ISSN 1021-4437, Russian Journal of Plant Physiology, 2018, Vol. 65, No. 4, pp. 490–497. © Pleiades Publishing, Ltd., 2018.
Published in Russian in Fiziologiya Rastenii, 2018, Vol. 65, No. 4, pp. 00000–00000.

RESEARCH PAPERS

Substrate Specificity of Acyl-Lipid Δ9-Desaturase from *Cyanobacterium* sp. IPPAS B-1200, a Cyanobacterium with Unique Fatty Acid Composition[1]

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Received July 14, 2017

Abstract—*Cyanobacterium* sp. IPPAS B-1200 is characterized by a high content of rare fatty acids (FAs), both myristic (14:0—30%) and myristoleic (14:1Δ9—10%) in the membrane lipids. Thus, short-chain FAs reach 40% of the sum of the all FAs in cells, which is unusual for Cyanobacteria. Monounsaturated palmitoleic acids (16:1Δ9) also reach 40% of the sum of the FAs. We determined the complete nucleotide sequence of the genome of this cyanobacterium and found the only gene for the acyl-lipid Δ9-desaturase, *desC1*. We cloned this gene and characterized its specificity to the length of the substrate using heterologous expression in *Escherichia coli*. The results show that DesC1 nonspecifically generates olefin bond in FAs with a length of 14, 16, and 18 carbon atoms. This finding confirms that all monoesterifed FAs in *Cyanobacterium* sp. IPPAS B-1200 are generated by one acyl-lipid Δ9-desaturase.

Keywords: Cyanobacterium, genome, desaturase, fatty acids, genes expression
DOI: 10.1134/S102144371804009X

INTRODUCTION

Cyanobacteria are one of the oldest organisms on Earth. They are widely distributed in various habitats. They also form a major part of oceanic and freshwater plankton. Various metabolites of cyanobacteria can be used in food, pharmacy, and fuel industries. One of the applications is as alternative fuel, such as biofuel, which consists of various organic compounds (biogas, alcohols, and methyl esters of FAs). Biofuels are produced from oils of plant biomass but not by chemical synthesis or mining [1]. In addition to traditional plant oil cultures, there are other perspective sources of related chemical compounds: the biomass of photoautotrophic microorganisms (microalgae and cyanobacteria), which are characterized by some features that make them useful for research and industrial applications [1, 2].

The ability of prokaryotic cyanobacteria to perform photosynthesis and the possibility to cultivate them in inorganic media allow the generation of huge amounts of biomass if compared to heterotrophic cultures [1]. The efficiency of biomass accumulation of different cyanobacterial strains can be enhanced either through routine microbiological methods (strain isolation, optimization of culture medium, and conditions of cultivation) or by methods of molecular biology and genetic engineering [2–4]. Changes in the metabolic pathways in a certain direction can increase the output of desired compounds [5, 6] or forward the production of some compounds nonspecific for an organism [7, 8].

FAs are the components of cell membrane lipids as well as the basic compounds of plant oils and animal fats. Thus, FAs perform structural, storage, and regulatory functions [9]. The diversity of FAs is determined by several enzymes responsible for the synthesis and modification of FAs. For example, acyl-lipid desaturases of cyanobacteria form double carbon bonds in the FAs of membrane lipids. This is the reason for changes in "geometry" of FAs and, consequently, for changes in their physical properties (toughness, melting temperature). Fatty acid desaturases change the qualitative composition of FAs, increase unsaturation, and, thus, regulate the tolerance of cells to low temperatures by preventing the cell membranes from rigidification. The principles of regulation of viscosity of biological membranes, which have been discovered in cyanobacteria, are also applicable for higher plants. For example, tobacco and potato transformed with

[1] The article was translated by authors.

genes for cyanobacterial acyl-lipid desaturases demonstrated an increased cold tolerance [6, 10].

