Applied Energy 111 (2013) 383–393

Contents lists available at SciVerse ScienceDirect

Applied Energy

journal homepage: www.elsevier.com/locate/apenergy

Hydrocarbon profiles and phylogenetic analyses of diversified cyanobacterial species

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HIGHLIGHTS

- The hydrocarbon profiles of 19 cyanobacterial species were studied.
- The conservation of the sequences of 16S rDNA and the two genes encoding the alkane-synthesizing enzymes was analyzed.
- The hydrocarbon distribution pattern in cyanobacteria was proposed with an evolutionary perspective.

ARTICLE INFO

Article history:
Received 7 February 2013
Received in revised form 1 May 2013
Accepted 4 May 2013

Keywords:
Biofuels
Hydrocarbons
Cyanobacteria
Acyl–acyl carrier protein reductase
Aldehyde deformylating oxygenase

ABSTRACT

The combination of environmental concerns and the growing demand for energy make the development of biofuels, an attractive alternative to fossil fuels, a goal for many researchers. The direct photosynthetic production of hydrocarbons, which are the major components of fossil fuels, is considered to be a promising and innovative strategy for the development of biofuels with advanced fuel properties and solar-driven energy input. Cyanobacteria have existed continuously since the early evolution of the biosphere and the biosynthetic pathways of hydrocarbons in these prokaryotes have been genetically and biochemically identified. In this study, the hydrocarbon compositions of 19 freshwater cyanobacterial species distributed among 13 genera were analyzed. Based on their hydrocarbon profiles, these cyanobacterial species were classified into 5 major subgroups. Combined with the previously reported hydrocarbon compositions in different cyanobacterial species, we found that branched-chain alkanes were limited predominantly in filamentous species but rarely in unicellular species. Phylogenetic analysis using traditional small-subunit ribosomal RNA (16S rDNA) of these strains presented clustering similar to their hydrocarbon production profiles. Acyl–acyl carrier protein reductase (AAR) and aldehyde deformylating oxygenase (ADO) are two key enzymes involved in the biosynthesis of hydrocarbons in cyanobacteria. A comparison of phylogenies revealed that the topology of 16S rDNA showed a general congruence with that of AAR but not with that of ADO. The results not only provide an evolutionary perspective with which to study the physiological function of cellular hydrocarbons but also display the engineering capacity to molecularly design diversified hydrocarbon fuel products in cyanobacteria.

1. Introduction

Together, rapidly growing energy demands and environmental concerns caused by the use of fossil fuels render the development of renewable and sustainable biofuels, particularly advanced biofuels with fuel properties similar to fossil fuels, increasingly attractive and urgent. Hydrocarbons produced from biomass resources or directly from solar energy and carbon dioxide through photosynthetic biological systems are becoming a significant goal of research and development in both academia and industry. Cyanobacteria, which are capable of the photosynthetic production of hydrocarbons and exhibit multiple adaptive morphological, biochemical and metabolic properties, have garnered particular attention because of their huge potential for renewable energies [1–3].

Cyanobacteria are common inhabitants of pristine terrestrial and aquatic environments on a global scale and include unicellular and colonial species, which form filaments, sheets or even hollow balls in natural environments [4]. They are known to be monophyletic but morphologically diverse, and traditionally, they are divided into five major subsections according to their

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http://dx.doi.org/10.1016/j.apenergy.2013.05.008

morphological distinctions [5,6]. Cyanobacterial strains of subsection I (e.g., *Chroococcales*, *Prochlorophytes* and *Gloeobacterales*) and II (e.g., *Pleurocapsales*) are unicellular. Subsections III, IV and V form filaments of various morphological complexities. It is reported that the strains of subsection III (e.g., *Oscillatoriales*) have only vegetative cells, but in subsections IV (e.g., *Nostocales*) and V (e.g., *Stigonematales*), vegetative cells can differentiate into morphologically distinct heterocysts [7]. To date, at least 126 cyanobacterial genomes have been sequenced which cover all five traditional subsections, the unicellular, baeocystous, filamentous, heterocystous and ramified phenotypes [6].

