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Precise characterization of GlnR Box in actinomycetes

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ABSTRACT

GlnR has been characterized as a central regulator governing most nitrogen metabolisms in many important actinomycetes. So far, the GlnR binding consensus sequences have been extensively studied, but with different motifs proposed, which has therefore brought confusion and impeded the understanding of the in-depth molecular mechanisms of GlnR-mediated transcriptional regulation. Here, a 30-nt GlnR-protected DNA sequence in the promoter of *glnA* in *Amycolatopsis mediterranei* was employed for precise characterization of GlnR binding consensus sequences. Site-by-site mutagenesis strategy combining with the Electrophoretic Mobility Shift Assay were employed, and a 5-nt GlnR Box was precisely defined as the basic unit for GlnR binding.

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1. Introduction

The Actinomycetaceae family is well-known for the production of a large number of bioactive metabolites, many of which are useful to human in both medicine and agriculture. Besides, this family also includes some important pathogens, *e.g. Mycobacterium* and *Nocardia*. Many evidences have demonstrated that nitrogen metabolism plays an important role in the physiology of actinomycetes, such as regulating the yield of the secondary metabolites in *Amycolatopsis mediterranei* [1] and affecting the bacterial pathogenicity of *Mycobacterium tuberculosis* [2,3].

Unlike the well-studied enterobacteria, which usually employs the NtrB-NtrC two-component system in the regulation of nitrogen metabolism, GlnR has been characterized as a global nitrogen regulator in many important actinomycetes, including the model *Streptomyces coelicolor* [4–6], the rifamycin-producing *A. mediterranei* [7,8] and the pathogenic *M. tuberculosis* [2]. GlnR, belonging to the OmpR/PhoB subfamily, is an orphan response regulator and its post-translational modification remains unclear [9]. So far, most nitrogen metabolism-associated genes are found to be transcriptionally regulated by GlnR, *e.g. glnA*, *glnII*, *amtB*, *nirB*, *nasA* and *ghdA*, and thus a GlnR regulon was proposed, firstly in *S. coelicolor* [4,10] and then in many other important actinomycetes, including *A. mediterranei* [7,11], *Mycobacterium smegmatis* [12,13], *Streptomyces venezuelae* [14] and so on. Moreover, GlnR has been found to co-regulate with other regulators, *e.g.* with PhoP in the transcriptional regulation of *amtB* in *S. coelicolor* [5,6]. Therefore, precise characterization of the GlnR binding consensus DNA sequences may not only facilitate understanding the mechanisms of GlnR-mediated transcriptional regulation but also help identification of other cooperative regulators.

Through aligning the promoter sequences of two GlnR targets of *glnA* and *amtB* in *S. coelicolor*, Fink et al. obtained a putative GlnR operator structure comprised of a 44-nt DNA sequence (GGTCAC-N5-CGAAAC-N5)$_2$ [15]. Then, based on Fink's work, Tiffert et al. used the MEME/MAST online search tools to identify 10 new GlnR targets and defined a more precise GlnR binding consensus sequence, comprised of the sequence of gTnAc-n6-GaAAc-n6-GtnAC-n6-GAAAc-n6 ("*a-b-a-b*" sites in abbreviation) [4]. Obviously, the consensus sequence shows great variability among some GlnR targeted promoters, *e.g. glnII*, *ureA*, *SCO0888* and *SCO2400* (Fig. 2A in *Ref.* [4]). More importantly, with more and more GlnR targets characterized, the number of GlnR binding sites varies, *i.e.* from zero to six in the promoter regions of different GlnR target

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Table 1
Summary of proposed GlnR-binding motifs.[a]

Authors	Sequences	Species	Refs	year
Tiffert et al.	gTnAc-n6-GaAAc-n6-GtnAC-n6-GAAAc-n6	*Streptomyces coelicolor*	[4].	2008
Pullan et al.	GTnAC-n6-GTnAC	*Streptomyces venezuelae*	[14].	2011
Jenkins et al.	AC/T-n9-AC	*Mycobacterium smegmatis*	[13].	2013
Sola-Landa et al.	11-nt Direct Repeat	*S. coelicolor*	[5].	2013

