ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2005, **37**(4): 221–226 **CN** 31-1940/Q

nifH Promoter Activity Is Regulated by DNA Supercoiling in *Sinorhizobium meliloti*

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Abstract In prokaryotes, DNA supercoiling regulates the expression of many genes; for example, the expression of *Klebsiella pneumoniae nifLA* operon depends on DNA negative supercoiling in anaerobically grown cells, which indicates that DNA supercoiling might play a role in gene regulation of the anaerobic response. Since the expression of the *nifH* promoter in *Sinorhizobium meliloti* is not repressed by oxygen, it is proposed that the status of DNA supercoiling may not affect the expression of the *nifH* promoter. We tested this hypothesis by analyzing *nifH* promoter activity in wild-type and *gyr*$^-$ *Escherichia coli* in the presence and absence of DNA gyrase inhibitors. Our results show that gene expression driven by the *S. meliloti nifH* promoter requires the presence of active DNA gyrase. Because DNA gyrase increases the number of negative superhelical turns in DNA in the presence of ATP, our data indicate that negative supercoiling is also important for *nifH* promoter activity. Our study also shows that the DNA supercoiling-dependent *S. meliloti nifH* promoter activity is related to the *trans*-acting factors NtrC and NifA that activate it. DNA supercoiling appeared to have a stronger effect on NtrC-activated *nifH* promoter activity than on NifA-activated promoter activity. Collectively, these results from the *S. meliloti nifH* promoter model system seem to indicate that, in addition to regulating gene expression during anaerobic signaling, DNA supercoiling may also provide a favorable topology for *trans*-acting factor binding and promoter activation regardless of oxygen status.

Key words *nifH*; DNA supercoiling; gyrase; *Sinorhizobium meliloti*; gene expression

In vivo, DNA superhelical density is determined by a balance between the activities of DNA topoisomerase I, which relaxes negative supercoiling, and DNA gyrase, which increases negative supercoiling [1–3]. When DNA negative superhelicity increases in *Escherichia coli* the *topA* gene is activated and the synthesized DNA topoisomerase I relaxes the negative DNA supercoiling. In contrast, when the superhelical density decreases, the *gyrA* and *gyrB* genes are activated, DNA gyrase is synthesized, and DNA supercoiling returns to its optimal levels. Recent studies have shown that topoisomerase IV plays a role in the unwinding process of double-stranded DNA [4]. In prokaryotes, negatively supercoiled genomic DNA promotes DNA recombination, replication and transcription [2,5]. In addition, DNA supercoiling plays an important role in the cellular perception of environmental signals. Changes in temperature, osmolarity and oxygen content directly affect DNA superhelical density via the action of gyrase [6–9]. In terms of oxygen content, anaerobic conditions activate bacterial gyrase, whereas aerobic conditions activate topoisomerase I. Indeed, it appears that the expression of aerobically-induced genes, such as peroxidase and superoxide dismutase (SOD) genes, requires DNA topoisomerase I, whereas gyrase favors the expression of anaerobically-induced genes, such as the hydrogenase gene.

In *Klebsiella pneumoniae*, the transcription of the *nifLA*

Received: January 10, 2005 Accepted: February 24, 2005

This work was supported by the grants from the National Natural Science Foundation of China (No. 30170512) and the Major State Basic Research Development Program of China (No. 2001cb108901)

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DOI: 10.1111/j.1745-7270.2005.00037.x

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operon requires DNA gyrase activity, while the transcription of other nitrogen fixation (*nif*) genes does not appear to be dependent on DNA gyrase activity [10]. Dixon *et al.* reported that *K. pneumoniae nifLA* promoter activity requires a certain level of DNA negative supercoiling, providing a connection between the regulation of *Kp nifLA* expression and the aerobic/anaerobic conditions known to alter supercoiling [11]. Our analysis of the anaerobic regulation mechanisms of the *Enterobacter cloacae nifLA* promoter revealed that an unknown *trans*-acting factor could bind the ferredoxin-NADP(H) reductase (FNR) binding site, leading to activation of *E. cloacae nifLA* expression under anaerobic conditions. In addition, we determined that DNA gyrase activity is essential for *E. cloacae nifLA* promoter expression [12]. *Sinorhizobium meliloti nifH* promoter has been reported to be activated by either NtrC (nitrogen regulation protein) or NifA, and both NtrC- and NifA-mediated types of activation do not respond to changes in oxygen level [13]. Therefore, it seems that *S. meliloti nifH* expression is not regulated by DNA supercoiling.

