Microbiology, Vol. 70, No. 1, 2001, pp. 73–78. Translated from Mikrobiologiya, Vol. 70, No. 1, 2001, pp. 86–91.

EXPERIMENTAL ARTICLES

A System of Oligonucleotide Primers for the Amplification of *nifH* Genes of Different Taxonomic Groups of Prokaryotes

A. I. Marusina*, E. S. Boulygina, B. B. Kuznetsov**, [1], T. P. Tourova***, I. K. Kravchenko***, and V. F. Gal'chenko*****

**Moscow Physicotechnical Institute, Institutskii proezd 9, Dolgoprudnyi, Moscow oblast, 141700 Russia*

***Bioengineering Center, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 1, Moscow, 117811 Russia*

****Institute of Microbiology, Russian Academy of Sciences, pr. 60-letiya Oktyabrya 7, k. 2, Moscow, 117811 Russia*

Received July 11, 2000

Abstract—Based on the analysis of the *nifH* gene nucleotide sequences from GenBank, a system of primers was developed that makes it possible to obtain 370- and 470-bp PCR fragments of the *nifH* gene of nitrogen-fixing bacteria and archaea. The effectiveness of the proposed system for revealing the presence of *nifH* genes was demonstrated by PCR on the DNA isolated from nitrogen-fixing prokaryotes for which the primary structure of these genes is known and which belong to different taxonomic groups. *nifH* sequences of nitrogen-fixing prokaryotes of the genera *Xanthobacter, Beijerinckia*, and *Methanosarcina*, for which the capacity for nitrogen fixation was demonstrated earlier, but no data existed on the nucleotide composition of these genes, were determined and deposited in GenBank.

Key words: nitrogen fixation, nitrogenase genes, *nifH*, PCR, oligonucleotide primers.

Modern trends of the development of microbiology include, along with the improvement of the conventional methods, an active use of molecular biological approaches to the study of microbiological processes and molecular diversity. In modern microbial ecology, the most popular is the approach based on determining 16S rRNA nucleotide sequences of the prokaryotes constituting the natural community studied [1]. At present, attention is focused on so-called functional genes, whose analysis allows the assessment of the diversity of microorganisms that perform certain ecological functions in an ecosystem. Among these genes, an important role is played by the nitrogenase complex, which is responsible for the transformation of molecular nitrogen into mineral and organic compounds available to microorganisms and plants.

Despite the great ecological and practical significance of the process of nitrogen fixation, the problems connected with quantitative estimates (dynamics of the number and activity of nitrogen fixers, the ratio of their actively metabolizing and resting forms), as well as the biodiversity of nitrogen-fixing prokaryotes in natural communities, are so far poorly understood. The results of the application of the conventional methods based on the use of selective nitrogen-free nutritive media reflect the potentials of the community, but not the activity in situ. A considerable part of the diazotrophs studied depend on starting nitrogen for biomass synthesis and, hence, they remain unaccounted for when nitrogen-free media are used.

The enzymatic nitrogenase complex consists of two subunits: a FeMo-protein encoded by *nifD* and *nifK* and an Fe-protein encoded by *nifH*. The *nifH* gene is one of the most rigorously conserved functional genes and the phylogeny of prokaryotes based on the analysis of this gene sequences agrees well with the data obtained when analyzing 16S rRNA [3, 4]. This allowed the molecular-biological approaches based on the analysis of the *nifH* gene sequences to be applied to the studies of biodiversity of both nitrogen-fixing isolates and the whole natural microbial community [5, 6].

To determine *nifH* gene nucleotide sequences, the oligonucleotide primers which make it possible to amplify fragments of these genes sufficient for identifying microorganisms have been proposed earlier. However, there are certain difficulties connected with the successful use of such primers. First, the wide phylogenetic diversity of nitrogen-fixing microorganisms results in considerable variability of even such con-

[1]Corresponding author (e-mail: borisk@biengi.ac.ru).