Early research in the 1960s showed that hydrocarbons could be produced in a variety of cyanobacterial strains (e.g., *Nostoc* sp., *Anacystis* sp., *Anacystis nidulans*, *Trichodesmium erythaeum*, *Microcoleus chthonoplastes*, *Plectonema terebrans*, *Oscillatoria williamsii*, *Lyngbya lagerhaimii*, etc.). This research also revealed that cyanobacteria mainly produced hydrocarbons with carbon chain lengths varying from C15 to C18 with a predominance of n-C17 compounds [8–10]. Two marine unicellular strains, *Agmenellum quadruplicatum* (currently named *Synechococcus* sp. PCC 7002) and *Coccochloris elabens* (currently named *Synechococcus* sp. PCC 7003), are known to synthesize C19 hydrocarbons with either one or two double bonds [8]. These initial investigations concerning hydrocarbon production in cyanobacteria were focused on their potential geochemical and taxonomic significance [11,12], and there were few further studies on hydrocarbon biosynthetic processes at the molecular level in the following decades.

Eukaryotic microalgae are an important resource for biofuel production [13–15]. Unlike microalgae, which accumulate large amounts of triacylglycerols (TAGs) through lipid metabolism involving several different cellular compartments, cyanobacteria contain few or no TAGs. Fatty acid metabolism in cyanobacteria is associated with a type II fatty acid synthesis (FASII) and is conducted by a multienzyme system in the cytoplasm. Fatty acid molecules must be activated to fatty acyl-thioesters by fatty acyl-CoA synthetase (ACS) or fatty acyl-ACP synthetase (AAS) before hydrocarbons can be synthesized. Free fatty acids exist mainly in the form of C18 with a small amount of C16 in cyanobacteria, thus the predominant hydrocarbon is C17. One precursor of hydrocarbon biosynthesis in cyanobacteria is thought to be fatty acyl–acyl carrier proteins (ACPs), which are essential metabolites for the production of lipid-based biofuels [3,16–18].

Until recent years, the common pathway for hydrocarbon production in cyanobacterial species was only preliminarily identified. This pathway consists of an acyl–acyl carrier protein (ACP) reductase (AAR), which reduces acyl-ACPs to aldehydes [1], and an aldehyde deformylating oxygenase (ADO), which converts aldehydes into alka(e)nes [1,19–23]. To our knowledge, there are four other hydrocarbon biosynthetic pathways in prokaryotes: (i) Isoprenoid biosynthesis involves the condensation of isoprene units to generate hydrocarbons with multiples of five carbon atom units (C10, C15, C20, etc.) [24]. (ii) In representative bacteria from diverse phyla (*Verucomicrobia*, *Planctomyces*, *Chloroflexi*, *Proteobacteria*, *Actinobacteria*, etc.), different types of long-chain olefinic hydrocarbons are synthesized from the head-to-head condensation of fatty acids, which depends on Ole proteins [25,26]. (iii) In *Jeotgalicoccus* sp. ATCC 8456, terminal olefins are synthesized through a direct fatty acid decarboxylation mechanism catalyzed by P450 fatty acid decarboxylase [27]. (iv) In the single-celled cyanobacterial strain *Synechococcus* sp. PCC 7002 mentioned above, hydrocarbon biosynthesis employs an elongation–decarboxylation mechanism, which is involved in the conversion of fatty acyl-ACPs to olefins [28]. The recent identification of different hydrocarbon biosynthetic pathways in diverse bacteria and cyanobacteria display the potential and feasibility to improve hydrocarbon production in cyanobacteria by employing genetic, enzymatic and metabolic engineering strategies. Our previous work demonstrated the viability of a cyanobacteria-based platform that targets fatty acid–based biofuels and discussed the possibility of controlling and regulating the properties of hydrocarbon fuels by choosing different starting cyanobacterial strains or by manipulating hydrocarbon biosynthesis pathways [2,29]. However, why cyanobacterial species produce hydrocarbons and how different types of hydrocarbons are synthesized are phenomena that have not yet been elucidated.

