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Expression Analysis of Genes in the *Nif* Cluster of *Clostridium beijerinckii*

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Abstract: The *nif* genes of *Clostridium beijerinckii* NRRL B593 occupy a region of about 16 kilobases. Besides the two *glnB*-like genes, five other genes are interspersed between the *nifNB* and the *nifVω* genes. An expression analysis of the *nif* genes in nitrogen-fixing and non-nitrogen-fixing cells with probes generated from various regions of the *nif* cluster by northern blot analysis revealed the presence of four different transcripts in nitrogen-fixing cells. Two of these transcripts had the predicted sizes spanning from *nifH* to *nifK* and from *nifVω* to *nifVα*, respectively. The other two transcripts did not have the expected sizes, but they suggested the presence of two other polycistronic mRNAs consisting of *nifE-nifNB* and *nirJ1-nirJ2-nirD-nirH*, respectively. The absence of the *nif* and *nif*-associated mRNAs in RNA samples from non-nitrogen-fixing cells indicated that *nif*-associated genes are regulated in parallel to *nif* genes.

Key Words: Nitrogen fixation, transcriptional analysis, *Clostridium beijerinckii*, northern blotting, *nif* cluster

Clostridium beijerinckii'da Bulunan *Nif* Genlerinin Ekspresyon Analizi

Özet: *Clostridium beijerinckii* NRRL B593'de bulunan *nif* genleri 16 kilobaz çiflik bir bölgeyi kaplamaktadır. Bilinen iki *glnB* benzeri genlerin yanısıra, beş adet farklı gen *nifNB* ve *nifVω* arasını işgal eder. Bu calışmada azot fiksasyonu yapan ve yapmayan iki farklı *C. beijerinckii* kültür tipinde *nif* gen grubundan düzenlenen problarla yapılan transkripsiyon analizinde dört farklı transkripsiyonun azot fikse eden kültürlerde varlığı northern blot analizi ile gösterildi. Bu transkriptlerden ikisi beklenen büyüklükte olup *nifH* geninden *nifK* genine ve *nifVω* geninden *nifVα* genine kadar olan bölgeyi içerdiler. Diğer iki transkrip beklenilen büyüklükte olmamasına rağmen bize *nifE-nifNB* ve *nirJ1-nirJ2- nirD-nirH* bölgelerini kapsayan iki ayrı polisistronik mRNA'nın varlığını gösterdi. *nif* ve *nif* bağlantılı genlerin mRNA'larının azot fikse etmeyen örneklerde bulunmayışı *nif* bağlantılı genlerin *nif* genleri ile beraber regüle edildiğinin belirtecidir.

Anahtar Sözcükler: Azot fiksasyonu, transkripsiyonel analiz, *Clostridium beijerinckii*, northern blotlama, *nif* gen grubu

The reduction of N_2 to ammonia is a trait widely distributed among representatives of the eubacteria and methanogenic archaea (1). For those bacteria and methanogenic archaea that are able to grow diazotrophically, the fixation of atmospheric nitrogen can be a major route of nitrogen assimilation. However, due to the high energy and reductant requirement for the process of nitrogen fixation, the nitrogen-fixing activity of diazotrophs is regulated in response to both the redox and nitrogen status of the cell, so that nitrogen fixation occurs only when it is both favorable and necessary (2). The primary mode of regulation of nitrogen fixation is by control of transcription of the *nif* genes. Some organisms also regulate the activity of the nitrogenase enzyme at a posttranslational level in response to ammonia and other fixed nitrogen sources (3).

In eubacteria and methanogens, the organization and expression of the *nif* genes have been described for several species (4). The Mo-nitrogenase structural genes *nifH, nifD* and *nifK* are typically found together in a single operon and are physically adjacent to other *nif* or *nif*-associated genes as part of a larger *nif* regulon. The genes *nifD* and *nifK* encode the subunits of the molybdenum-iron (MoFe) protein or dinitrogenase, an α_2b_2 heterotetramer. The *nifH* gene codes for the iron protein or dinitrogenase reductase. Downstream of *nifK*, the genes *nifE, niN* and *nifV* are often found in separate operons. The genes *nifE* and *nifN* encode subunits of a

scaffold structure upon which the essential iron-molybdenum cofactor (FeMoCo) for nitrogenase is assembled, and the gene *nifV* encodes homocitrate synthase, which catalyzes the synthesis of homocitrate, the organic component of the FeMoCo (5).