0026-2617/01/7001-0073$25.00

Table 1. Bacterial strains used in this work

Organism	Strain	Source
Azotobacter chroococcum	126	Dep. Microbiol., Moscow State Univ.
Azotobacter vinelandii	59	The same
Sinorhizobium meliloti	201	″
Rhizobium trifolii	199	″
Xanthobacter autotrophicus	7c (DSMZ 432)	Inst. Microbiol., Russ. Acad. Sci.
Beijerinckia indica subsp. *indica*	KS1-7 (ATCC 21423)	The same
Nostoc (Anabaena) sp.	PCC 7120 (ATCC 27893)	″
Anabaena variabilis Kitzing	IUCC 1444 (ATCC 29413)	Dep. Microbiol., Moscow State Univ.
Methanosarcina lacustera		Inst. Microbiol., Russ. Acad. Sci.
Escherichia coli	DH5α	Bioengineering Center, Russ. Acad. Sci.
Bacillus thuringiensis	696	The same

Note: DSMZ denotes Deutsche Sammlung von Mikroorganismen und Zellkulturen Braunschweig, Germany; ATCC, American Type Culture Collection, Manassas, USA.

Table 2. Taxonomic diversity of microorganisms used when designing primers specific to the *nifH* gene

Phylogenetic groups of microorganisms	Representatives of the genera
Green sulfur bacteria	*Chlorobium*
Gram-positive bacteria with a low G+C content	*Bacillus, Clostridium, Paenibacillus*
Gram-positive bacteria with a high G+C content	*Frankia*
Cyanobacteria	*Calothrix, Chlorogloeopsis, Cyanothece, Dermocarpa, Fischerella, Gloeothece, Lyngbya, Microcoleus, Myxosarcina, Phormidium, Plectonema, Pseudanabaena, Scytonema, Synechococcus, Anabaena, Trichodesmium, Nostoc, Xenococcus*
Proteobacteria: α-subgroup	*Acetobacter, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Rhizobium, Rhodobacter, Rhodospirillum, Azospirillum*
Proteobacteria: β-subgroup	*Alcaligenes, Herbaspirillum, Azoarcus*
Proteobacteria: γ-subgroup	*Azotobacter, Klebsiella, Vibrio, Marichromatium, Pseudomonas, Thiobacillus*
Proteobacteria: σ-subgroup	*Desulfobacter, Desulfovibrio, Desulfonema*
Archaea	*Methanobacterium, Methanococcus, Methanosarcina, Methanobrevibacter*

served genes as *nifH*. That is why the set of conserved sites of these genes suitable for constructing oligonucleotide primers is rather limited. Second, the high degree of degeneracy of the *nifH* gene nucleotide sequences virtually rules out the possibility of the existence of sufficiently extended invariant sequence sites. Therefore, most of the currently existing oligonucleotide primers were designed to amplify *nifH* genes of individual taxonomic groups of prokaryotes or even for individual microorganisms, particularly cyanobacteria [7–10]. Apart from this, the pool of the *nifH* nucleotide sequences available from GenBank is relatively small (in comparison with the number of available 16S rRNA sequences). This also hinders the development of primers that are sufficiently universal.

The aim of this work was to design a system of primers allowing amplification of *nifH* gene fragments from as wide a spectrum of prokaryotes as possible.

MATERIALS AND METHODS

Microorganisms and cultivation conditions. The microorganisms used in this work and the sources from which they were obtained are presented in Table 1.

Fig. 1. Nucleotide sequences of oligonucleotide primers. Abbreviations used: y = T, C; r = A, G; m = A, C; k = T, G; w = A, T; s = G, C; b = T, G, C; v = A, G, C; d = A, T, G; h = A, T, C; n = G, A, T, C. * Numbering is by the nucleotide sequence of *Azotobacter vinelandii* M20568 *nifH* gene.

