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EXPERIMENTAL ARTICLES

Molecular Genetic Analysis of New *Anabaena* Strains Isolated from a Plant–Cyanobacterial Community

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Abstract—Ecosystems of rice paddies are good sources of new strains of heterocyst-forming cyanobacteria that can be used in biotechnological systems for production of photohydrogen. The morphological and physiological properties of two novel epiphytic strains of cyanobacteria, *Anabaena* sp. 182 and *Anabaena* sp. 281, were studied. DNA typing of these strains based on PCR amplification of hydrogenase-encoding genes and DNA analysis using RAPD and Rep primers was carried out. The properties of the genome of strain *Anabaena* sp. 281 differed considerably from those of two reference strains (*Anabaena variabilis* ATCC 29413 and *Nostoc* sp. PCC 7120) with sequenced genomes, whereas strain *Anabaena* sp. 182 was found to be a close relative of *A. variabilis* ATCC 29413. Due to a number of physiological and biochemical advantages, *Anabaena* sp. 182 may be considered a new promising model for molecular and genetic engineering studies aimed at the development of H_2 producers.

Key words: cyanobacteria, *Anabaena,* hydrogen photoproduction, genotyping, DNA repeats

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Cyanobacteria are an ancient group of photosynthetic microorganisms and occur widely in various ecological niches. They provide an initial material for screening of strains producing bioactive compounds, ammonium, and photohydrogen. The strains involved in biotechnological processes include filamentous nitrogen-fixing cyanobacteria of the genera *Anabaena* and *Nostoc*, both free-living and isolated from symbiotic associations with plants and fungi. Heterocyst-forming cyanobacteria and mutants thereof are the most likely candidates that might be used in advanced systems for hydrogen photoproduction [1–3].

The aim of the present work was to study two novel epiphytic cyanobacterial strains of the genus *Anabaena*, which were isolated from the cyanobacterial associations with rice, using molecular genetic techniques. These isolates were compared with the model strain of the free-living cyanobacterium *Anabaena variabilis* ATCC 29413 with a completely sequenced genome (http://genome.jgi-psf.org/microbial/anava.home.html). The main emphasis was placed on analysis of the properties that make these strains potential producers of molecular hydrogen.

MATERIALS AND METHODS

In our study, we used the laboratory strain ATCC 29413 of the nitrogen-fixing cyanobacterium *Anabaena variabilis* and two novel strains, *Anabaena* sp. 182 and *Anabaena* sp. 281, isolated from rice leaves (IR22 variety) in Vietnam (the strains were kindly provided to us by Prof. Nguyen Than Khyen, Hanoi University). In our molecular genetic studies, we used the strain *Nostoc* (*Anabaena*) sp. PCC 7120 with a completely sequenced genome (http://wiki.kazusa.or.jp/Kazusa:CyanoBase:Anabaena_sp._PCC_7120) kindly provided to us by Prof. P. Wolk and strain *Anabaena siamensis* isolated from the soil of Thailand rice paddies and kindly provided to us by Prof. S. Boussiba (Israel) as reference strains.

The cultures were grown at 30°C and under fluorescent light at an illumination intensity of 40 $\mu E(m^{-2}s^{-1})$ on the BG11 [4] and $BG11_o$ (without $NaNO_3$) media in conical flasks with 40–50 ml of the medium. The growth rate was determined on a spectrophotometer at 540 nm.

The cultures for semiquantitative assessment of the hydrogen production (preformed in 8-ml graduated tubes with rims) were grown under microaerophilic conditions. Two-day nitrogen-fixing cultures were concentrated by centrifugation up to an OD_{540} of 1.5–2.0 in $BG11_o$ medium supplemented with 5 mM of fructose and 2×10^{-5} of dichlorophenyl dimethylurea (DCMU). The tubes were filled to capacity leaving no air bubbles and incubated for 24–48 h at an illumination intensity of 50 $\mu E(m^{-2}s^{-1})$.

