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SHORT COMMUNICATIONS

A New System of Degenerate Oligonucleotide Primers for Detection and Amplification of *nifHD* Genes

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Biological nitrogen fixation is a process of dinitrogen reduction to assimilable ammonium [1, 2]. Many prokaryotes fix nitrogen by means of nitrogenase, a highly conservative enzyme complex consisting of dinitrogenase (MoFe-protein) and dinitrogenase reductase (Fe-protein) encoded by the *nifDK* and *nifH* genes, respectively [1]. The most convenient method of assessing the nitrogen-fixing ability in different prokaryotes is amplification of nitrogenase structural genes (*nif* genes) using degenerate oligonucleotide primers in polymerase chain reaction (PCR). Nitrogen fixation can be assessed by other methods, such as acetylene reduction, hybridization of genome DNA with labeled probes, or methods using ^{15}N. However, these methods require expensive equipment and reagents, and their results are not always reliable.

The *nifH* gene is most often used as a marker of nitrogen fixation, and the representative database formed to date contains the sequences of the *nifH* genes of bacteria from various habitats [2]. Several systems of "universal" oligonucleotide primers for amplification of the *nifH* gene are known [3–6].

Another structural gene of nitrogenase, *nifD*, may also be a marker of the nitrogen-fixing ability of an organism, but relatively few systems of primers for its amplification are known as of yet [4, 7]. However, it is necessary to mention that NifD protein is less conservative than NifH; hence, selection of degenerate primers for detection and amplification of the *nifD* gene implies significant problems.

Most methanotrophic bacteria have been found to be diazotrophs [3, 4, 8]. Methylobacteria, another physiological group of methylotrophs that are incapable of CH_4 utilization, are less studied in this respect. For primary detection of the nitrogen fixation genes in methylobacteria, we have compared the nucleotide sequences of diazotrophic bacteria of different taxonomic positions with the sequences of primers F1 and R6 taken from work [3]. It has been shown that primer F1 is absolutely suitable for amplification of this gene in diverse prokaryotes, whereas primer R6 has significant mismatches in the 3'-region with the *nifH*- genes of *Clostridium acetobutylicum*, *Rhodospirillum rubrum*, and *Methanosarcina barkerii*; PCR is therefore not always accomplished. Moreover, as the calculated annealing temperature for primer R6 is much lower than that for F1, PCR is carried out in a long and complicated temperature–time mode.

The goal of this work was to develop an efficient and reliable system of primers for amplification of the array of *nifHD* genes, covering the greatest possible diversity of diazotrophs, including methylobacteria.

Primer F1 (5'-TAYGGIAARGGIGGIATIGGIAARTC-3') was used [3]. In addition, the following primers were developed for *nifHD* amplification on the basis of the full sequences of the *nifHD* genes and proteins NifH and NifD presented in the GenBank database: nifH-2f 5'-GMRCCIGGIGTIGGYTGYGC-3' corresponding to positions 280–299 of *Bradyrhizobium japonicum* USDA 110 *nifH* gene (GenBank NC_004463) and nifH-3r 5'-TTGTTGGCIGCRTASAKIGCCAT-3' (positions 469–491 of *B. japonicum* USDA 110 *nifH* gene). For *nifD* amplification, the following probes were developed: nifD-1f 5'-GIGGITGCGCYTAYGCCGG-3' (positions 194–212 of *B. japonicum* USDA 110 *nifD* gene), nifD-2f 5'-CATCGGIGACTACAAYATYGGYGG-3' (positions 699–722 of *B. japonicum* USDA 110 *nifD* gene), and nifD-1r 5'-CCCAIGARTGCATYTGICGGAA-3' (positions 1339–1360 of *B. japonicum* USDA 110 *nifD* gene). PCR amplification was carried out in an Eppendorf Mastercycle Personal amplifier (Germany). PCR fragments with different pairs of primers were obtained in the temperature–time modes presented in the table. The reaction mixture (10 μl) contained: 1× *Taq*-polymerase buffer (Fermentas, Lithuania), 2.5 mM of $MgCl_2$, 40 μM of each dNTP (Fermentas, Lithuania), 10 ng of DNA, 5 pM of each of the corresponding primers, and 0.5 U of DNA polymerase. The products were separated by electrophoresis in agarose gel.

The above system of primers was tested with 14 collection strains of diazotrophic bacteria of different taxonomic positions (figure). With primers F1–nifH-3r, a