The cultures of *Nostoc* and *Anabaena* were grown on BG11 mineral medium for freshwater algae [11] at room temperature (20–23°C) at an illumination of 50 klx for 12 h. *Xanthobacter* and *Beijerinckia* were grown on Plate Count Agar (Difco, USA), *Rhizobium* and *Sinorhizobium* were grown on mineral medium with mannitol and yeast extract [12]; and *Azotobacter* was grown on agarized Ashby's nitrogen-free medium with mannitol [13].

The design of primers. *nifH* gene nucleotide sequences of prokaryotes belonging to different taxonomic groups were searched for in the international GenBank database. Sequence alignment procedures were carried out using the Internet and the Basic Genebee Clustal W1.75 program (http://www.genebee.msu.su/clustal).

DNA isolation. DNA was isolated according to the Miniprep protocol (Promega, USA) with slight modifications. The DNA concentration in the preparations obtained was 5–7 μg/ml. RNA was present in the preparations in trace amounts (less than 1%, according to electrophoretic data).

Amplification, isolation, and cloning of *nifH* fragments. Amplification was carried out using the primer pairs designed by us (F1–R6, F2–R6) on a Cetus 480 device (Perkin-Elmer, Sweden) using the thermostable polymerase BioTaq (Dialat LTD, Moscow) according to the recommendations of the manufacturer. The reaction mixture volume was 20 μl. The temperature–time PCR profile was as follows. The 1st cycle: 3 min at 94°C, 3 min at 50°C, and 3 min at 72°C; the subsequent five cycles: 30 s at 94°C, 2 min at 50°C, and 30 s at 72°C; the last 30 cycles: 30 s at 94°C, 30 s at 40°C, and 30 s at 72°C; the final polymerization: 7 min at 72°C. PCR products were analyzed by electrophoresis in 1% agarose gel. The bands were visualized in UV light after staining the gels with ethidium bromide, and the result was photographed using a BioKom (Moscow) transilluminator. Fragments were isolated and purified from low gelling temperature agarose using the Wizard PCR Preps kit (Promega) according to the manufacturer's recommendations. The fragments were cloned in pGEM-3Zf(+) vector in *E. coli* DH5α cells.

Sequencing of cloned fragments. Sequencing was performed using Sanger's Silver Sequencing method (Promega, USA) [14] according to the manufacturer's recommendations with slight modifications. Electrophoresis was carried out using the Macrophore (Pharmacia, Sweden) and SQ3 Sequencer (Hoefer, USA) devices, with 0.19 mm thick polyacrylamide gel. The sequencing used both the universal plasmid primers (SP6 and T7) and the primers designed by us.

Depositing nucleotide sequences. The nucleotide sequences of the cloned *nifH* gene fragments obtained during this work were deposited in the GenBank database under the accession numbers AF296348–AF296358.