Thus, a study of cyanobacterial desaturases is important not only for academic research but also for biotechnological and agrarian applications. With extensive knowledge and modern approaches, one can change the productivity of cyanobacterial cultures and increase the range of compounds produced by them. This is a reason to study microorganisms with unique FAs composition and the enzymes involved in their biosynthesis.

Cyanobacterium sp. IPPAS B-1200 was isolated from salt Lake Balkhash, Kazakhstan (46°32′27″ n.l. 74°52′44″ e.l.) [3]. The strain synthesized only monounsaturated FAs and it was assigned to Group 1 according to FAs classification [11]. Availability of monounsaturates with double bounds at position Δ9 implied that only Δ9-desaturase(s) should be active in this organism. Unlike the majority of cyanobacteria that usually accumulate C16 or C18 FAs, *Cyanobacterium* sp. IPPAS B-1200 accumulates large amounts of C14 (myristic and myristioleic) FAs [3]. This unique FAs composition makes this object particularly attractive not only for academic studies but also for industrial applications. Earlier, we analyzed the FAs profiles of this culture grown at different temperatures and did not observe any changes in quantitative or qualitative FA composition. This fact is rather unusual, because the induction of desaturases is a feature of cyanobacteria subjected to cold stress.

Previously, we determined and assembled the whole nucleotide sequence of the *Cyanobacterium* genome [12] with Ion Torrent PGM™ [13]. The aim of this work is to identify the gene(s) for fatty acid desaturase(s) and to characterize its (their) substrate specificity by heterologous expression in *Escherichia coli*.

MATERIALS AND METHODS

Strain *Cyanobacterium* sp. IPPAS B-1200 was isolated from Lake Balkhash, Kazakhstan [3] and was cultivated in Zarruck's medium [2].

Genome sequencing. Genomic DNA were isolated according to Williams [14] with an additional purification step [15]. The quantity of DNA was estimated with a NanoDrop 1000 spectrophotometer (NanoDrop Technologies Inc., United States). The quality of DNA was estimated by agarose gel electrophoresis (1% agarose, Tris-Acetate buffer). A genomic DNA library was prepared using an Ion Xpress™ Plus Fragment Library Kit (Thermo Fisher Scientific Inc., United States). We used a Qubit® 3.0 fluorimeter (Thermo Fisher Scientific, United States) for accurate estimation of quantity of double-stranded DNA. Genomic DNA (1 μg) was treated with a mixture of nucleases to obtain a size of 150–250 bp. Fragmented DNA was ligated to adapter sequences. The resultant library was treated by Agencourt™ AMPure™ XP magnetic beads according to a protocol (https://www.neb.com/protocols/1/01/01/size-selection-e6270) and eluted into 25 μL of sterile deionized water.

The dilution factor was calculated according to the recommendations of the manufacturer. The final library concentration was equal to 100 pM.

Emulsion PCR was performed with Ion One-Touch™ 2 (Thermo Fisher Scientific, United States). Procedure of emulsion PCR and enrichment of samples with amplicon-containing spheres was performed with PGM™ Hi-Q™ OT2 Kit (Thermo Fisher Scientific, United States).

Sequencing was carried out with an Ion PGM Hi-Q Sequencing Kit and Ion 316™ Chip v2 BC microchips. All procedures were carried out strictly in accordance with the manufacturer's protocols (Thermo Fisher Scientific, United States).

Genome assembling and annotation. The program SPAdes 3.1.0 included in Torrent Suite was used to de novo assemble the genome of *Cyanobacterium* [16]. The genome was annotated at NCBI with Prokaryotic Genome Annotation Pipeline (PGAP). The genes for the acyl-lipid desaturases were searched for among all genes of *Cyanobacterium*. The determined translated sequence of the desaturase was aligned with desaturases whose function was earlier characterized or suggested in silico. The procedure was performed with the Muscle algorithm [17, 18] with basic parameters. CLC Sequence Viewer (QIAGEN Bioinformatics, United States) was used to visualize the results [19].