[a] All of the GlnR binding motifs were obtained with bioinformatics analyses of multiple GlnR targeted genes.

genes [6,7,10,14], reflecting the complexity of GlnR binding consensus sequences. Nevertheless, the "*a*-*b*-*a*-*b*" motif has been widely used for a long while until recently different GlnR binding motifs have been proposed in *S. venezuelae*, *M. smegmatis* and even in *S. coelicolor*. For example, in *S. coelicolor*, 11-nt direct repeats were obtained through analysis of a total of 14 GlnR-binding sites (50 direct repeat units) [5]. In *M. smegmatis*, a consensus motif of AC/T-n9-AC was identified and was shown to present once in all 53 GlnR binding sites [13]. While in *S. venezuelae*, the most common motif was a single sequence of GTnAC-n6-GTnAC in the promoters of GlnR target genes. Because these GlnRs show high homology, especially in their C-terminal DNA binding domains [9,12], it is rather confusing to have so diverse GlnR binding motifs (Table 1). Notably, all these motifs were obtained on the basis of bioinformatics analyses such as aligning the promoter regions of GlnR targeted genes, and the predicted consensus, including both the sequence and the length, have never been experimentally verified.

To address this issue, a 30-bp DNA sequence in *A. mediterranei* U32 *glnA* promoter was employed and the GlnR binding consensus sequences were experimentally defined.

2. Material and methods

2.1. Electrophoretic Mobility Shift Assay (EMSA)

Chemically synthesized Oligos were purified through Polyacylamide Gel Electrophoresis (PAGE) and listed in supplementary Table S1. Pairs of 30-nt reverse complementary oligos were denatured and annealed to form double-stranded fragments, which were then labeled with γ-P^{32} with T4 polynucleotide kinase (PNK) (NEB) followed by oligo purification with Zeba™ Spin Desalting Column (Thermo). *A. mediterranei* U32 that was cultured for 24 h in Bennet medium supplemented with 80 mM potassium nitrate before being harvested at 4 °C. Cells were broken using sonication treatment and cell debris was removed *via* high speed centrifugation (12,000 rpm, 30 min) at 4 °C as described before [16]. Labeled oligos were then used as probes to incubate with different amounts of cell extracts following the conditions exactly the same as described before [16]. The intensities of the bands in EMSA pictures were firstly quantified with Quantity One (Bio-rad). Linear regressions were obtained with two parameters of the y and x. y = the ratio of (the intensity of shifted bands)/(the intensity of total bands, including shifted and unshifted bands). x = the amount (mg) of U32 cell extracts used in a reaction volume of 20 μL. Original data were listed in Fig. S1.

3. Results and discussion

A 30-nt DNA sequence within the *glnA* promoter in *A. mediterranei* U32 (designated as glnACIS30(WT)) was previously defined to be protected by GlnR [8]. To precisely characterize the GlnR binding sequences, glnACIS30(WT) was employed for EMSA analysis, combing with site-by-site mutagenesis. Because *glnA* is transcriptionally activated by GlnR in the presence of nitrate, U32 was cultured in Bennet medium with 80 mM nitrate supplemented and cell extracts were prepared for EMSA to mimic the *in vivo* GlnR binding conditions. With the increase of cell extracts, an obviously shifted band could be observed, just as previously described [17].