In *C. pasteurianum*, three consecutive groups of *nif* genes are present. The first group consists of structural genes (*nifH1, nifD* and *nifK*) for Mo-nitrogenase. The second group contains *nifE* and the fused *nifNB* genes, and the third group contains the split *nifVω* and *nifVα* genes for FeMo biosynthesis. There are two intervening open reading frames (*modA* and *modB*) present between *nifNB* and *nif Vω,* and their protein products are possibly involved in molybdate transport (5). In *C. acetobutylicum*, the *nif* cluster consists of nine genes (6): *nifH, nifD* and *nifK* for nitrogenase polypeptides; *nifE, nifN-B, nifVω* and *nifVα* for the synthesis of the FeMoCo; and $nifI_1$and $nifI_2$ for GlnB-like proteins that may be necessary for regulation of nitrogen-fixing activity (7). In *C. beijerinckii* NRRL B593, in addition to $nifI_1$ and $nifI_2$, four *nir* genes (*nirJ1 J2 H D*) and the *fdxA* gene are present in the *nif* cluster (Figure 1). *nir* genes may have a role in synthesis of heme d_1 (8), and *fdxA* encodes a protein similar to *C. pasteurianum* 2Fe:2S ferredoxin that may have a role in regulation of nitrogen fixation (9).

The nitrogen-fixing activity of *C. beijerinckii*, a potential candidate for industrial production of *n*-butanol and isopropanol, was reported in 1949 (10). An analysis of the *nif* genes of *C. beijerinckii* may be useful for the realization of the biotechnological potential of this organism. In this study, an expression analysis of the *nif* genes was performed in nitrogen-fixing and non-nitrogen-fixing cells with probes generated from various regions of the *nif* cluster. HRP-labelled DNA probes, specific for *nifH,* glnB-like 2-*nifD, nifE, nifNB, fdxA, nirJ1, nirJ1-nirJ2, nirD* and *nifVω-nifVα*, were used in northern blots to investigate gene expression. The relative locations of these probes are shown in Figure 1 and their corresponding sequences are given in Table 1. For northern blot analysis, total RNA was isolated both from nitrogen-fixing and non-nitrogen-fixing cells and six mg of the total RNA were resolved on a 0.7% formaldehyde-agarose gel (11,12). The RNA species were then transferred to positively charged nylon membranes, and the membranes were incubated with 100 ng of HRP-labelled DNA probes. The sizes of RNA fragments were estimated by using two different RNA ladders (New England Biolabs, Inc., Gibco BRL Life Tech.). The probes for analysis of the *nif* transcripts were generated by PCR using the sequences in the *nif* cluster of *C. beijerinckii*. PCR amplification of the gene fragments was performed in a 50 mL reaction volume as described (13).

The transcripts of the *nif* and *nif*-associated genes were only detected in the RNA samples isolated from nitrogen-fixing cells, with the exception of *nifH*, which can also be detected in non-nitrogen-fixing cells. Hybridizations with *nifH* and *glnB*-like 2-*nifD* probes (probe 1 and 2 of Figure 1) generated a distinct band with an estimated size of 4.9 kb (Figure 2A). The presence of a major transcript of 4.9 kb suggests that *nifH, glnB*-like 1, *glnB*-like 2, *nifD* and *nifK* are expressed