Cloning the gene encoding Δ9-desaturase from *Cyanobacterium* sp. IPPAS B-1200. To perform heterologous expression, we constructed a vector expressing the gene for the acyl-lipid Δ9-desaturase, *desC1*. The gene was amplified from genomic DNA of *Cyanobacterium* using PCR and synthetic nucleotides as primers with created restriction sites: *Nco* I (ATAACCATGGCAGTTTCAAC) and *Hin*d III (TTTGAAGCTTTTATTATGC). Primers were designed with Vector NTI software. The PCR reaction mix included primers (0.1 μM each), genomic DNA (5 ng), dNTPs (0.2 mM), $MgCl_2$ (2 mM), and DNA-polymerase Phusion® High-Fidelity DNA Polymerase (New England Biolabs, United States). PCR included the following steps: preliminary DNA denaturation (30 sec, 98°C); 30 cycles that included DNA denaturation (10 sec, 98°C), primers annealing (30 sec, 57°C), DNA chain synthesis (2 min, 72°C); and, finally, additional DNA chain synthesis (5 min, 72°C).

At the end of PCR, we changed the concentration of $MgCl_2$ to 10 mM and added suitable restriction endonuclease. Samples were incubated for 1 h at 37°C.

The obtained DNA fragments were purified by electrophoresis and isolated from an agarose gel with a GeneJet™Gel Extraction Kit (Thermo Fisher Scientific, United States). DNA fragments were eluted with 25 μL of water.

PCR products and pTrc99a vector were digested with *Nco* I and *Hin*d III. The vector was dephosphorylated by alkaline phosphatase FastAP (Thermo Fisher Scientific, United States) and purified as described above for DNA fragments obtained by PCR. The vector was ligated with a DNA fragment by T4 DNA ligase (Fermentas, Lithuania). The resultant construction for the expression was designated as pTrc99A-*desC*.

Heterologous expression. Cells of *E. coli* (strain Rosetta) were transformed by pTrc99A-*desC*. A single growing colony were inoculated into 100 mL of M9 medium supplemented with 1 μM $MgSO_4$, 0.5 μg/mL of vitamin B1, 0.2% glucose, 0.1% casamino acids, 100 μg/mL of ampicilin, 10 μM $FeCl_3$, and grown at 37°C to reach an optical density of 0.5 at 660 nm. After that, isopropylthiogalactoside (IPTG) was added to the culture at a final concentration of 100 μM, and incubation continued for 4 h.

The appearance of DesC in *E. coli* cells was checked in 0.75 mm thick SDS-PAGE [20] with Mini-PROTEAN™ Tetra Cell (Bio-Rad, United States). The concentration of acrylamide was 12.5% in a separating gel and 5% in a concentrating gel.

Chromatographic analysis of FAs. Cells were washed by PBS-buffer and resuspended in 10 mL of isopropyl alcohol that contained ionol at 20 mg L^{-1}; thus, samples were fixed.

Total FAs were trans-esterified in a mixture of methanol and acetyl chloride (9 : 1) for 60 min at 70°C. The resultant fatty acid methyl esters (FAMEs) were analyzed with a GC-MS Agilent 7890A gas-liquid chromatography system with the mass spectrometric detector Agilent 5975C (Agilent Technology Systems, United States). The 60-m capillary column DB-23 (∅ 0.25 mm; Fischer Scientific, Loughborough, United Kingdom) was filled with 50% cyanopropyl methylpolysiloxane. Other separation conditions for FAMEs were as follows: helium pressure in the injector of 245 kPa, flow rate of 1 mL min^{-1}, volume of the sample solution was 1 μL, flow divider 1 : 5, and evaporation temperature of 260°C. Program of column gradient temperature was the following: jump from 130 to 170°C at 6.5°C per min; jump from 170 to 215°C at 2.75°C per min; holding at 215°C for 25 min, jump from 215 to 240°C at 40°C per min; and holding at 240°C for 50 min. Operating temperature of MS detector was 240°C at the ionization energy of 70 eV.