The nitrogenase (NG) activity was determined as described earlier [5] by the rate of acetylene reduction during incubation of the culture (2 ml) in air atmosphere supplemented with 10% acetylene in hermetically sealed 14-ml vials at an illumination intensity of

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Table 1. Primers used for PCR amplification

Primers	Forward/Reversed nucleotide sequence (5' ⟶ 3')	Annealing temperature chosen for the PCR reaction	Size of the amplified fragment of the DNA of *A. variabilis**, bp
1–2 hupS	CCTGGTGGTAGTTCTTTGGGT/ CGAAGAACCGACAGTATGCGA	50	810
3–4 hupL	TGAACTCGTGCAAGTTGACG/ GGTTTCTATTGGCGTATGGG	50	497
5–6 hupSLMid	GCTAAGATGGCGTTGCG/ GGACAACGCAAAGGCTG	48	659
7–8 hoxH	GAAGGTCACGCCAAGATTAGTAT/ GATTCCTGCTTCTACACGATTGA	50	1299
9–10 hoxYH	CCACTATTACAAGGCAAAACACCAC/ CTTCACTCACACCAACGGCTTCTAA	54	1969
11–12 orf2 hox	TGGGATTATTAGTGAGAGCGA/ GCGATTTTGCTAACTTTTTCA	48	414
Rep1	GCTACAACGGGGGGAAC	50	1648
Rep2	GGATACTGTTGGCGAAT	48	1818, 3375
Rep3	TCGTAGGGTGGGCAATG	50	1396 + (1095, 1121, 1750)**
CRA22	CCGCAGCCAA	40	N.d.
CRA25	AACGCGCAAC	40	"
OPA 11	CAATCGCCGT	40	"

Notes: * The fragment sizes were determined using the BLASTN software package on the basis of the primer localization in the DNA of *A. variabilis*.
** Possible amplification products obtained using 5'-truncated primers of at least 11 bp.

50 μE($m^{-2}s^{-1}$) for 1–2 h. Prior to the addition of acetylene, the vials were preincubated under the same conditions under illumination for 15 min.

The cells were lysed in 1N NaOH at 37°C for 20 h, and the protein content was determined by the Lowry method.

The occurrence frequency of heterocysts in the cultures was determined by microscopic examination in which at least 1000 cells were counted.

The primers were selected on the basis of the nucleotide sequence of the *Anabaena variabilis* ATCC 29413 genome (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) using the Oligo software package. Table 1 and Figure 1 show the list of the RAPD-primers, primers complementary to the repetitive nucleotide sequences (Rep), and primers for the genes required for hydrogen metabolism.

The search for repetitive sequences was carried out using a software package designed at the Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences (http://wwwmgs.bionet.nsc.ru/mgs/programs/lzcomposer) [6]. The search for homologous sequences was carried out using the

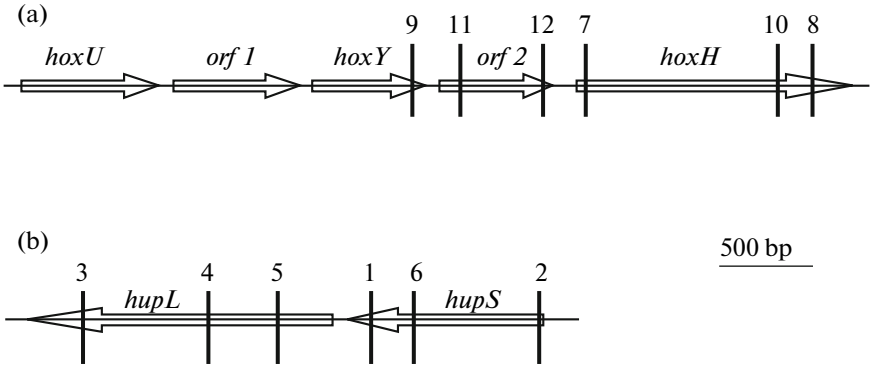

Fig. 1. Genetic scheme of the *hox* (a) and *hup* (b) gene clusters of *A. variabilis* specifying the location of specific primers (1–12).