Mutagenesis of the 30-nt DNA sequence was performed by usage of synthesized mutated oligonucleotides. Mutated sequences were further analyzed with EMSA, employing glnACIS30(WT) as a positive control. Considering the conformational block of DNase I digestion, which usually produces a relatively larger region than the protein actually binds, the first 5 nts and the last 7 nts were firstly mutated together, and the mutated DNAs showed no obvious decrease in the binding affinity by GlnR (U32glnASM1, U32glnASM2, U32glnASM21 and U32glnASM22 in Fig. S1). Then, site-by-site mutagenesis of the sequences from 4th to 23rd nucleotides was performed, *i.e.* each base is individually mutated to the rest three bases (Fig. S1). EMSA figures were quantified and the data were analyzed with linear regression. As a positive control, the wild type glnACIS30(WT) has a slope of 4.12 with the R-square of 1.0 (U32glnACIS(WT) in Fig. S1). Mutations only located in two 5-nt regions, *i.e.* from T6 to C10 and from G17 to C21, severely damaged the GlnR binding, which could be reflected by their sharply decreased slopes, while the 6-nt interval of the two regions could be freely mutated without harming the GlnR binding efficiencies (Fig. 1). Therefore, one can conclude that the 5-nt region (*a*/*b* site) is the minimal unit for GlnR to bind.

Although the internal 6-nt can be freely mutated, based on previous findings, the interval distance between two sites are not fixed and may vary among different promoters [6,7,10,14]. Meanwhile, bases within these *a*- and *b*-sites were not irreplaceable, *e.g.* T6G, A8T, A8C, C10T, A18T, A18C, A19T, A19C and A21T could be well bound by GlnR. When combinatory mutagenesis of multiple sites is performed, more tolerant mutations might be identified. Therefore, the length of the *a*- and *b*-sites but not the precise sequences is fixed. Moreover, considering the fact that *a*- and *b*-sequences are highly homologous and the numbers and arrangement of these sites vary among different GlnR targeted promoters [6,7,10,14], the 5-nt minimal unit is renamed as "GlnR Box", for simplicity.

Notably, the fourth position of "A" in GlnR Box seems extremely conserved and mutation of this site would result in total loss of

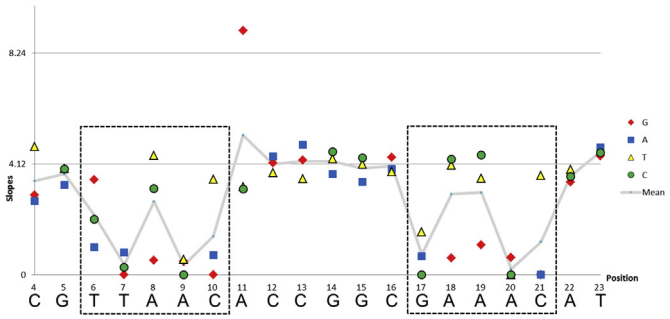

Fig. 1. EMSA efficiencies with mutated DNA sequences. The slope represented the EMSA efficiencies and DNA sequences from the 4th to the 23rd were shown. The wild-type promoter was used a positive control with the slope value of 4.12. Detailed information could be found in "EMSA Methods".

GlnR binding activities. This information is very important for further precise characterization of GlnR binding sites. For example, in the promoter region of *nas* operon in *A. mediterranei* U32, GlnR was proved to bind three GlnR binding sites of *a1*, *b1* and *b2* (three GlnR Boxes) [7]. In the previous study, the *b2* site was ever defined as "TAACA" (Fig. S2B in Ref. [7].), following the rules of 6-nt interval distance. Although the site of "TTAAC", which was 1-nt left shifted from the original site, was proposed for *b2* (Fig. S2C in Ref. [7].), the proposition is difficult to be accepted due to the lack of evidences. Considering the fact that the fourth nucleotide should be "A", the *b2* site is thus defined as "TTAAC" in the promoter of *nas* operon.

Because GlnRs show high homology in both amino acid sequences and the three dimensional structures among actinomycetes [9,12], the GlnR Box characterized in *A. mediterranei* surely sheds light on the GlnR binding consensus sequences in other actinomycetes. More importantly, the GlnR binding model is no longer confusing, and the research into the molecular mechanism of GlnR-mediated transcriptional regulation can thus be expedited.

Conflict of interest

The authors, including Jin WANG, Ying WANG, and Guo-Ping ZHAO declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.010.

Transparency document

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