RESULTS AND DISCUSSION

Sequencing and Annotation of the Genome of Cyanobacterium sp. IPPAS B-1200

Despite partial loading of a chip, we got an optimal allotment of read, which was sufficient for the assembly of the whole genome. Finally, the amount of information reached 444 Mbp and provided the coverage of more than 100× per genome (~4 Mbp). Such coverage confirms the reliability of the sequencing results.

The genome was assembled with SPAdes version 3.1.0 with the following parameters: –k (21, 33, 55, 77, 99, 127), -s, -iontorrent. Number of contigs were 119. The quality of the assembly of the draft genome was analyzed by QUAST (6). The draft genome's median coverage was approximately 200x and its *N*50 value was 80222 bp. The approximate genome size was estimated at 3.4 Mb, with an estimated average G+C content of 37.7%. [12]. As a result, the draft genome sequence of *Cyanobacterium* sp. IPPAS B-1200 was obtained (accession number in GenBank NZ_LWHC00000000.1). The draft genome is represented by a sum of contigs that are generated as the first assembly of unique DNA sequences but not the repeated sequences [21]. Therefore, the draft genome sequence is suitable for the search of genes of interest.

The genome was automatically annotated by the open-source software. The genome contained a total of 3119 genes, with 2934 genes coding for proteins, 137 pseudogenes, four rRNA-coding sequences, 40 tRNAs, and four noncoding RNAs. Two CRISPR (clustered regularly interspaced short palindromic repeats) arrays were found in the genome. The analysis revealed only one gene that encodes the acyl-lipid FA desaturase.

Alignment of the amino acid sequence of the determined FA desaturase (Fig. 1) with known cyanobacterial desaturase sequences (for example, *Synechocystis* sp. PCC 6803: DesA, DesB, DesC, and DesD) [22–24] showed that the desaturase of *Cyanobacterium* belongs to a group of DesC1. This group includes the enzymes that catalyze the reaction of dehydrogenation of Δ9-bond in FAs in both the *sn*-1 positions of membrane lipids [25].

Thus, bioinformatic analysis demonstrated that *Cyanobacterium* sp. IPPAS B-1200 has only one desaturase, which belongs to acyl-lipid Δ9 desaturases of the DesC1 group (Fig. 1). These results correlate with an earlier observation that pointed to the existence of only one gene for the FAs desaturase in the genome of a related cyanobacterium, *Cyanobacterium stanierii* PCC 7202 [26]. The analysis of FAs composition of *Cyanobacterium* sp. IPPAS B-1200 also showed only one type of monounsaturated FAs at position Δ^9 [3].