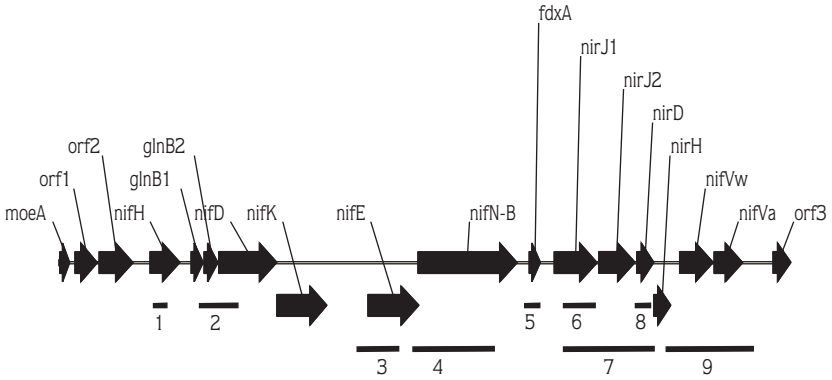

Figure 1. Schematic representation of a 19-kb region of *C. beijerinckii* NRRL B593 chromosome containing the *nif* and *nif*-associated genes. The ORFs are shown as filled thick arrows. The locations of the probes (1 through 9) used for transcriptional analysis of the *nif* cluster are shown as solid black lines underneath the ORFs.

Table 1. Sequences of the primer pairs used to generate probes from the *nif* cluster of *C. beijerinckii* NRRL B593 for analysis of the *nif* transcripts. The NCBI sequence accession number for the shown 19-kb region is AF266462.

Location of the first base	Designed from the sequence of	Sequence
2557	*nifH*	5'-GGWTGTGAYCCWAAGGCWG
2916	*nifH*	5'-AKWGCCATCATYTCWCC
3810	*glnB-like* 2	5'-AACTGGAGAAAAAGGTGC
4852	*nifD*	5'-GTTCTGCTGATTGAGATAC
8096	*nifE*	5'-GGCTTTGCCACAATACGGAAC
9223	*nifE*	5'-ATCCTCAAGCCCCACAAAAC
9617	*nifNB*	5'-TGGGAGTTGCCTTTTGTG
11830	*nifNB*	5'-GGGCTTTTTTTGTTACTTCCTC
12657	*fdxA*	5'-GAAGAAATGCCTACAGCC
13064	*fdxA*	5'-TACTACTTTTCCGCCTTC
13707	*nirJ1*	5'-GGGCATTATTGTTTTCTGGAG
14575	*nirJ1*	5'-GGATGATTTTTGGGCAGAGG
13707	*nirJ1*	5'-GGGCATTATTGTTTTCTGGAG
16177	*nirJ1*	5'-GGGTCACTAATCATTTGTATCATCC
15675	*nirD*	5'-CATTCCAAAACTTTATGCGG
16082	*nirD*	5'-AACTCATTATTTCTTCCAAACCAG
16505	*nifVω*	5'AACTGCAGCACGAGAAAGATAAGGAAAG
18884	*nifVα*	5'-AAGGATCCCCCACGCAATAAAATAAG

as a unit. The length of the 4.9-kb band correlates closely with the predicted length of the transcript extending from *nifH* to *nifK*. The transcriptional linkage of the *glnB*-like genes with the nitrogenase structural genes may imply a role for the *glnB* genes in the regulation of nitrogen fixation in *C. beijerinckii*.

When total RNA from the nitrogen-fixing cells was tested with a *nifVω* - *nifVα* probe (probe 9 in Figure 1), a major band of 1.7 kb was detected (Figure 2B), suggesting that *nifVω* and *nifVα* are also contained on a single message. The size of 1.7 kb also correlates closely to the predicted length of a transcript extending from *nifVω* to *nifVα.*

A clear band of 2.5 kb was detected when a 2.5 kb probe (probe 4 in Figure 1) covering part of *nifN-B* gene was used (Figure 2C). It is possible that the 2.5-kb message only contains the *nif N-B* mRNA. If this were the case, then one would expect to detect another transcript when *nifE* was used as the probe in northern hybridization. However, efforts to detect the *nifE* message failed when a DNA probe (probe 3 in Figure 1) covering part of the *nifE* gene was used. Therefore, it is possible that the 2.7-kb signal arose from degradation of a larger transcript, which extends from *nifE* to *nifNB*, and the *nifE* region was more susceptible to nuclease action.

To examine the transcription of *nif*-associated genes in the *nif* cluster, DNA probes generated from *fdxA* (probe 5 in Figure 1), *nirJ1* (probe 6 in Figure 1) and *nirD* (probe 8 in Figure 1) genes were used. These probes failed to detect a transcript in RNA samples isolated from either nitrogen-fixing or non-nitrogen-fixing cells.