Table 2. Morphological, physiological, and biochemical properties of the *Anabaena* strains

Strain	Growth rate in a liquid medium in the light*, OD_{540}			Heterocyst frequency in liquid medium **, %		Nitrogenase activity under aerophilic conditions***	Hydrogen production under microaerophilic conditions	Capacity for production of motile hormogonia during growth on solid medium		
								light		dark
	$BG11_o$	$BG11_o$	$BG11_o$ + 5 mM fructose	$BG11_o$	BG11	$BG11_o$	$BG11_o$ + 5 mM fructose; + 10^{-5} M DCMU	$BG11_o$	$BG11_o$ + 5 mM fructose	$BG11_o$ + 5 mM fructose
A. variabilis	1.20	1.25	4.5	6.25	2.0	0.54	+	–	+	+
Anabaena sp. 182	1.51	1.53	4.2	7.20	<0.5	0.65	++	–	–	–
Anabaena sp. 281	1.30	1.25	4.0	3.50	<0.2	0.42	–	–	–	+

Notes: * 5-day cultures (mid-exponential phase), initial $OD_{540} = 0.1$.
** 3-day cultures.
*** 2-day cultures, μmol C_2H_4/(h mg protein).

BLASTN software package (http://blast.kazusa.or.jp/blast_dearch/cyanobase/genomes).

Genomic DNA was extracted from the cells frozen at –20°C. For this purpose, the cells were treated mechanically with glass beads in the presence of sodium dodecyl sulfate and chloroform [7].

PCR (polymerase chain reaction) amplification with a 25 μl reaction volume overlaid with mineral oil was performed in an Amply4 amplifier (Biokom, Russia). The reaction mixture was prepared using the PCR Master Mix kit (Fermentas, Lithuania) and contained 1 U of *Taq* polymerase, 2 mM of $MgCl_2$, 0.2 mM of each deoxynucleotide, 10 ng of template DNA, and 2 pmol of primers (100 ng of DNA and 10 pmol of primer for RAPD analysis). Amplification with standard and Rep primers was performed using the following program: initial denaturation, 94°C for 2 min; 30 cycles: 94°C for 60 s, annealing temperature of the specific primer for 60 s, 72°C for 2 min; and final synthesis at 72°C for 10 min. The PCR amplification with RAPD primers was performed using the techniques for RAPD analysis of cyanobacterial DNA [8], with minor modifications: initial denaturation, 94°C for 2 min; 94°C for 30 s, 40°C for 30 s; 72°C for 60 s, 30 cycles; and final synthesis at 72°C for 5 min. When performing the multiplex RAPD–PCR analysis, equimolar concentrations of the two primers were used. All amplification reactions were performed in at least three repetitions. The obtained amplification products were separated by agarose gel electrophoresis according to the standard techniques. The TAE buffer contained 0.7% agarose for analysis of the PCR products using the standard primers and 1.5% agarose for PCR analysis with the STRR and RAPD primers. A 1-kb marker (Fermentas) was used as a molecular standard for determining the size of the DNA fragments.

RESULTS AND DISCUSSION

Table 2 shows the growth, morphological, and physiological properties of three *Anabaena* strains. Study of the their capability of utilizing exogenous mono- and disaccharides during mixotrophic ($BG11_o$ medium in the light) of heterotrophic ($BG11_o$ medium in the dark) growth revealed that all three strains are facultative heterotrophs and grow well on fructose (≥5 mM) in the dark. Strain *Anabaena* 281 is also capable of heterotrophic growth on glucose.