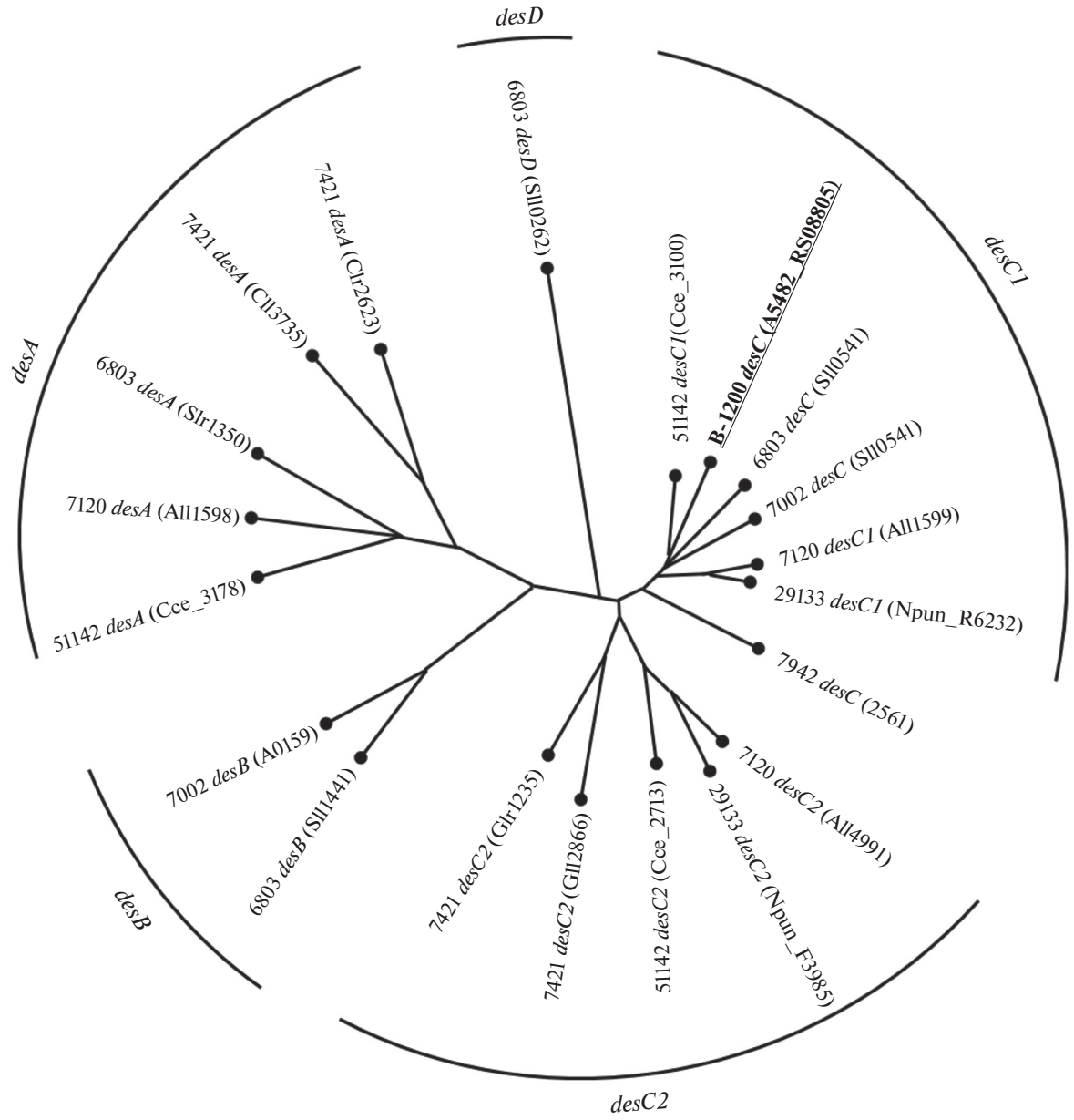

Fig. 1. Functional groups of fatty acid desaturases of different cyanobacteria. The tree was built on the basis of the alignment of amino acid sequences of known FA desaturases. The following designations have been used: 1200—*Cyanobacterium* sp. IPPAS B-1200; 6803—*Synechocystis* sp. PCC 6803; 7002—*Synechococcus* sp. PCC 7002; 7120—*Nostoc* sp. PCC 7120; 51142—*Cyanothece* sp. ATCC 51142; 29133—*Nostoc punctiforme* ATCC 29133; 7942—*Synechococcus elongatus* PCC 7942; 7421—*Gloeobacter violaceus* PCC 7421. The names of the reading frames (in parentheses) are indicated in accordance to gene designations in the annotated genomes.