In this study, the hydrocarbon composition of 19 freshwater cyanobacterial species distributed among 13 genera was surveyed. By structurally characterizing the species of hydrocarbons from each strain and subsequently comparing the strains and their composition to previously reported work, we found that most fatty alka(e)nes with branched chains appear to exist predominantly in filamentous strains but rarely in unicellular strains. Additionally, we examined the evolutionary relationship of AAR and ADO with hydrocarbon biosynthesis in cyanobacteria, which might provide an evolutionary perspective on the hydrocarbon biosynthetic pathways in cyanobacteria.

2. Materials and methods

2.1. Chemicals and reagents

Eicosane was obtained from Sigma–Aldrich (USA). All other chemicals were obtained from either Merck (Germany) or Amaresco (USA). Oligonucleotide synthesis and sequencing were performed by Sunny (Shanghai, China). *Taq* and *Pfu* DNA polymerases were purchased from Fermentas (Canada). The kits used for molecular cloning were from Omega or Takara (Japan).

2.2. Strains, culture conditions and growth curves

Synechocystis sp. PCC 6803, *Anabaena* sp. PCC 7120 and *Synechococcus elongatus* PCC 7942 were generous gifts from Professor Xudong Xu of the Institute of Hydrobiology, Chinese Academy of Sciences. The *Nostoc punctiforme* strain ATCC 29133 was kindly provided by Professor John C. Meeks of the University of California, Davis. All other cyanobacterial strains used in this study were purchased from the Freshwater Algal Culture Collection of the Institute of Hydrobiology (FACHB), Chinese Academy of Sciences.

The original source of all the strains used in this study is FACHB monocultures. Three methods were used to ensure that there was no contamination. First, each strain was regularly inspected via microscopy for the entire duration of the experiments to confirm that they were not contaminated by other algae. Second, during the 16S rDNA analysis, three replicates of sequenced 16S rDNA fragments were consistent with one another for each strain; this showed that there was no contamination at the molecular level. Third, we used Luria–Bertani medium to check for contamination by heterotrophic bacteria and found that each strain was axenic.

The cyanobacterial strains used in this study were grown at 30 °C in 500 mL flasks, each of which contained 300 mL of air-infused BG11 medium [30] under 30–50 $\mu E/m^2/s$ of white light. The dry cell weights (DCWs) of cyanobacterial cells were used for the determination of cell growth curves. The seed cultures were grown under the conditions mentioned above for approximately 5 days using 5% (v/v) inoculums. Every 2 days, 5 mL of the thoroughly mixed culture was filtered onto dried and pre-weighed nitrocellulose filter membranes (25 mm in diameter, 0.22 μm in mesh) and then dried at 110 °C for approximately 24 h to constant weight; the DCW was calculated by subtracting the initial weight of the filter. The DCW data shown in Table 2 represents the means ± standard deviations of the values from three replicates.

2.3. Light microscopy and morphology analyses of cyanobacterial strains

Images of cyanobacterial strains were captured using an Olympus CX31 microscope equipped with an Olympus DP-72 digital camera (Olympus, Tokyo, Japan) using the software DP2-BSW (version 2.2). The exposure time was set at 20 ms. At 100× magnification on the light microscope, six digital images of cyanobacteria were randomly taken. The morphology of the cyanobacterial cells was ascertained using these images.

2.4. Hydrocarbon extraction and analytical methods

Cyanobacterial cells were harvested from 200 mL of culture by centrifugation (6000×*g*, 5 min). The cells were suspended in 10 mL of TE buffer (pH 8.0) and then lysed by sonication. The lysate was extracted for 2 h at room temperature with 10 mL of chloroform–methanol (v/v, 2:1). Prior to extraction, 30 μg of eicosane (for hydrocarbon analysis) was added to the cell lysate as the internal standard. The organic phase was separated following centrifugation (3000×*g*, 5 min) and evaporated to dryness under nitrogen at 55 °C. The solute was then dissolved in 1 mL of n-hexane and a 1 μL aliquot was analyzed by GC–MS using an Agilent 7890A system equipped with a HP-INNOWax (30 m × 250 μm × 0.25 μm). Helium (constant flow 1 mL/min) was used as the carrier gas. For hydrocarbon analysis, the temperature of the injector was 250 °C. The following temperature program was applied: 40 °C for 1 min, an increase of 5 °C/min to 200 °C, and then an increase of 25 °C/min to 240 °C for 15 min. The internal standard was used to determine the product yield. The hydrocarbon yields shown in Table 2 represent the means ± standard deviations of the values from three replicates.