The growth rate of *Anabaena* sp. 182 was higher in a nitrogen-free medium, which correlates well with the high rate of heterocyst formation and the high level of nitrogenase activity under these conditions. The strains *A. variabilis* and *Anabaena* sp. 182 were able to produce large amounts of hydrogen under microaerophilic conditions (in the presence of DCMU) during incubation on fructose as an energy and electron source [9]. Fructose-dependent production of hydrogen was observed under these conditions at a decreased growth rate of the culture. The results obtained revealed a similarity between the systems responsible for regulation of nitrogen fixation and coupled to it the process of hydrogen uptake in *A. variabilis* and *Anabaena* sp. 182. However, in the presence of available nitrogen sources in the growth media, the patterns of inhibition of heterocyst (and nitrogenase) production of the novel strains, as well as their ability to form motile hormogonia, differ from those of *A. variabilis*. During growth on solid media, the cells of strain *Anabaena* sp. 182 did not form hormogonia. No significant differences were found between the sizes of the cells in hormogonia formed by *Anabaena* sp. 281 and the sizes of the cells of vegetative trichomes, which is a typical morphological trait of the genus *Anabaena* [4]. Hormogonia formed by *A. variabilis* consist of very small cells, that is typical of *Nostoc* strains [10]. In the case of *A. variabilis*, this fact corresponds to the data on the high level of relatedness between this strain and

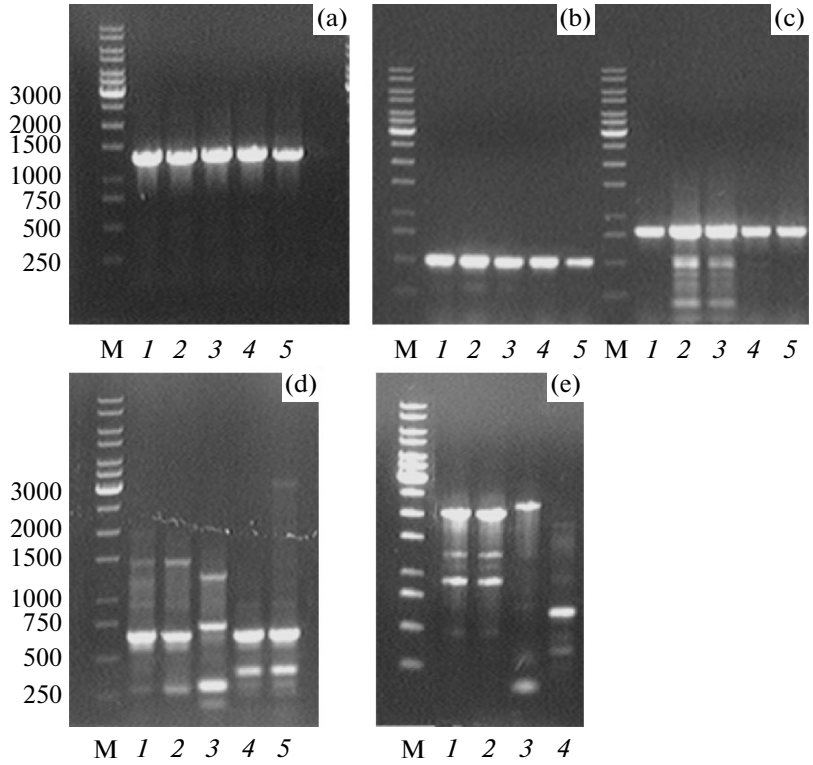

Fig. 2. Profiles of the PCR products obtained using the primers 7–8 (hoxH) (a), 3–4 (hupL) (b), 1–2 (hupS) (c), (hupSLMid) (d), and 9–10 (hoxYH) (e) of the DNA of the following cyanobacterial strains: A. variabilis (*1*), *Anabaena* sp. 182 (*2*), *Anabaena* sp. 281 (*3*), *A. siamensis* (*4*) and *Nostoc* sp. PCC 7120 (*5*); M, a 1-kb marker.

members of the genus *Nostoc* and, in particular, between *A. variabilis* and *Nostoc* sp. PCC 7120, as demonstrated by the results of proteomic and phylogenetic analyses [11, 12] which allowed more precise determination of the phylogenetic position of strain *A. variabilis* ATCC 29413 deposited in the Pasteur Culture Collection as *Nostoc* PCC 7937.