In the present research, we found that DNA supercoiling can regulate *S. meliloti nifH* promoter activity, and this regulation might be associated with the binding of the *trans*-acting factor to the *nifH* promoter.

Materials and Methods

Strains and plasmids

The strains and plasmids used in this study are listed in **Table 1**.

DNA manipulation

DNA isolation, restriction analysis, ligation and transformation were all performed according to the methods described by Sambrook *et al.* [15].

Construction of plasmid pNTRC230

To construct the plasmid carrying the constitutively expressed *ntrC* of *E. coli*, we designed specific primers according to the *E. coli ntrC* gene sequence (forward 5′-CTCGAGAATAATCAATCTTTACACACAAGC-3′ and reverse 5′-CTCGACTCGGTTTACCTGCCTATCAG-3′). These primers were used to amplify the *ntrC* gene, including the Shine-Delgarno (SD) sequence, using pGln53Y DNA as the template. The amplified fragment was cloned into the *Xma*I site of plasmid pKT230 to yield pNTRC230, encoding the *ntrC* gene driven by the kanamycin-resistant phosphoacetyltransferase gene promoter.

β-Galactosidase activity assay

Bacteria transformed by various recombinant plasmids were grown aerobically for 24 h at 28 °C in nitrogen-free minimal medium supplemented with 0.01% casein hydrolysate, 1 mg/ml vitamin B_1, 20 mg/ml glutamine and appropriate antibiotics. Bacterial cells were pelleted, washed

Table 1 Strains and plasmids used in this study

Strain/plasmid	Relevant characteristics	Source or reference
E. coli YMC9	*endA1 thi-1 hsdR17 supE44 ΔlacU169*	[14]
E. coli YMC12	*endA1 thi-1 hsdR17 supE44 ΔlacU169 glnG::Tn5*	[14]
E. coli DH5α	*supE44 ΔlacU169 (Φ80 lacZ ΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	[15]
pGD926	*ter*^{R}, *IncP*, *lacZ* fusion vector	[16]
pMB210	*Sm nifHp-lacZ* fusion in pGD926	[13]
pPW926	*Ec nifLAp-lacZ* fusion in pGD926	[12]
pDO531	*Kp nifLAp-lacZ* fusion in pRK248	[17]
pMK90	*E. coli gyrA* cloned in pKC16	[18]
pMK47	*E. coli gyrB* cloned in pKC16	[18]
pXD1	*Kp nifA* constitutively expressed under *kan*^{R} promoter in pKT230	[19]
pNTRC230	*E. coli ntrC* constitutively expressed under *kan*^{R} promoter in pKT230	This study
pGln53Y	*amp*^{R}, *ter*^{R}, derivative of pGln53	[17]
pKT230	*kan*^{R}, *str*^{R}	[20]

These strains and plasmids were kept in our laboratory. *Sm*, *Sinorhizobium meliloti*; *Ec*, *Enterobacter cloacae*; *Kp*, *Klebsiella pneumoniae*.

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and resuspended in the same medium, and then grown under aerobic or anaerobic (flashing with nitrogen) conditions for 8 h at 28 °C. β-Galactosidase activity was assayed as described by Miller [21].

Electrophoretic analysis of supercoiled plasmid DNA

Supercoiled plasmid DNA samples were separated according to their superhelical densities, using the method previously described [22]. Briefly, the pUC18 plasmid DNA was purified from *E. coli* strain YMC9 grown anaerobically in LB medium with or without the gyrase inhibitor, novobiocin [15]. The purified DNA samples were subjected to electrophoresis on a horizontal slab gel consisting of 1.5% agarose and 15 μg/ml chloroquine. Electrophoresis was performed for 12 h at 25 °C with a voltage gradient of 3 V/cm. The gel was stained with ethidium bromide (1 μg/ml) and photographed under UV transillumination at 302 nm.