Cloning and Expression of the desC1 Gene in E. coli

We constructed the expression vector pTrc99A::*desC* that expressed DesC1 under the control of the IPTG-inducible P_{trc} promoter. First step of clone selection was done by PCR-screening of the transformed cells. We performed 16 independent PCR-reactions, including two negative and one positive controls, and 14 reactions demonstrated the presence of the desired insert (Fig. 2a). The size of all cloned PCR-products correlated with the expected value of 828 bp. The vectors from PCR-positive clones were tested by restriction analysis with *Nco* I and *Hin*d III restriction endonucleases. An empty pTrc99A vector was employed in this test as a negative control. Typical results of this restriction analysis are shown in Fig. 2b. A size of one of the bands is ~800 bp, which is the proof of the presence of the insert in a vector. Calculated sizes of short and long fragments are 828 and 4128 bp, respectively,

Table 1. Fatty acid composition of the total lipids of *E. coli* Rosetta cells transformed with the *desC* gene from *Cyanobacterium* sp. strain IPPAS B-1200

FA	Fatty acids, mole %	
	pTrc99a	pTrc99a::*desC*
12:0	6.2	2.8
14:0	13.4	4.9
14:1Δ9	0	1.1
16:0	40.3	7.5
16:1Δ9	1.9	43.6
Me-17:0	23.2	8.1
18:0	0.5	2.1
18:1Δ9	0	0.2
18:1Δ11	4.7	28.9
Me-19:0	9.8	0.8

pTrc99a—FAs of cells transformed with an empty vector pTrc99a; DesC—FAs of cells transformed with the recombinant pTrc99a::*desC*. Showed basic FAs or synthesized de novo. Relative deviation: 2–4% for basic FAs; 0.1–0.5% for minor components.

give a sum of 4956 bp, which comprise the size of the whole recombinant vector.

PAGE showed the appearance of a protein of ~30 kDa in samples that expressed *desC*. That protein was not detectable in other probes (data not shown). This is a confirmation of the expression of *desC* in *E. coli*. Low level of expression and differences with calculated molecular weight are characteristic for the membrane desaturases expressed in heterologous systems [27].

Chromatographic and MS Analysis of FAs

Samples obtained after heterologous expression have been analyzed by GC-MS. Chromatographic profiles are presented in Fig. 3, and the calculated amounts (%) of individuals FAs in each sample are presented in Table 1.

The analysis of FAs composition was performed to reveal the specificity of DesC1 desaturase of *Cyanobacterium* sp. IPPAS B-1200 to the position of a double bond as well as to its preferences towards the length of a carbon chain. Cells of *E. coli* contain enough amounts of saturated C14 and C16 FAs that may serve as the substrates for the cyanobacterial acyl-lipid Δ9 desaturase. However, the content of stearic acid is very low in *E. coli*. This is the reason for low amounts of oleic acid in cells that expressed DesC.

E. coli cells do not have monounsaturated C18 FAs with a double bond at position Δ^9 [28]. The C18 monounsaturate in *E. coli* is represented by *cis*-vaccenic acid (18:1Δ11), which is formed in anaerobic pathways through a reaction of 16:1Δ9 elongation by the FA synthase. A double bond at position Δ^9 in palmitoleic acid is also formed during the synthesis of FAs in *E. coli* but not via the reaction of O_2-dependent desaturation [29].