2.5. DNA extraction

The DNA extraction protocol was based on previously described procedures for extracting total DNA from lake cyanobacterial samples [31]. A volume of 15 mL of cyanobacterial cells in the exponential phase was collected by centrifugation, washed with 10 mL of TE (10 mmol/L Tris.Cl, 1 mmol/L EDTA, pH 8.0) and then resuspended in 1 mL of TE. The cells were lysed with 1% SDS at 37 °C for 1–2 h. After digestion with 100 μg/mL proteinase K at 37 °C for an additional 2 h, the cell lysate was extracted twice with phenol–chloroform and once with chloroform. Genomic DNA was precipitated with ethanol, and RNA was removed with RNase A. All DNA extracts were dissolved in sterilized double-distilled water (ddH_2O) and stored at −20 °C.

2.6. Degenerate oligonucleotide design and touchdown polymerase chain reactions (PCR)

Protein sequences with a similarity to *Synechocystis* sp. PCC 6803 ADO and AAR were retrieved using BLASTp from Cyanobase (http://genome.kazusa.or.jp/cyanobase). Multiple sequence alignments were performed using ClustalX v2.0 software [32], and degenerate oligonucleotide primers were designed based on the conserved regions. All primers used in this study are listed in Table 3. A mixture of *Taq* and *Pfu* (1:1) DNA polymerases was used in the PCR reactions. Touchdown PCR was performed with an initial denaturation step at 95 °C for 5 min followed by 10 cycles of 0.5 min at 95 °C, 0.5 min at 55–45 °C (decreasing 1 °C every cycle), 2 min at 72 °C, 20 additional cycles at 95 °C for 0.5 min, 45 °C for 0.5 min and then 72 °C for 2 min. The tubes were then incubated for 10 min at 72 °C. After the addition of dA to their ends with *Taq* DNA polymerase, the DNA fragments were cloned into a pMD18-T vector and sequenced.

2.7. Phylogenetic analyses

The sequences of 16S rDNA *ado* and *aar* were chosen for the phylogenetic analyses. For 5 model species (*Syneochcystis* sp. PCC 6803, *Synechococcus elongetus* PCC 7942, *Anabaena* sp. PCC 7120, *Microcystis* sp. PCC 7806, and *Nostoc punctiforme* ATCC 29133), the sequenced genomes were retrieved from Cyanobase (http://genome.kazusa.or.jp/cyanobase). For the other 14 FACHB cyanobacterial strains, genome sequences were amplified by degenerated PCR and sequenced. Multiple sequence alignments were performed using software packages ClustalX v2.0 [32]; the results were manually confirmed. Phylogenetic trees were constructed using the neighbor-joining algorithm within MEGA v5.1 and were verified using the maximum likelihood method [33]. The Jukes–Cantor model with frequencies (for the neighbor-joining method) and the WAG substitution model (for the maximum likelihood method) were used with a gamma-distributed rate of variation across sites. To assure statistical significance, 1000 bootstraps were used in the computation of each tree, and bootstrap values greater than 70% were shown.

2.8. Nucleotide sequence accession number

The partial sequences of 16S rDNA *ado* and *aar* of 14 FACHB strains obtained in this study were deposited in GenBank. The associated accession numbers are listed in Table S1.