Results

Negative DNA supercoiling is required for *S. meliloti nifH* promoter activity

To confirm whether DNA supercoiling regulates the activity of the *nif* gene promoter, we analyzed the activity of the *Sm nifH* promoter in *E. coli* in the presence and absence of gyrase, and compared the results with those obtained using the *Kp nifLA* and *Ec nifLA* promoters. A plasmid carrying the *Sm nifHp-lacZ* (pMB210), *Kp nifLAp-lacZ* (pDO531) or *Ec nifLAp-lacZ* (pPW926) fusion gene was introduced into the *gyr*$^+$ strain YMC9 or *gyrA*$^-$ strain DH5α separately. We then analyzed the relative promoter activity in different transformants by measuring the relative β-galactosidase activities under anaerobic and aerobic conditions.

We found that, in strain YMC9 *Sm nifH* promoter activity was not affected by oxygen content, whereas the *Kp nifLA* and *Ec nifLA* promoters were inhibited under aerobic conditions. In the *gyrA*$^-$ strain DH5α, the activity of all tested promoters was lower than their expression in *gyrA*$^+$ strain YMC9 (**Table 2**). The introduction of a plasmid carrying constitutively expressed *gyrA* into DH5α cells containing each of the constructs restored the β-galactosidase activity in each case. However, introduction of a *gyrB*-expressing clone did not have the same effect. As gyrase increases the negative superhelical density, our results indicate that *Sm nifH* promoter activity requires a certain level of negative supercoiling, although its expression is not affected by oxygen content.

DNA gyrase inhibitors decrease *Sm nifH* promoter activity

To confirm that *Sm nifH* promoter activity requires a certain level of negative supercoiling, we evaluated the sensitivity of the *Sm nifH*, *Kp nifLA* and *Ec nifLA* promoters to gyrase inhibitors. Expression of the promoter-

Table 2 Expression of *Sm nifHp-lacZ*, *Ec nifLAp-lacZ* and *Kp nifLAp-lacZ* translation fusions in *E. coli*

Strain/plasmid [a]	Relative characteristics	β-Galactosidase activity [b]	
		$+O_2$	$-O_2$
E. coli YMC9/pGD926	promoterless *lacZ*	2	3
E. coli YMC9/pMB210	*gyrA*$^+$*/Sm nifHp-lacZ*	648	638
E. coli DH5α/pMB210	*gyrA96/Sm nifHp-lacZ*	118	117
E. coli DH5α/pMB210 pMK47	*gyrA96/Sm nifHp-lacZ gyrB* [c]	124	113
E. coli DH5α/pMB210 pMK90	*gyrA96/Sm nifHp-lacZ gyrA* [c]	621	595
E. coli YMC9/pPW926	*gyrA*$^+$*/Ec nifLAp-lacZ*	127	1296
E. coli DH5α/pPW926	*gyrA96/Ec nifLAp-lacZ*	3	15
E. coli DH5α/pPW926 pMK47	*gyrA96/Ec nifLAp-lacZ gyrB* [c]	5	12
E. coli DH5α/pPW926 pMK90	*gyrA96/Ec nifLAp-lacZ gyrA* [c]	74	854
E. coli YMC9/pDO531	*gyrA*$^+$*/Kp nifLAp-lacZ*	65	268
E. coli DH5α/pDO531	*gyrA96/Kp nifLAp-lacZ*	10	15
E. coli DH5α/pDO531 pMK47	*gyrA96/Kp nifLAp-lacZ gyrB* [c]	12	10
E. coli DH5α/pDO531 pMK90	*gyrA96/Kp nifLAp-lacZ gyrA* [c]	48	240

[a] bacteria were cultured in basic media; [b] unit, Miller; [c] constitutively expressed.