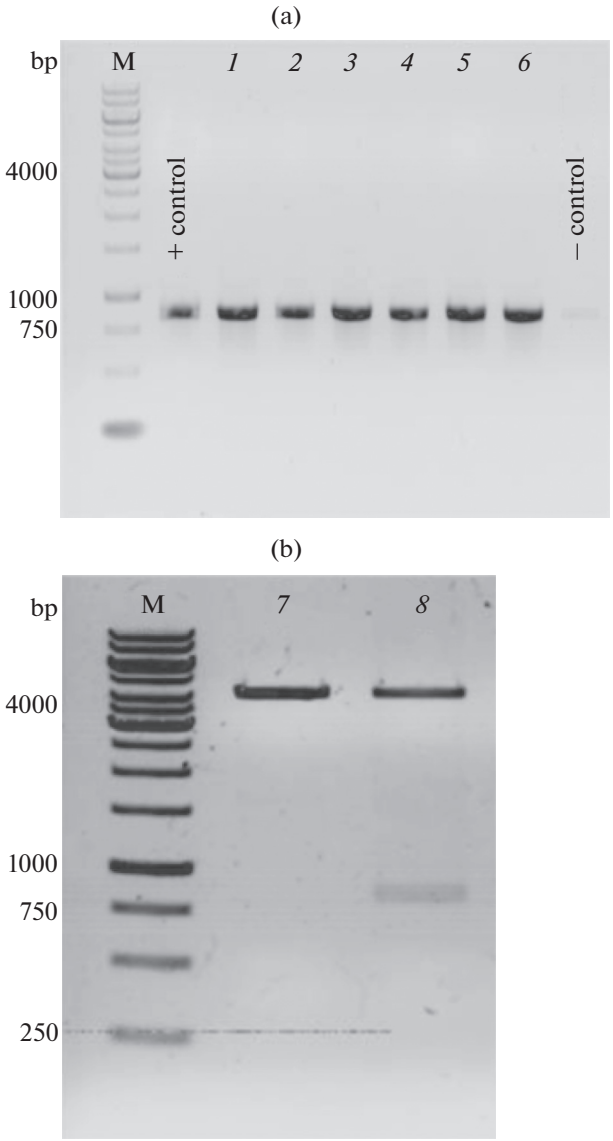

Fig. 2. (a) PCR-products from *E. coli* transformed with pTrc99A::*desC*. M—marker of DNA; "+ control"—PCR-product amplified from genomic DNA of *Cyanobacterium* sp. IPPAS B-1200; "– control"—PCR-product from medium of cultivation. (*1–6*) product of amplification from vectors. (b) DNA fragments obtained after digestion of the recombinant pTrc99a::*desC* plasmid with *Nco* I and *Hin*d III. M—marker of DNA; pTrc99a—vector (*7*); pTrc99a::*desC*—vector with insert DNA, cloning from *Cyanobacterium* sp. IPPAS B-1200 (*8*). DNA fragments were resolved in 1% agarose by gel electrophoresis and stained with ethidium bromide.

