Acta Biochim Biophys Sin, 2020, 52(8), 910–913
doi: 10.1093/abbs/gmaa057
Advance Access Publication Date: 8 June 2020
Lab Note

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Sinorhizobium meliloti NtrX interacts with different regions of the *visN* promoter

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The bacterial flagellum is a motile organelle consisting of a transmembrane multi-protein apparatus composed of the flagellins, the flagellin export complex, and the basal body with motor proteins. Flagellum biosynthesis is regulated by a hierarchical system in *Escherichia coli* [1]. Different from *E. coli*, *Sinorhizobium meliloti*, as a plant-symbiotic soil bacterium, induces the formation of nitrogen-fixing root nodules on the host plant alfalfa when combined nitrogen is limited. *Sinorhizobium meliloti* contains a 'flagellum regulon' that consists of 41 genes, including 10 chemotaxis (*che*) genes, 4 motor (*mot*) genes, 15 *flg*, *flh*, and *fli* genes encoding the components of the protein export complex and the basal body, 4 flagellin (*fla*) genes, and 4 unknown genes [1]. Similar to *E. coli*, flagellum biosynthesis in *S. meliloti* depends on a hierarchical system that is composed of the Class IA regulators, VisN and VisR, the Class IB regulator, Rem, Class II FliM, MotABC, and Class III flagellin and chemotaxis proteins [2]. Recently, several other regulators involved in flagellum biosynthesis have been reported, including the tow-component regulatory system ExoS/ChvI with the suppressor ExoR and the LuxR regulator ExpR in *S. meliloti* [3,4].

VisN and VisR, belonging to LuxR family regulators, are important activators of flagellum production, motility, and chemotaxis in *S. meliloti* [1]. They were proposed to work as a heterodimer for transcription activation of *rem* (encoding an OmpR-like transcriptional activator) by recognizing a motif, and their activities may depend on an unknown signal [2]. Constitutive expressions of *visN* and *visR* were suggested in *S. meliloti*, meaning that their transcription is not altered by transcriptional factors.

The two-component regulatory system NtrY/NtrX, first identified in *Azorhizobium caulinodans*, is required for nitrogen metabolism, nodule formation, and nitrogen fixation on host plants [5]. In *Rhizobium tropici*, this system was found to participate in symbiotic nodulation [6]. NtrY is a transmembrane histidine kinase containing a PAS domain, responsible for sensing the oxygen level as characterized in *Brucella abortus* [7]. NtrX is a response regulator, constituted by a REC domain with a conserved aspartate residue and a DNA binding domain. As a transcriptional regulator, NtrX regulates nitrogen metabolism in several bacterial species [8]. We previously found that the *S. meliloti* NtrX regulates symbiotic nitrogen fixation on host plants, succinoglycan biosynthesis, flagellum generation, and motility [8]. The transcriptional downregulation of *flaA*, *flaD*, *visN*, and *visR* genes was found in the *ntrX*-knockdown mutant (SmLL1) compared with the wild-type strain (Sm1021) [8]. This result was confirmed by microarray and qRT-PCR data in the newly isolated *S. meliloti* strain [9]. However, it remains elusive that NtrX, as an upstream transcriptional factor, directly regulates transcription of *visN*/*visR* by recognizing specific DNA motifs on the promoter.

To answer this question, we analyzed the promoter DNA sequence of the *visN* gene using a bioinformatics program. The DNA sequence between *fliF* and *visN* was downloaded from the website of https://iant.toulouse.inra.fr/bacteria/annotation/cgi and was loaded into the BDGP (Berkeley Drosophila Genome Project Searches Neural Network Promoter Prediction) server. The program for prokaryotes, and the forward strand and 0.8 of the minimum promoter score (between 0 and 1) were selected. Two highly conserved promoters were predicted (scores were 0.99 and 0.96, respectively). The promoters are shown as the italic and underlined letters in Fig. 1A, indicating that the *visN* gene has two independent promoters. To determine whether the promoter region interacts with the NtrX protein *in vivo*, the chromatin-immunoprecipitation (ChIP) was performed using anti-NtrX antibodies (Wenyuange Biotech, Shanghai, China), and ChIP product in the promoter sequence was applied for qPCR assays. The results showed that the promoter DNA fragment (blue letters in Fig. 1A) was significantly enriched in the sample of Sm1021 compared with that of SmLL1, the *ntrX*-knockdown mutant (Fig. 1B [8]), suggesting that the NtrX protein binds to a *visN* promoter region *in vivo*.