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Table 3 Effect of courmermycin and novobiocin on the expression of *Sm nifHp-lacZ*, *Kp nifLAp-lacZ* and *Ec nifLAp-lacZ* fusion genes

Strain/plasmid	Fusion gene	DNA gyrase inhibitor	β-Galactosidase activity [a]	
			$+O_2$	$-O_2$
E. coli YMC9/pMB210	*Sm nifHp-lacZ*	No inhibitor	648	638
		Courmermycin A1 [b]	358	433
		Novobiocin [c]	305	311
E. coli YMC9/pPW926	*Kp nifLAp-lacZ*	No inhibitor	169	1122
		Courmermycin A1 [b]	81	278
		Novobiocin [c]	83	167
E. coli YMC9/pDO531	*Ec nifLAp-lacZ*	No inhibitor	68	228
		Courmermycin A1 [b]	61	68
		Novobiocin [c]	48	46

[a] unit, Miller; [b] 50 μg/ml of courmermycin; [c] 800 μg/ml of novobiocin.

lacZ fusion gene in bacteria cultured in nitrogen-free medium containing a gyrase inhibitor (novobiocin or courmermycin) revealed that the activity of all tested promoters was inhibited by the gyrase inhibitors. The *Sm nifH* promoter activity was reduced by a smaller extent than that of the *Kp nifLA* or *Ec nifLA* promoters (**Table 3**). Normally, the bacteria grow in 800 μg/ml of novobiocin. Under these conditions, the *nifLA* promoter activity was only 10% of the control activity, while the *nifH* promoter activity was 50% of the control activity.

We assayed the DNA superhelicity following the addition of novobiocin by using agarose gel electrophoresis in the presence of chloroquine. The results show that DNA superhelical density is reduced by novobiocin treatment, the degree of reduction being dependent on the dose (**Fig. 1**). In addition, anaerobically activated *Kp* or *Ec nifLA* promoters were found to be more sensitive to DNA supercoiling than the oxygen-insensitive *Sm nifH* promoter (**Fig. 2**).

Collectively, these results provide evidence that DNA supercoiling is required for the activity of both *nifH* and *nifLA* promoters, and indicate that sensitivity to DNA supercoiling differs among various *nif* gene promoters.

NtrC-mediated activation of the *Sm nifH* promoter is more sensitive to DNA supercoiling than NifA-mediated activation

NifA and NtrC are critical for regulating biological nitrogen fixation. Previous work has shown that the *Sm nifH* promoter can be activated by *E. coli* NtrC and *K. pneumoniae* NifA. To test the effect of DNA supercoiling on *Sm nifH* promoter activity that was activated by *nifA*

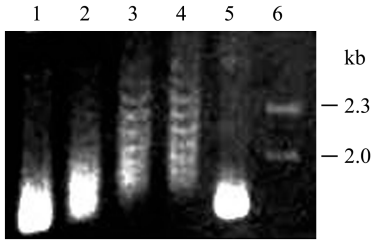

Fig. 1 Reduction of DNA superhelical density by using the DNA gyrase inhibitor, novobiocin

Samples were prepared as described in the "Materials and Methods". 1–4, novobiocin treatment at 0, 50, 100 and 250 μg/ml, respectively, under anaerobic conditions; 5, novobiocin non-treatment and aerobic conditions; 6, Lambda/*Hin*dIII DNA marker.