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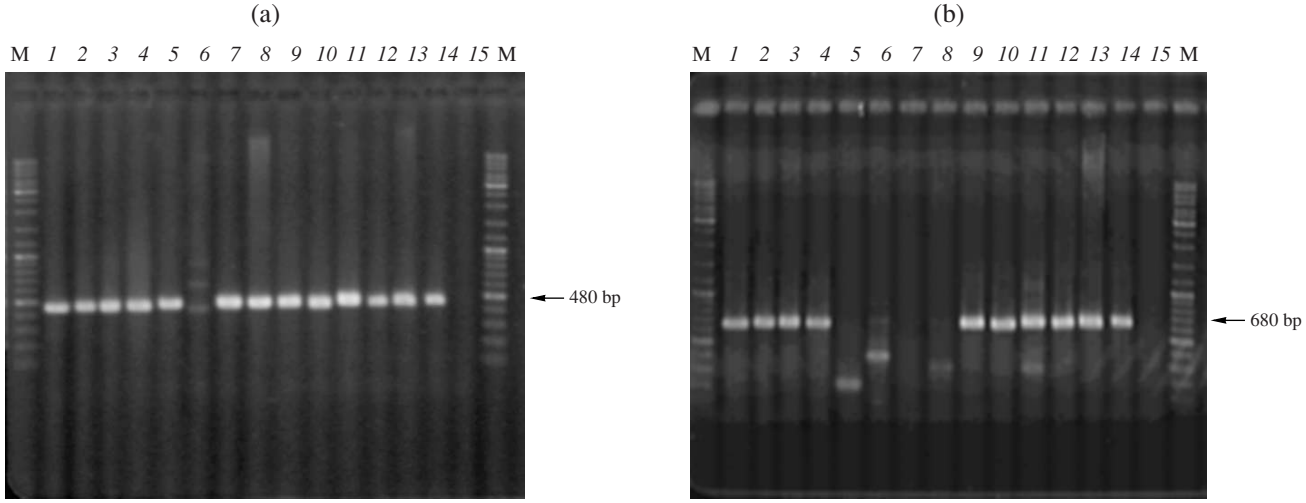
Temperature–time modes for amplification of PCR products of the *nif* genes with different combinations of primers

Temperature mode	Temperature, °C	Number of cycles	Set of primers; time, s				
			nifH-2f–nifH-3r	F1–nifH-3r	nifD-2f–nifD-1r	nifD-lf–nifD-1r	F1–nifD-1r
Initial denaturing	96	1	180	180	180	180	180
Denaturing	94		1	1	1	5	10
Annealing of primers	50–55	30	10	10	10	10	10
Elongation	72		5	10	15	30	70
Final polymerization	72	1	60	60	60	120	180

specific PCR product of 480 bp was obtained from DNA of all of the studied diazotrophs (figure, a). This fact showed the above pair of primers to be quite suitable for *nifH* amplification in different classes of prokaryotes (α-, β-, γ-, and δ-*Proteobacteria*, as well as in archaea). Positive results were obtained with the pair of primers nif-2f–nifH-3r for all samples. With primers nifD-2f–nifD-1r, the amplicons of expected length were not obtained in the case of *Desulfovibrio desulfuricans*, *Methanosarcina barkerii*, and *Methanothermobacter thermoautotrophicus* which is consistent with the analysis of alignments of the *nifD* gene sequences of these prokaryotes and the corresponding primers. In all other cases, the specific product was present (figure, b). A similar result was obtained with primers nif-1f–nifD-1r. Moreover, PCR with primers F1–nifD-1r can yield fragments containing nearly complete sequences of the *nifHD* genes.

As a result, 18 collection strains of methylobacteria of different taxonomic position were analyzed, which belong to 15 species with different pathways of C_1 assimilation: *Hansschlegelia plantiphila* strains S_1, S_2, and S_4, *Paracoccus methylutens*, *Angulomicrobium tetraedrale*, *Albibacter methylovorans*, *Beijerinckia mobilis*, and *Xanthobacter autotrophicus* (ribulose biphosphate pathway); *Methylobacterium extorquens* G10 and TK0001, *Methylobacterium dichloromethanicum*, and *Methylopila capsulata* (serine pathway); *Methylovorus mays* C, *Methylophaga murata*, *Methylobacillus pratensis*, *Methylovorus glucosetrophus*, *Methylophilus quaylei*, and *Methylobacillus glycogenes* (ribulose monophosphate pathway). However, specific PCR products were revealed only for *Beijerinckia* and *Xanthobacter*, for which nitrogen fixation had been shown previously [9, 10]. Hence, unlike methanotrophs, very few methylobacteria are able to fix nitrogen.



The *nif* gene PCR fragments from different diazotrophs with combinations of primers F1–nifH-3r (a) and nifD-2f–nifD-1r (b). *1*, *Azospirilum brasilense* Sp7; *2*, *Beijerinckia mobilis; 3, Bradyrhizobium japonicum; 4, Burkholderia* sp. R22; *5*, *Desulfovibrio desulfuricans; 6, Erwinia carotovora; 7, Methanothermobacter thermoautotrophicus; 8, Methanosarcina barkerii; 9, Methylobacter marinus* 7C; *10*, *Methylococcus capsulatus* Bath; *11*, *Methylomonas methanica* S_1*; 12*, *Methylosinus trichosporium* OB3b; *13*, *Rhizobium leguminosarum* VF39; *14*, *Xanthobacter autotrophicus*; *15*, control PCR without DNA; M, DNA molecular weight marker GeneRuler DNA Ladder Mix 100–10000 bp (Fermentas, Lithuania).

Thus, the developed system of primers can be effectively used in the screening of nitrogenase genes in bacteria isolated in pure cultures, for which nitrogen fixation has not been definitely proved, and in the study of diversity of the *nifHD* genes in different diazotrophic communities.

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