In *B. abortus*, NtrX has been found to recognize a motif of $CAAN_{3\text{-}5}TTG$ on the promoter of *ntrY* [10]. In the *visN* promoter region, we found that there are two putative motifs of $CAAN_XTTG$ (**CAA**TGC**TTTG** and **CAA**GG**TTG**; Fig. 1A). To test whether *S. meliloti* NtrX directly binds to the DNA fragment containing the putative motifs, we synthesized a DNA probe (bold and underline letters in Fig. 1A) to perform an electrophoretic mobility shift assay (EMSA). The results indicated that the phosphorylated NtrX protein

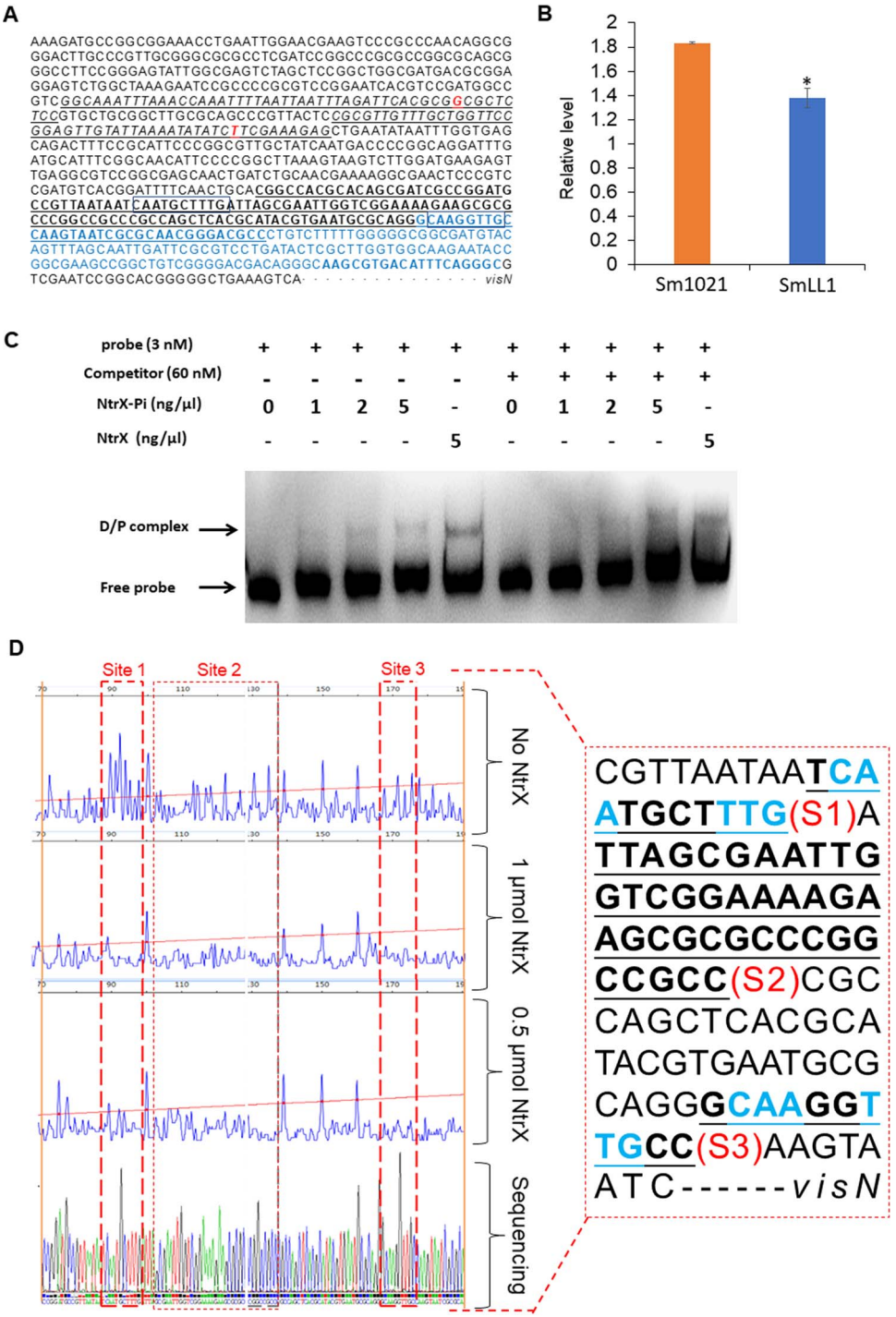

Figure 1. ***Sinorhizobium meliloti*** **NtrX protein directly interacts with the promoter region of** ***visN*** (A) The promoter region of *visN*. Two promoters as predicted using the BDGP server, are shown in the italic and underlined letters. The red italic letter is the predicted start site of transcription. The ChIP-PCR product is shown in blue. The EMSA probe (used in C) is indicated on the underlined letters. The boxed nucleotides indicate possible motifs of NtrX recognition [10]. (B) Evaluation of NtrX interaction with the promoter of *visN in vivo* by ChIP-PCR. Data were analyzed by Student's *t*-test, and the asterisk indicates a $P<0.05$. (C) NtrX binding to the promoter region *in vitro* as evaluated by EMSA. (D) The DNA regions protected by NtrX from the foot-printing assay. Three DNA regions found to be protected by the NtrX protein *in vitro* were S1, S2, and S3. S1 and S3 both contain a $CAAN_xTTG$ motif.