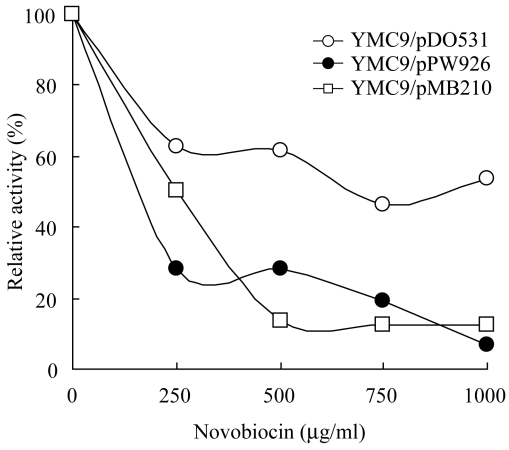

Fig. 2 Effect of novobiocin on *nif* gene promoter activity

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or *ntrC*, we introduced plasmids carrying constitutively expressed *nifA* (pXD1) or *ntrC* (pNTRC230) into *ntrC*⁻ strain YMC12 harboring a plasmid carrying the *Sm nifHp-lacZ* fusion gene, pMB210. We then measured the *nifH* promoter activity in the presence and absence of novobiocin. The NifA-stimulated *nifH* promoter activity was unchanged in the presence of 500 μg/ml novobiocin, but decreased slightly in the presence of 750 μg/ml novobiocin (**Fig. 3**). In contrast, the NtrC-stimulated *nifH* promoter activity decreased to 60% of the control level in the presence of novobiocin with concentrations as low as 250 μg/ml (**Fig. 3**). These results show that NtrC-mediated activation of the *nifH* promoter is more sensitive to supercoiling changes than NifA-mediated activation, and indicate that the various *trans*-acting factors of the *nifH* promoter have different requirements for DNA supercoiling conformation.

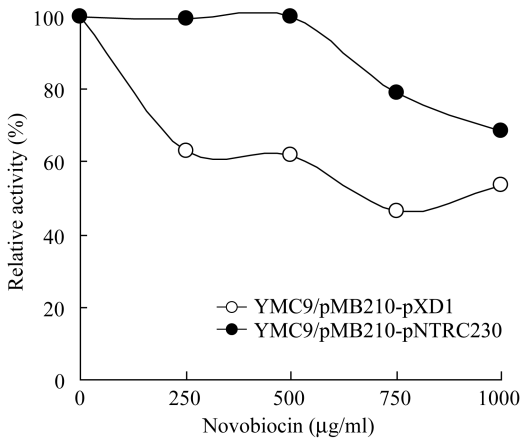

Fig. 3 Effect of novobiocin on NtrC- or NifA-mediated activation of the *Sm nifH* promoter

Discussion

Here, we will compare the effect of gyrase on the activity of the *nifH* and *nifLA* promoters. We found that both the oxygen-insensitive *nifH* promoter and the anaerobically induced *nifLA* promoter required DNA supercoiling for optimal expression of the *nifH* and *nifLA* genes. These results suggest that promoter activities may thus require a certain level of DNA supercoiling, regardless of their regulation by oxygen content. We further observed that the DNA negative superhelical density in the presence of novobiocin (250 μg/ml) and under anaerobic conditions was lower than that under aerobic conditions without novobiocin (**Fig. 1**), indicating that the superhelical density under aerobic conditions may be sufficient to trigger *nifH* expression. The further decrease of DNA superhelicity following the addition of novobiocin led to an observable decrease in *Sm nifH* promoter activity, which suggests that, in this case, promoter sensitivity to supercoiling maybe base on oxygen-independent changes in DNA topology.

Our data suggest that the sensitivity of a promoter to DNA supercoiling is dependent on *cis*-elements and *trans*-acting factors associated with the promoter. Our analysis of the anaerobic regulation mechanisms of the *Enterobacter cloacae nifLA* promoter revealed that an unknown *trans*-acting factor could bind the FNR-binding site, leading to activation of *Ec nifLA* expression under anaerobic conditions, and that both an FNR-like *trans*-acting factor and supercoiled conformations are necessary for *Ec nifLA* promoter expression under anaerobic conditions [12]. Furthermore, *Ec nifLA* promoter activity under anaerobic conditions and in the presence of 250 μg/ml novobiocin was 50% (**Fig. 2**). In contrast, *Kp nifLA* promoter activity under anaerobic conditions and in the presence of 250 μg/ml novobiocin was only 30% of the control level (**Fig. 2**), indicating that the *Kp nifLA* promoter is more sensitive to changes in DNA supercoiling compared to the *Ec nifLA* and *Sm nifH* promoters. Collectively, these results indicate that different oxygen-controlled regulation mechanisms affect the *Kp nifLA* and *Ec nifLA* promoters.

Finally, we observed that the *Sm nifH* promoter shows differential sensitivity to changes in supercoiling when activated by different *trans*-acting factors. DNA supercoiling seems to directly facilitate gene transcription from the tested promoters, indicating the possible importance of a specific topological conformation during promoter activation. Therefore, these novel data indicate that DNA supercoiling may not only transmit oxygen signals, but also provide an oxygen-independent favorable topography for initiation of *nif* gene expression.

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