Genotyping and determination of the taxonomic position of natural isolates of cyanobacteria are usually based on the results of comparative analysis of their 16S rRNA gene sequences, conservative genes, and intergenic spacer regions [13, 14]. The results of molecular genetic studies of the genes responsible for different stages of the hydrogen metabolism of heterocyst-forming cyanobacteria provide much information as well [1, 2].

Based on the results of analysis of the nucleotide sequences of the sequenced genome of *A. variabilis* ATCC 29413, specific primers (Fig. 1, Table 1) were selected and PCR analysis was performed in order to detect the presence and elucidate the structure of the nucleotide sequences encoding the genes responsible for the hydrogen metabolism of the reference and the two novel strains of cyanobacteria (Fig. 2, Table 3). It was demonstrated that, in all of the strains studied, amplification with the primer targeting the internal sites of the *hupS, hupL*, and *hoxH* genes encoding the uptake hydrogenase subunits hupL and hupS, as well as the bidirectional hydrogenase subunit hoxH, yielded the PCR products analogous to the fragments formed on the template DNA of *A. variabilis*.

With the primers targeting other sites of the *hox* and *hup* clusters, the more extensive and/or less conservative sites (due to inclusion of intergenic sequences), *orf2 hox, hoxYH,* and *hupSLMid*, some differences in the fragment sizes and amounts of PCR products were detected (Fig. 2, Table 3). However, in the case of *A. variabilis* and *Anabaena* sp. 182, PCR amplification with all specific primers produced the same results, which suggests a high similarity between the studied nucleotide sequences of the *hox* and *hup* clusters of these cyanobacterial strains.

To detect and assess the genomic diversity, as well as to perform genotyping of new isolates, PCR amplification with primers based on repetitive DNA sequences, Hip1 [15], STRR, LTRR [16], and Rep [17], widely distributed in the genomes of filamentous cyanobacteria, is frequently used. The profiles of PCR products obtained using these primers are analogous to DNA fingerprinting and demonstrate the variability of intergenic regions and the gene positions in the genome due to the fact that many repetitive DNA sequences are usually detected in variable intergenic regions.

The genome of *A. variabilis* contains approximately 1450 inverted repeats of 20 or more base pairs [18],

Table 3. PCR amplification of the fragments of the *hox* and *hup* clusters using the primers targeting the genome of *A. variabilis*

Strain	Primers, amplified loci, and size of the PCR product*					
	hupS 810 bp (1–2)	*hupL* 497 bp (3–4)	*hupSLMid* 659 bp (5–6)	*hoxH* 1299 bp (7–8)	*hoxYH* 1969 bp (9–10)	*orf2 hox* 414 bp (11–12)
A. variabilis	+	+	+	+	+	+
Anabaena sp. 182	+	+	+	+	+	+
Anabaena sp. 281	+	+	≈740 bp, ≈300 bp,	+	≈2150 bp	+
A. siamensis	+	+	+, ≈350 bp	+	≈600 bp	+
Nostoc sp. PCC 7120	+	+	+, ≈350 bp	+	NT	NT

Notes: * the numbers of primers according to Fig. 1 are given in parentheses.
+, the presence of the PCR fragment mentioned in the upper row of the table.
NT, not tested (the primers showed low homology).