nonspecifically bound to the probe because the competitor DNA fragment (the probe without a biotin label) did not apparently reduce the protein–DNA complex (Fig. 1C). Interestingly, the dephosphorylated NtrX formed more protein–DNA complexes compared with the phosphorylated protein (Fig. 1C) and the complex appeared to be reduced after addition of the competitor, suggesting that this DNA region can be specifically recognized by the dephosphorylated NtrX protein.

To confirm whether the dephosphorylated NtrX protein directly recognizes the putative motif of $CAAN_xTTG$ in the DNA fragment

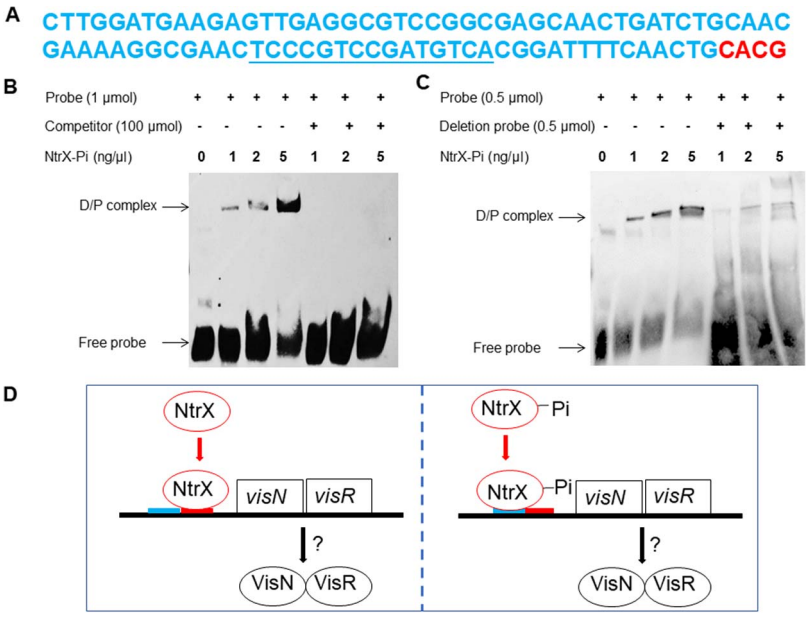

Figure 2. NtrX specifically interacts with the DNA fragment without a CAAN$_x$TTG motif (A) DNA sequence of the second probe. The red letters are overlapping with the first probe shown in the Fig. 1C. The underlined letters were deleted in the third probe used for the experiment shown in Fig. 2C. (B) Phosphorylated NtrX proteins specifically bound to the second probe as evaluated by EMSA. (C) Phosphorylated NtrX proteins weakly interacted with the truncated probe. (D) A possible model of NtrX binding to the *visN* promoter. The dephosphorylated NtrX recognizes CAAN$_x$TTG motifs (the red region), as the phosphorylated protein binds to the second region (blue).

