positively regulated by GlnR in response to the nitrogen-limited condition. In that direction, it is interesting to notice that a *glnR* miss-sense mutant *S. coelicolor*, strain F55 [17] was able to reduce nitrate into nitrite, which was then extruded into the supernatant [2]. Because this miss-sense mutant GlnR is unable to activate the expression of *glnA* [17], it would not maintain the ability of activating the expression of *nasA* either. Therefore, the activity of the non-assimilatory nitrate reductase in *S. coelicolor* [6,9] might account for the observed reduction of nitrate into nitrite in strain F55.

Identification of the GlnR protected cis-element within the putative nasA promoter region

The *nasA* gene was oriented in the same direction with its 5′ vicinity gene SCO2472 in transcription and there was only 103 nucleotides (nts) in between. To determine whether the two adjacent genes were co-transcribed, RT-PCR was performed with primers designed to generate PCR products encompassing both SCO2472 and *nasA* gene. Because no PCR products were detected (data not shown), the *nasA* gene was unlikely to be co-transcribed with SCO2472 but rather transcribed from its own promoter.

His-tagged *S. coelicolor* GlnR was purified to SDS–PAGE homogeneity and was shown to bind to the putative *nasA* promoter region *in vitro* (Fig. 3). DNase I footprinting assay employing a ^{32}P labeled oligonucleotide complimentary to the 5′-terminus of the coding strand was carried out to determine the precise DNA sequences protected by GlnR in the putative *nasA* promoter region (Fig. 4). Two separate protected regions within the *nasA* promoter were identified (region I, 5′-GTCC**CC**GCCGTGTAACAGGGC-3′ and region II, 5′-GTCGCGTAACACGGCA-3′) with two hypersensitive sites (in region I, the underlined bold letters).

GlnR was proposed to regulate the expression of almost all of the important genes related to nitrogen metabolism via specific binding to the promoters of the respective target genes in *S. coelicolor*, and the GlnR regulon was thus designated [1,2]. Including of *nasA* as a target gene subject to GlnR regulation shown by this study apparently completed the whole spectrum of the GlnR regulon in addition to the target genes responsible for nitrite reduction (*nirB*), ammonium transportation (*amtB*) and assimilation (*gdhA*, *glnA*) as well as the urea utilization (*ureA*) [2].

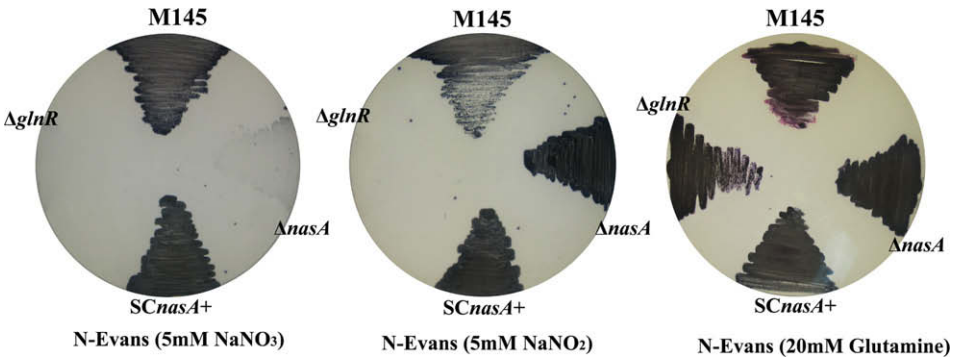

Fig. 1. Growth phenotypes of *S. coelicolor* strains on nitrogen-limited N-Evans medium with either 5 mM nitrate or 5 mM nitrite or 20 mM glutamine. Strains were grown at 30 °C for 4 days and photos were taken from the bottom of the plates. M145Δ*glnR* was proven unable to grow on minimal medium and was used as a control.

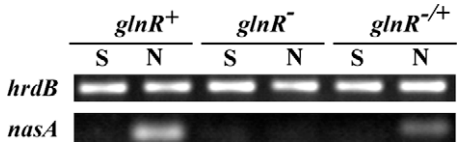

Fig. 2. RT-PCR analysis of *S. coelicolor nasA* transcripts. No DNA bands of PCR products were observed when total RNA without reverse transcription was used as the templates, indicating no contamination of DNA in the RNA samples (data not shown). Symbols used here: *glnR*$^{+}$, M145; *glnR*$^{-}$, M145Δ*glnR*; *glnR*$^{-/+}$, SC*glnR*+; S, S medium; N, N-Evans medium with 5 mM nitrate.