Table 4. Characterization of inverted repeats in the genome of *A. variabilis* used for primer selection

Repeat	Nucleotide sequence 5' ⟶ 3' (+DNA strand)	Position within the genome	Number of copies in the genome	Localization
Rep1 = 87 hp	GAAGAAGACGCGACGCCAGTC***GCTACAACGGGGGGGAAC***CCCCCGCAACGCGCTGGCTCAT-GAATCGCGTCTCTACAAATGGTTTATTT	4582720–4582779	3 + at least 12*	Intergenic
	AAATAAACCATTTGTAGAGACGCGATTCATGA GCCAGCGCGTTGCGGGGG***GTTCCCCCCCGTTG-TAGC***GACTGGCGTCGCGTCTTCTTC	4584332–4584418		
Rep2 = 27 hp	CTCCTACAA***GGATACTGTTGGCGAATT***	296065–296091	3 + 33*	Right and left ends of the IS5 element
	A***ATTCGCCAACAGTATCC***TTGTAGGAG	297874–297900		
Rep3 = 24 hp	***TCGTAGGGTGGGCAATG***CCCACCA	4389523–4389546	2 + at least 103*	Intergenic
	TGGTGGG***CATTGCCCACCCTACGA***	4390896–4390919		

Notes: Primer locations are shown in bold italics; the underlined sequences refer to (a) inverted repeats at the ends of the IS5 elements in the Rep2 sequence and (b) palindromic sequences in the Rep1 and Rep3 repeats.
* the number of truncated copies of the repeats containing the listed consensus nucleotide sequences (see explanations in the text).

some of which are located at a distance not exceeding 3500 bp from each other and can be used as single primers for amplification of the DNA fragments between these inverted repeats.

In this study, new markers (the inverted repeats Rep1, Rep2, and Rep3 (Table 4)) have been selected for genotyping. These markers provided the basis for designing primers for PCR amplification of small fragments of genomic DNA. The strains *A. variabilis* and *Anabaena* sp. 182 produced almost the same profiles of the PCR products generated with these primers. These profiles differed considerably from those of *Anabaena* sp. 281, *A. siamensis*, and *Nostoc* sp. PCC 7120 (Fig.3).

More detailed analysis of the *A. variabilis* genome using the BLASTN software package allowed us to detect additional copies of the complete Rep1, Rep2, and Rep3 sequences, as well as of certain consensus nucleotide blocks within these inverted repeats (Table 4). The Rep1 and Rep3 repeats with a high G + C content and the palindromic structure typical for the elements mediating transcription termination are located only in intergenic regions. The Rep2 repeats are located only in the terminal regions of IS5 elements, which, in the *A. variabilis* genome, are represented by 18 full-sized copies. One of these copies is merged with the sequence of the IS66 element and yields a fragment of more than 3300 bp upon amplification (Table 1). According to the data obtained using the ISfinder database (http://www-is.biotoul.fr), the genome of *Nostoc* PCC 7120 does not contain any IS5 elements, which was confirmed by the results of PCR amplification with the Rep2 primer (Fig. 3).

The fourth column of Table 4 shows the numbers of complete and truncated copies of the three studied types of repeats detected in the genome of *A. variabilis*. In the case of the Rep2 repeat, 33 additional copies are represented by sequences consisting of at least 18 nucleotides corresponding to the consensus sequence of the terminal inverted repeats in the terminal regions of the IS5 elements in the genome of