3. Results

3.1. Evaluation of the hydrocarbon-producing capability of different cyanobacterial strains

In this study, 19 common cyanobacterial strains distributed among 13 genera were autotrophically grown with continuously filtered air and then evaluated for hydrocarbon production. Hydrocarbons were then identified using GC–MS-based analyses of the organic solvent extracts of cell lysates. Almost all strains tested entered the stationary phase after growing for 12–16 days (Table 2). The biomass concentration of different strains varied greatly, with the lowest value of 0.7 g/L dry cell weight (DCW) in *Nostoc punctiforme* FACHB 252 and the highest value of 1.3 g/L DCW in *Nostoc spongiaeforme* FACHB 130. The growth rate was determined using DCW per liter, and all unicellular strains grew faster than filamentous strains before entering the exponential phase (data not shown). The hydrocarbon content of 9 strains exceeded 1 mg/g DCW with the maximum content of 1.8 mg/g DCW observed in *Nostoc spongiaeforme* FACHB 130. The hydrocarbon yield per gram DCW at the late stationary phase ranged from 0.2 to 1.8 mg, revealing a great variation among the different cyanobacterial strains distributed among 13 genera (Table 2). The maximum hydrocarbon yield was found in *Nostoc spongiaeforme* FACHB 130, which has the ability to grow in a homogenously suspended mode.

3.2. Hydrocarbon production profiles of unicellular and filamentous cyanobacterial strains

The hydrocarbon composition of each chosen cyanobacterial strain is shown in Fig. 1 as a weight percentage. The hydrocarbon profiles of the 19 strains studied here were generally classified into 5 major types, and heptadecane occurred in all 5 types. In addition to heptadecane, all 6 strains of type 1 contained 8-methylheptadecane in varied amounts. Two members of *Nostoc* in type 2 were first detected to produce 4-ethyltetradecane in cyanobacteria based on a preliminary GA-MS analysis. The strains classified as type 3, including two *Microcystis* species and other filamentous

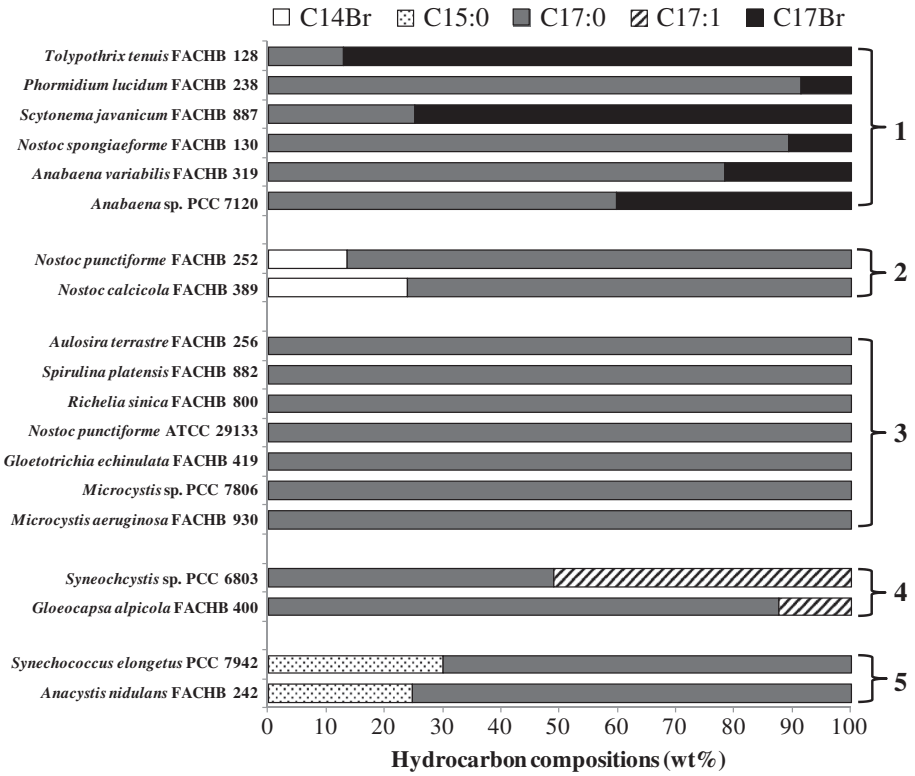

Fig. 1. Different hydrocarbon profiles of the 19 cyanobacterial strains studied here. (Numbers before the colon indicate the numbers of carbon atoms and numbers after the colon indicate the numbers of double bonds; C14Br and C17Br indicate ethyltetradecane and methylheptadecane, respectively; The data are presented as the weight percentage of the corresponding hydrocarbons).