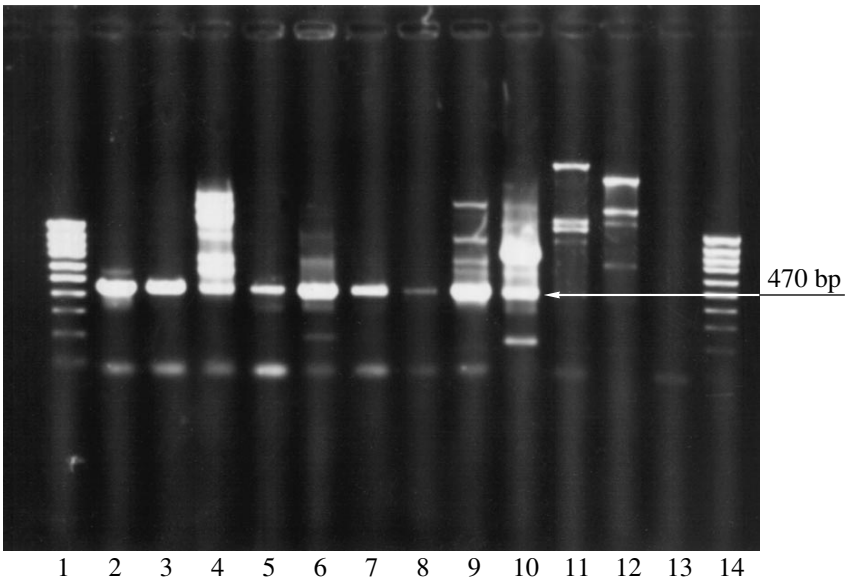

Fig. 2. Electrophoretic analysis of the products of PCR on the DNA preparations from different nitrogen-fixers using the F1–R6 primers (the size of the expected PCR product is 470 bp). Lanes: (1) the DNA molecular mass marker GeneRuler 100 bp (Fermentas); (2) *Sinorhizobium meliloti*; (3) *Rhizobium trifolii*; (4) *Azotobacter vinelandii*; (5) *Azotobacter chroococcum*; (6) *Nostoc (Anabaena)* sp.; (7) *Anabaena variabilis*; (8) *Xanthobacter autotrophicus*; (9) *Beijerinckia indica* subsp. *indica*; (10) *Methanosarcina lacustera*; (11) *Escherichia coli*; (12) *Bacillus thuringiensis*; (13) control PCR in the absence of the template; (14) the DNA molecular mass marker GeneRuler 100 bp (Fermentas).

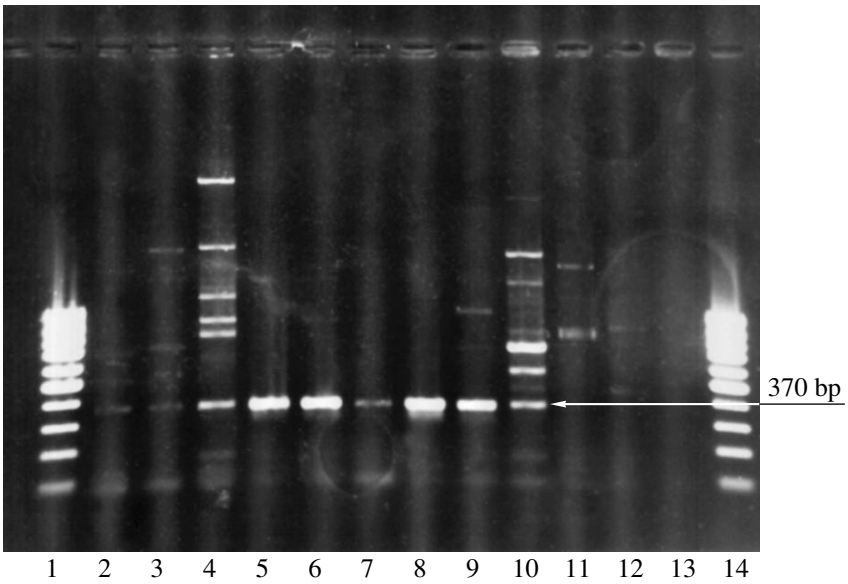

Fig. 3. Electrophoretic analysis of the products of PCR on the DNA preparations from different nitrogen-fixers using the F2–R6 primers (the size of the expected PCR product is 370 bp). Lanes: (1) the DNA molecular mass marker GeneRuler 100 bp (Fermentas); (2) *Sinorhizobium meliloti*; (3) *Rhizobium trifolii*; (4) *Azotobacter vinelandii*; (5) *Azotobacter chroococcum*; (6) *Nostoc (Anabaena)* sp.; (7) *Anabaena variabilis*; (8) *Xanthobacter autotrophicus*; (9) *Beijerinckia indica* subsp. *indica*; (10) *Methanosarcina lacustera*; (11) *Escherichia coli*; (12) *Bacillus thuringiensis*; (13) control PCR in the absence of the template; (14) the DNA molecular mass marker GeneRuler 100 bp (Fermentas).