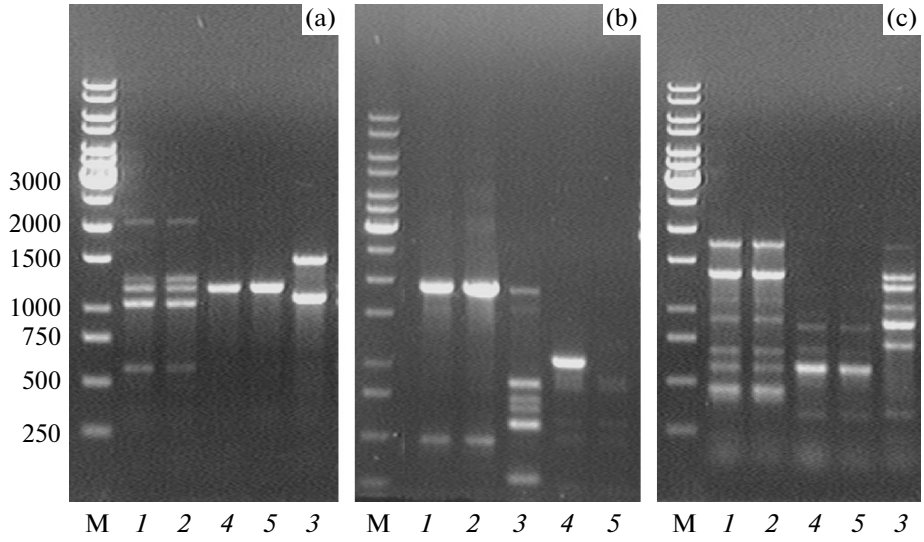

Fig. 3. Profiles of the amplification products obtained using the primers Rep1 (a), Rep2 (b), and Rep3 (c) for the DNA of the following strains of cyanobacteria: *A. variabilis* (*1*), *Anabaena* sp. 182 (*2*), *Anabaena* sp. 281 (*3*), *Anabaena siamensis* (*4*), and *Nostoc* sp. PCC 7120 (*5*); M, an 1-kb marker.

A. variabilis. A total of 103 additional copies of the Rep3 repeat whose terminal regions were only one or two nucleotides short (positions 3–22 of the repeat) were detected, including 38 copies that were completely homologous in this region of the initial sequence and 65 copies that contained one or two nucleotide substitutions, primarily in the center of the palindromic sequence (AAT) of the Rep3 repeat. It seems likely that the structure of the large Rep1 repeat is complex, since the complete 87-bp sequence was detected in the genome of *A. variabilis* three times, the sequence of the region (4–7)–87 bp with several nucleotide substitution was detected five times, and the shorter fragments of the Rep1 repeat in the region (3–26)–(45–53) bp, which contained a palindromic sequence, were detected seven times. The terminal fragment of the Rep1 repeat located between the positions (53–62) and (82–87) was detected in the genome at least 55 times. The Rep1 primer containing a fragment of the palindromic sequence of the Rep1 repeat amplifies, for some unknown reason, a fragment of about 2150 bp, instead of a 1648-bp fragment, as follows from the results of the analysis of homologous sequences of the *A. variabilis* genome performed using the BLASTN software package (Table 1). Nevertheless, PCR amplification using the Rep1 primer allowed us to detect a number of genotypic differences between *A. variabilis* and *Anabaena* sp. 182 and three other cyanobacterial strains.

The additional bands on the amplification profiles obtained using Rep primers were probably generated during amplification of additional fragments as a result of the multiplicity of sites homologous to the 3'-ends of shorter primers [19], which was confirmed by the results of the analysis of the *A. variabilis* genome for the presence of the inverted sequences homologous to 5'-truncated derivatives of the Rep3 primer of at least 11 bp (Tables 1 and 4). The Rep3 primer was found to be most promising for genotyping of cyanobacterial strains, since it yields accurate and reproducible results, and the profiles of amplification products are highly strain-specific.

Using the RAPD–PCR method for analysis of the genetic heterogeneity of closely related organisms, we can achieve repeatability of results in the case of cyanobacteria [8, 20]. Figure 4 demonstrates the profiles obtained by amplification of the PCR products using three RAPD primers, OPA 11, CRA 22, and CRA 25. According to the similarity levels between the PCR products of these and 12 other tested RAPD primers (data not presented), the studied cyanobacterial strains may be divided into three clusters:

(1) *A. variabilis* and *Anabaena* sp. 182;

(2) *Anabaena* sp. 281; and

(3) *A. siamensis* and *Nostoc* sp. PCC 7120.