(probe 1) in the *visN* promoter, we performed footprinting assays by cloning the DNA fragment into the pMD-19 T vector and reacting with the purified His-NtrX fusion protein *in vitro*. The sequencing results indicated that three DNA regions were protected by the dephosphorylated NtrX protein, including S1, S2, and S3 (Fig. 1D). Noticeably, two protected regions, S1 and S3, contain the CAAN$_x$TTG motif (**CAA**TGCT**TTG** and **CAA**GG**TTG**), whereas the S2 region did not (Fig. 1D). These data suggested that the CAAN$_x$TTG motif can be recognized by NtrX in *S. meliloti*.

The observation that phosphorylated NtrX protein nonspecifically bound to the probe (Fig. 1C) promoted us to design a new probe adjacent to the first (Fig. 2A). The EMSA results showed that this probe was specifically recognized by the phosphorylated NtrX protein because the protein–DNA complex completely disappeared after addition of enough competitor DNA (Fig. 2B). Furthermore, for the third probe with a 15-bp deletion (TCCCGTCCGATGTCA), the protein–DNA complexes were apparently reduced in comparison with the parent probe (Fig. 2C), suggesting that the size or the composition of the DNA fragment is required for the recognition of the phosphorylated NtrX. These results revealed that the phosphorylated NtrX may recognize a new motif, different from CAAN$_x$TTG.

In *S. meliloti*, flagellum biosynthesis is sequentially regulated by the transcriptional factors VisN and VisR (Class IA) and Rem (Class IB). Transcription of *visN* and *visR* was downregulated in the *S. meliloti ntrX* mutant [8,9], suggesting that the NtrX protein may activate the expressions of *visN* and *visR*. Here, we further provide biochemical evidence that NtrX is an upstream regulator of *visN/visR* by directly interacting with the *visN* promoter DNA. Since the NtrX homologs from several bacteria are involved in nitrogen metabolism, it is possible that they link nitrogen metabolism/level to control bacterial flagellum biosynthesis and motility.

Brucella abortus NtrX, with or 'without phosphorylation modification, can bind to the probe of the *ntrY* promoter [10]. However, results of competition tests in retardation assays are found in the literature. Therefore, it is hard to judge whether the observed protein–DNA interactions are specific in the case. In this study, we found that the phosphorylated NtrX protein of *S. meliloti* can specifically bind to one DNA fragment of the *visN* promoter (Fig. 2A,B). Interestingly, the dephosphorylated NtrX appeared to specifically bind to another DNA fragment of the promoter (Fig. 1C). Our results provide a possibility that NtrX, with or without a phosphorylation modification, can recognize different motifs in the *visN* promoter region, which may be associated with the promoter transcriptional activity.

The phosphorylated NtrX protein of *B. abortus* was found to protect three DNA regions containing each CAAN$_x$TTG motif [10]. Differently, the dephosphorylated NtrX protein of *S. meliloti* protects three DNA regions containing two CAAN$_x$TTG motifs (Fig. 1D). It is possible that both phosphorylated and de-phosphorylated NtrX can recognize the motif of CAAN$_x$TTG in bacteria. Noticeably, the phosphorylated NtrX of *S. meliloti* may recognize a new DNA motif that is different from CAAN$_x$TTG (Fig. 2A,B). We do not exclude the possibility that the second probe may contain a derivative of CAAN$_x$TTG (such as **CAA**CTGATC**TG**), which will be tested in future.

Taken together, our results suggest that the NtrX protein from *S. meliloti* activates the expression of *visN/visR* to control flagellum biosynthesis and motility by binding to specific DNA regions in the promoter.

Funding

This work was supported by the grant from the National Natural Science Foundation of China (No. 31570241).

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