RESULTS AND DISCUSSION

The first stage of the work was the computer analysis of nitrogenase gene sequences data, which is available in modern databanks. The preliminary sample volume was 360 sequences pertinent to *nifH* genes. After discarding the gene sequences of the alternative vanadium nitrogenase, *nifH*-like or homologous sequences, and the *nifH* gene sequences of unidentified microorganisms, the number of the sequences to be analyzed was reduced to 130. This reduction of the sample vol-

ume did not affect its representation in the taxonomic diversity of nitrogen-fixing prokaryotes (Table 2).

After thorough analysis of the aligned *nifH* gene sequences, the most conserved regions were selected corresponding to positions 19–56, 115–131, and 457–494 of the *Azotobacter vinelandii nifH* gene. This enabled us to design a system of primers (two forward, F1 and F2, and one reverse, R6) for amplifying the *nifH* gene fragments whose length is approximately 470 bp for primers F1–R6 and 370 bp for primers F2–R6. The sequences of these primers, as well as of those available in the literature [7, 11–13], are shown in Fig. 1.

The designed system of primers was tested with DNA preparations isolated from microorganisms that are known to be capable of nitrogen fixation (Figs. 2, 3). *E. coli* and *B. thuringiensis* DNA preparations were used as negative controls. As seen in the photographs, *nifH* fragments were revealed in all nitrogen fixers included in the experiment and the band intensity varied with the use of different pairs of primers (F1–R6 and F2–R6). Thus, when the first pair of primers was used, the yield of the amplification product for *Sinorhizobium meliloti*, *Rhizobium trifolii*, and *Nostoc (Anabaena)* sp. PCC7120 was substantially higher than for *Xanthobacter autotrophicus.* When the F2–R6 pair of primers was used, the amplification pattern was reversed. Thus, these pairs of primers are complementary.

To confirm the fact that the amplificates obtained are indeed *nifH* fragments, direct sequencing of the PCR fragments was carried out for *Nostoc (Anabaena)* sp. PCC7120 and *Sinorhizobium meliloti*, whose *nifH* sequences are available from GenBank under the accession numbers M55229.1 and AF012326.1, respectively. More than 95% homology was found between the sequences we determined and the sequences available from GenBank. Thus, the PCR fragments were confirmed to correspond to *nifH* genes.

The designed system of primers was used for determining the nucleotide sequences of cloned *nifH* fragments of nitrogen-fixing (eu)bacteria and archaea for which the data on the primary structure of the relevant genes were absent. The sequencing of five clones derived from *Beijerinckia indica* ATCC 9093, two *Xanthobacter autotrophicus* DSMZ 432 clones, and four *Methanosarcina lacustera* clones was performed. The nucleotide sequences of clones derived from the same species differed between one another within the sequencing error (by no more than 1%).

Thus, the proposed system of primers reveals *nifH* fragments in a wide range of microorganisms. This allows for more detailed studies of the microbial communities of different ecosystems, including both (eu)bacteria and archaea, to be performed without cultivation in laboratory conditions. An important continuation of our study will be the analysis of *nifH* genes in microorganisms whose involvement in the nitrogen fixation process has not yet been conclusively confirmed. Among them are methane-oxidizing and photosynthesizing bacteria, which are the object of our current investigations.

The number of *nifH* gene sequences deposited in the GenBank is steadily increasing, and their consensus is changing accordingly. It is therefore necessary to constantly improve the proposed system of primers in accordance with the emerging information.

ACKNOWLEDGMENTS

We are grateful to N.N. Kolotilova, Z.A. Arkad'eva (the Faculty of Biology, Moscow State University), S.N. Dedysh, M.V. Siman'kova (Institute of Microbiology, Russian Academy of Sciences), and N.F. Ryabchenko (Bioengineering Center, Russian Academy of Sciences) for kindly providing microbial cultures.