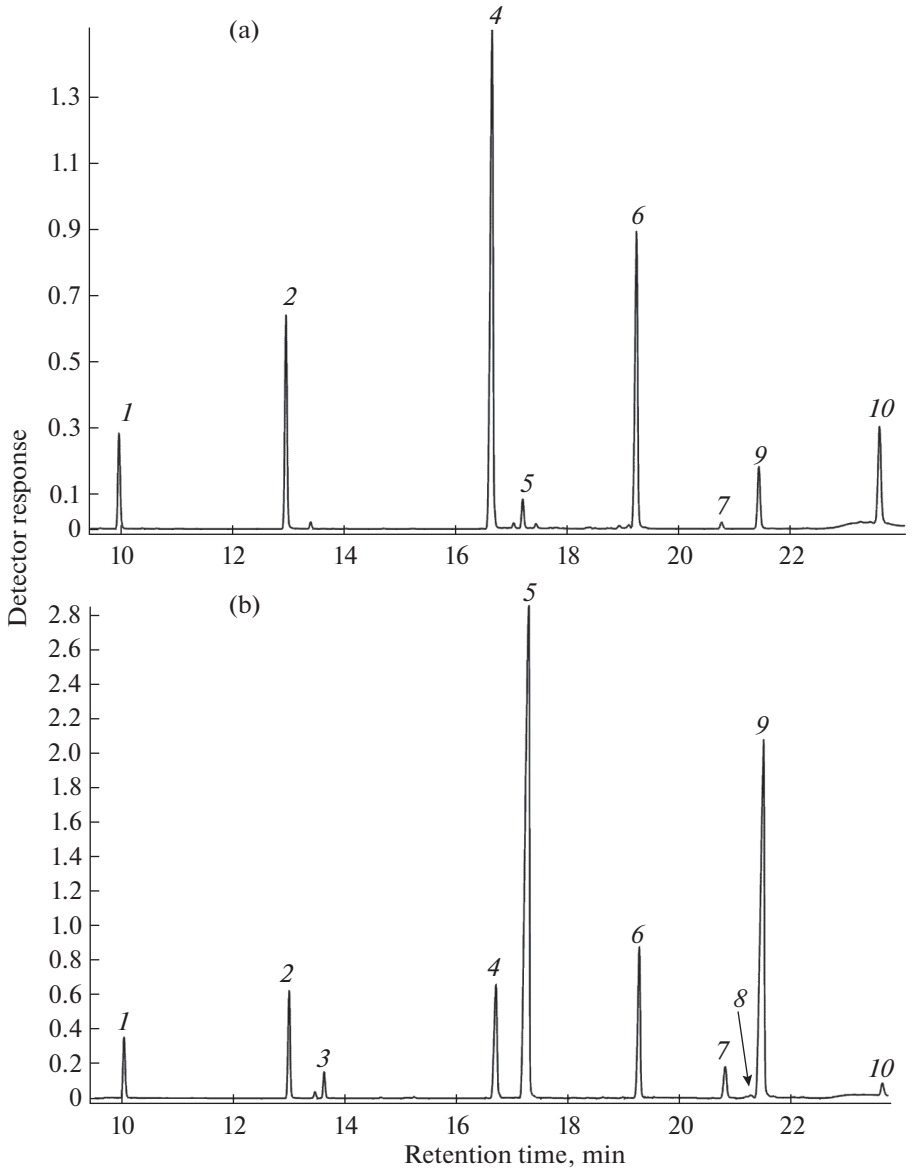

Fig. 3. Qualitative changes in FAs composition of *E. coli* cells expressed DesC1: (a) cells transformed by empty pTrc99a vector; (b) cells transformed by pTrc99a::desC and induced by IPTG. Fatty acids: *1*—lauric (12:0); *2*—myristic (14:0); *3*—myristoleic (14:1Δ9); *4*—palmitic (16:0); *5*—palmitoleic (16:1Δ9); *6*—*cis*-9,10-methylen gexdecanoic (Me-17:0); *7*—stearic (18:0); *8*—oleic (18:1Δ9); *9*—vaccenic (18:1Δ11); *10*—lactobacillic (Me-19:0).

Monosaturated FAs (18:1Δ9 and 18:1Δ11) are distinctly separated by GC, and 18:1Δ9 can be easily detected in a chromatographic profile. In transformed *E. coli*, FAs composition contains more unsaturated FAs, while we see the prevalence of saturated FAs in the control (Table 1, Fig. 2). Data showed that desaturase of *Cyanobacterium* sp. IPPAS B-1200 can use C14, C16, and C18 FAs as substrates.

Thus, expression of the Δ9-desaturase of *Cyanobacterium* sp. IPPAS B-1200 in *E. coli*, provides activity to C14:0, C16:0, and C18:0, and create FAs with double bonds at position Δ9 (14:1Δ9, 16:1Δ9, 18:1Δ9). It confirms that a length of a FA chains in *Cyanobacterium* sp. IPPAS B-1200 is determined by the activity of FA synthase, and that the reaction of desaturation at position Δ9 is nonspecific in relation to the length of the FA chain.

This result correlates with data of our previous work, which is devoted to substrate specificity of one of the FA desaturasea of *Prochlorothrix hollandica*. This organism can synthesize a large amount of saturated and monosaturated C14 FAs. Desaturase of *P. hollandica* is also nonspecific to the length of the carbon chain and forms Δ9-bonds in C14–C18 substrates [30].

ACKNOWLEDGMENTS

The work was supported by grants of the Russian Science Foundation, no. 14-24-00020 (D.A. Los), and of the Ministry of Education and Science of the Republic of Kazakhstan, no. 1582/GF4 (B.K. Zayadan).

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