Hence, analysis of genomes by RAPD–PCR confirms the possibility of clustering of the studied strains according to their genetic similarity revealed by gene markers, as well as by the location of the Rep repeats in their genomic DNA. Irrespective of the general similarity between the distributional patterns of PCR fragments with all primers in *A. variabilis* and *Anabaena* sp. 182, the genetic polymorphism between these strains (Fig. 4b; the arrows point to the position of the "mismatched" fragments of these two strains) can be revealed only by RAPD–PCR. Thus, a close genotypic relatedness between the strains *A. variabilis* and *Anabaena* sp. 182, which may be considered independent isolates of one species of cyanobacteria with similar genome structures, was revealed. The molecu-

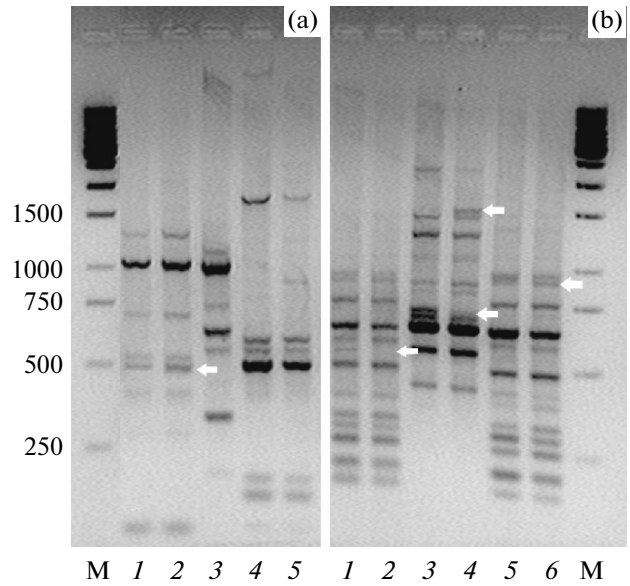

Fig. 4. Profiles of the PCR products obtained using RAPD primers: (a) amplification with the primer OPA 11 for the DNA of the following strains of cyanobacteria: A. variabilis (*1*), *Anabaena* sp. 182 (*2*), *Anabaena* sp. 281 (*3*), *A. siamensis* (*4*), and *Nostoc* sp. PCC 7120 (*5*). (b) Amplification using CRA22 (*1, 2*), CRA 25 (*3, 4*), and CRA 22 + CRA 25 (*5, 6*) DNA primers for the DNA of *A. variabilis* (*1, 3, 5*) and *Anabaena* sp. 182 (*2, 4, 6*). The arrows point to the location of the fragments in which the profiles of the PCR products obtained for the strains *A. variabilis* and *Anabaena* sp. 182 differ from each other; M, a 1-kb marker.

lar genetic properties of strain *Anabaena* sp. 281 differs considerably from those of all the cyanobacterial strains studied in this work. The similarity between the obtained PCR profiles with the majority of primers used for amplification of the gene fragments of *A. siamensis* and *Nostoc* sp. PCC 7120 (the characteristic traits of which are obligatory photoautotrophy and the absence of hormogonia) is noteworthy.

We were the first to obtain *A. variabilis* ATCC 29413 mutants capable of producing molecular hydrogen under aerobic conditions [5]. Later, these strains were effectively used in photobioreactors in other laboratories [3, 21]. The strategy for construction of efficient hydrogen-producing strains of cyanobacteria is based on the search for initial lines with high nitrogenase activity, as well as on isolation of strains with mutant genes mediating hydrogen metabolism [21, 22]. The results of the present work indicate that strain *Anabaena* sp. 182, closely related to *A. variabilis* ATCC 29413, may be considered a promising subject for studying the genetic control of hydrogen metabolism and a potential hydrogen producer. The nitrogenase and hydrogen-producing activities of *Anabaena* sp. 182 are higher than those of *A. variabilis*; its colonies developing on solid media are more round and compact. These and other physiological properties make this strain a convenient model for molecular and genetic engineering studies aimed at the development of mutant strains that can be used in photobiotechnology.

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