This work was supported by the Russian Foundation for Basic Research, project no. 00-04-48801a.

REFERENCES

1. Stackebrandt, E. and Goebel, B.M., Taxonomic Note: A Place for DNA–DNA Reassociation and 16S rRNA Sequence Analysis in the Present Species Definition in Bacteriology, *Int. J. Syst. Bacteriol.*, 1994, vol. 44, pp. 846–849.
2. Young, J.P.W., Phylogenetic Classification of Nitrogen-Fixing Organisms, *Biological Nitrogen Fixation*, Stacey, G., Burris, R.H., and Evans, H.G., Eds., New York: Chapman and Hall, 1992, pp. 43–86.
3. Ueda, T., Suga, Y., Yashiro, N., and Matsuguchi, T., Remarkable N_2-Fixing Bacterial Diversity Detected in Rice Roots by Molecular Evolutionary Analysis of *nifH* Gene Sequences, *J. Bacteriol.*, 1995, vol. 177, pp. 1414–1417.
4. Zehr, J.P., Mellon, M., Braun, S., Litaker, W., Steppe, T., and Paerl, H.W., Diversity of Heterotrophic Nitrogen Fixation Genes in a Marine Cyanobacterial Mat, *Appl. Environ. Microbiol.*, 1995, vol. 61, pp. 2527–2532.
5. Ben-Porath, J. and Zehr, J.P., Detection and Characterization of Cyanobacterial *nifH* Genes, *Appl. Environ. Microbiol.*, 1995, vol. 60, pp. 880–887.
6. Okhuma, M., Noda, S., Usami, R., Horikoshi, K., and Kudo, T., Diversity of Nitrogen Fixation Genes in the Symbiotic Intestinal Microflora of the Termite *Reticulitermis speratus*, *Appl. Environ. Microbiol.*, 1996, vol. 62, pp. 2747–2752.
7. Kirshtein, J.D., Paerl, H.W., and Zehr, J., Amplification, Cloning, and Sequencing of a *nifH* Segment from Aquatic Microorganisms and Natural Communities, *Appl. Environ. Microbiol.*, 1991, vol. 54, pp. 2645–2650.
8. Zehr, J. and McReynolds, L., Use of Degenerate Oligonucleotide Primers for Amplification of the *nifH* Gene from the Marine Cyanobacterium *Trihodesmium thiebautii*, *Appl. Environ. Microbiol.*, 1989, vol. 55, pp. 2427–2532.

9. Ohkuma, M., Noda, S., and Kudo, T., Phylogenetic Diversity of Nitrogen Fixation Genes in the Symbiotic Microbial Community in the Gut of Diverse Termites, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 4926–4934.

10. Widmer, F., Shaffer, B.T., Porteous, L.A., and Seidler, R.J., Analysis of *nifH* Gene Pool Complexity in Soil and Litter at a Douglas Fir Forest Site in the Oregon Cascade Mountain Range, *Appl. Environ. Microbiol.*, 1999, vol. 65, pp. 374–380.

11. Balows, A., Trüper, H.G., Dworkin, M., Harder, W., and Schleifer, K.-H., *The Prokaryotes*, New York: Springer-Verlag, 1992, vol. 2, pp. 2065–2066.

12. Jarvis, B.D.W., Gillis, M., and De Ley, J., *Rhizobium loti*, a New Species of Legume Root Nodule Bacteria, *Int. J. Syst. Bacteriol.*, 1982, vol. 32, pp. 378–380.

13. *Metody pochvennoi mikrobiologii i biokhimii* (Methods in Soil Microbiology and Biochemistry), Zvyagintsev, D.G., Ed., Moscow: Mosk. Gos. Univ., 1991, p. 304.

14. Sanger, F., Nicklen, S., and Coulson, A.R., DNA Sequencing with Chain-Terminating Inhibitors, *Proc. Natl. Acad. Sci. USA*, 1977, vol. 84, pp. 5463–5467.