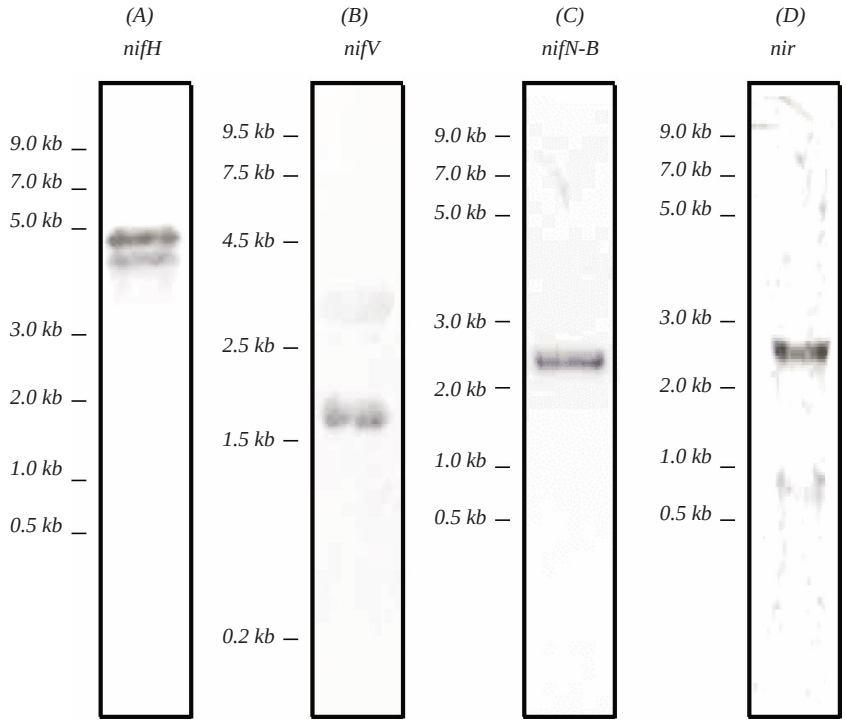

Figure 2. Northern blot analysis of the nif mRNA from nitrogen-fixing cells of *C. beijerinckii* NRRL B593.

Therefore, a larger probe (2.5 kb) covering *nirJ2,* most of *nirJ1* and part of *nirD* (probe 7 in Figure 1) genes was used in northern blot analysis. The blot revealed a weak but distinct 2.5 kb signal only in the RNA samples isolated from nitrogen-fixing cells (Figure 2D). The size of the signal (2.5 kb) was smaller than the predicted size (3.2 kb). One might argue that the 2.5 kb signal might arise from degradation of mRNA during isolation. However, defined mRNA bands of expected sizes were detected when the same RNA preparations were characterized with other DNA probes. Therefore, the smaller transcripts might not be due to the degradation of mRNA during isolation. Partial degradation of a transcript, which may have a short half-life, may generate hybridizing bands of a smaller size on the blots. Therefore, it may be speculated that the transcripts of *nir* genes are relatively unstable and can be degraded shortly after translation. By examining the organization of the *nif* cluster and the spacing between genes, it is possible to predict that the *nir* genes may be on another polycistronic mRNA. However, the *fdxA* gene can be cotranscribed either with the *nir* genes or the *nifE* and *nifNB* genes or be transcribed by itself. The absence of this signal in RNA samples isolated from non-nitrogen-fixing cells suggested that *fdxA* and *nir* genes may only be expressed under nitrogen-fixing conditions. In addition, the absence of the signal in RNA samples isolated from cells grown in ammonia-supplemented nitrogen-fixing medium indicated that *nir* expression might be regulated in parallel with the *nif* genes.

With the completion of the genome sequence of *C. beijerinckii* NCIMB 8052 (http://genome.ornl.gov/microbial/cbei/, sequences can be found in contig 149), we were able to see a striking conservation of organization of the *nif* operons between *C. beijerinckii* NRRL B593 and *C. beijerinckii* NCIMB 8052. Whether such conservation implies a similar transcriptional regulation in both species is a question of investigation.

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