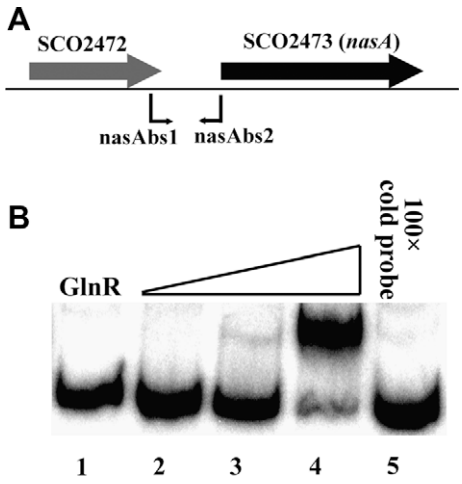

Fig. 3. GMSA analysis of *nasA* promoter with purified His-tagged *S. coelicolor* GlnR. The *nasA* promoter region was amplified with primers nasAbs1 and nasAbs2 (A), 0.04 pmol of which was used to bind with various amount of GlnR protein (B) (0 μg in lane 1, 0.025 μg in lane 2, 0.1 μg in lane 3, and 0.4 μg in lane 4). In lane 5, 0.4 μg GlnR was pre-incubated with 100 times unlabeled cold probe for a specific competitive assay. Two micrograms sheared salmon sperm DNA was added in lanes 2–4, preventing non-specific binding.

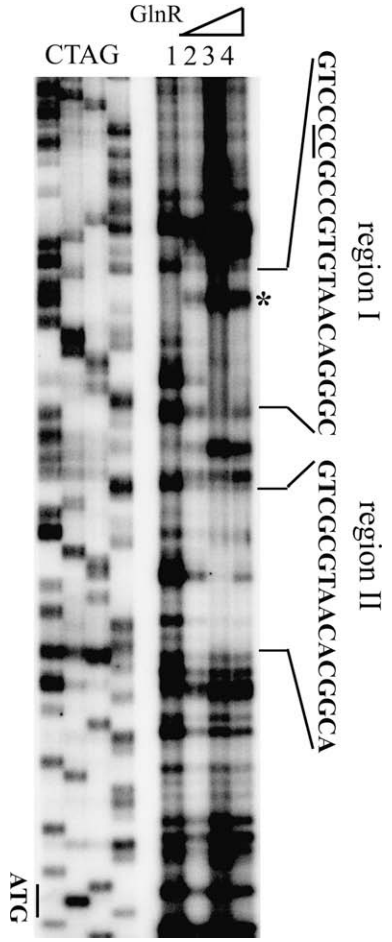

Fig. 4. Identification of the GlnR protected *cis*-element in *S. coelicolor nasA* promoter using DNase I footprinting assay. DNA sequences protected by GlnR from DNase I cleavage were shown as "region I" and "region II". Two DNase I-hypersensitive sites were denoted with asterisks and shown with underlined letters. The translation start site ("ATG") for *nasA* was indicated by a vertical line. Various amount of GlnR was added and proper time was used for DNase I digestion: lane 1, 0 μg GlnR (30 s); lane 2, 0.5 μg GlnR (60 s); lane 3, 1.0 μg GlnR (90 s); and lane 4, 2.0 μg GlnR (120 s).

What is the precise cis-element required for GlnR binding and regulation in S. coelicolor?

The *cis*-element required for *S. coelicolor* GlnR binding has been a subject of research over the years. Through aligning several promoter sequences of the GlnR regulated target genes, Fink et al. proposed a 44-bp *S. coelicolor* GlnR binding motif (GGTCAC -N_5-CGAAAC-N_5)$_2$ [1]. Based on Fink's work, Tiffert et al. [2] identified 10 new GlnR target genes and deduced a much shorter 22-bp consensus sequence specific for GlnR binding (gTnAc-n_6-GaAAc-n_6) comprised of an "a-site" of "gTnAc" and a "b-site" of "GaAAc".

The *cis*-element in the *nasA* promoter that we characterized is somehow different from the previously defined consensus sequence. Therefore, it is no surprise that when we tried to use the MAST tool [18], queried with the 22-bp consensus sequence, to search into the genome of *S. coelicolor* for potential target genes subject to GlnR regulation, the SCO2473 (*nasA*) was apparently missed, as shown in the previous trials [2]. A careful comparison indicated that two "a-site" sequences could be found within the GlnR protected sequences in the *nasA* promoter ("GTAAC" in both regions I and II, Supplementary data, Fig. S1) and they are separated by 18 nts. In fact, this configuration could be derived from the previously reported consensus sequence composed of a 2× 22-bp tandem repeat [2] similar to the GlnR binding motif suggested by Fink et al. [1] although it would reveal a 17 nts sequence, instead of the 18 nts defined by this study, located in between the two a-sites. However, a new question about the necessity of having a "b-site" sequence downstream of an "a-site" should be raised to account for the minimum *cis*-element(s) required for GlnR binding and/or regulation. Therefore, this finding, *i.e.*, only two "a-site" sequences segregated by 18 nts is sufficient for GlnR binding, has brought us a new insight into future studies. With more GlnR target genes identified in *S. coelicolor*, new forms of GlnR binding sequence and the *cis*-element essential for GlnR binding and regulation will surely be better characterized not only in their precise compositions and configurations but also for the mechanisms of action.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.05.147.

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