strains, were showed only heptadecane. In addition to heptadecane, C17:1 and C15:0 hydrocarbons were also found in those strains belonging to type 4 and type 5, respectively. When the 19 strains were morphologically grouped, we found that type 1 and 2 are all filamentous and are capable of producing branched hydrocarbons (methylheptadecane or ethyltetradecane). Fig. 2 showed the photomicrographs and GC–MS profiles of *Anabaena variabilis* FACHB 319 (Fig. 2A), *Nostoc cacicola* FACHB 389 (Fig. 2B), *Richelia sinica* FACHB 800 (Fig. 2C), *Synechocystis* sp. PCC 6803 (Fig. 2D), and *Anacystis nidulans* FACHB 242 (Fig. 2E), which were representatives of each of the 5 types of cyanobacterial strains present in this study. The previous reports on the production of C17 and other hydrocarbons in cyanobacteria have been summarized together (Table 1). The analysis of hydrocarbon production profiles and the morphologies of these cyanobacterial strains showed a tendency for ethyltetradecane and methylheptadecane to be present in filamentous cyanobacterial strains.

3.3. Identification of an ado–aar pathway of hydrocarbon biosynthesis in all cyanobacterial strains used in this study

Two pairs of degenerate oligonucleotide primers (*ado*-1/*ado*-2, *aar*-1/*aar*-2, Table 3) were designed to amplify the major sequences of *ado* and *aar* based on multiple sequence alignments (Fig. 3). Because *ado* and *aar* in most cyanobacteria are co-located genes retrieved from Cyanobase (http://genome.kazusa.or.jp/cyanobase), we chose *ado*-1 for the forward primer and *aar*-2 for the reverse primer to amplify the possible *ado–aar* operon. For *Nostoc spongiaeforme* FACHB 130, *Nostoc punctiforme* FACHB 252, *Gloeocapsa alpicola* FACHB 400 and *Microcystis aeruginosa* FACHB 930, both the *ado* and *aar* partial sequences were amplified with *ado*-1/*ado*-2 and *aar*-1/*aar*-2, respectively. By DNA sequencing and multiple sequence alignments, we finally obtained the major sequences of both genes in all cyanobacterial strains in this study. The length of *ado* partial sequences with a complete 3′ end was approximately 621bp (except *Nostoc spongiaeforme* FACHB 130, *Nostoc punctiforme* FACHB 252, *Gloeocapsa alpicola* FACHB 400 and *Microcystis aeruginosa* FACHB 930, which lack a complete 3′ end), and the length of the *aar* partial sequences with a complete 5′ end was approximately 942bp. The *ado–aar* pathway of hydrocarbon biosynthesis is present in all strains used in this study.

3.4. Analyses of the phylogenetic distribution of 16S rDNA, ADO and AAR in cyanobacterial strains

The 16S rDNA regions were amplified by PCR with genomic DNA as a template using degenerate primers CYA106F and CYA781R(a)/CYA781R(b) (Table 3) as described by Nubel et al. [34]. An approximate 663bp sequence of 16S rDNA (Table S1) was cloned and sequenced, and dendrograms based on these sequences were constructed. Of the 20 cyanobacterial strains, 13 strains are filamentous and the other 7 strains are unicellular (Fig. 4A and Fig. S1A). *Phormidium lucidum* FACHB 238 and *Spirulina platensis* FACHB 882 are filamentous strains belonging to the traditional subsection III taxa that lack the capability of heterocyst formation. In 16S rDNA trees, these two strains are placed with unicellular species, indicating polyphyletic origins of filamentous morphology within cyanobacteria, as previously reported [5]. When morphological examination and the analyses of the hydrocarbon profiles were combined with the phylogenetic analysis based on 16S rDNA sequences of 20 cyanobacterial strains, we found that strains with type 1 and type 2 hydrocarbon profiles were all filamentous, whereas the strains with type 4 and type 5 hydrocarbon profiles were all unicellular. The strains with a type 3 hydrocarbon profile were placed within a group of mixed

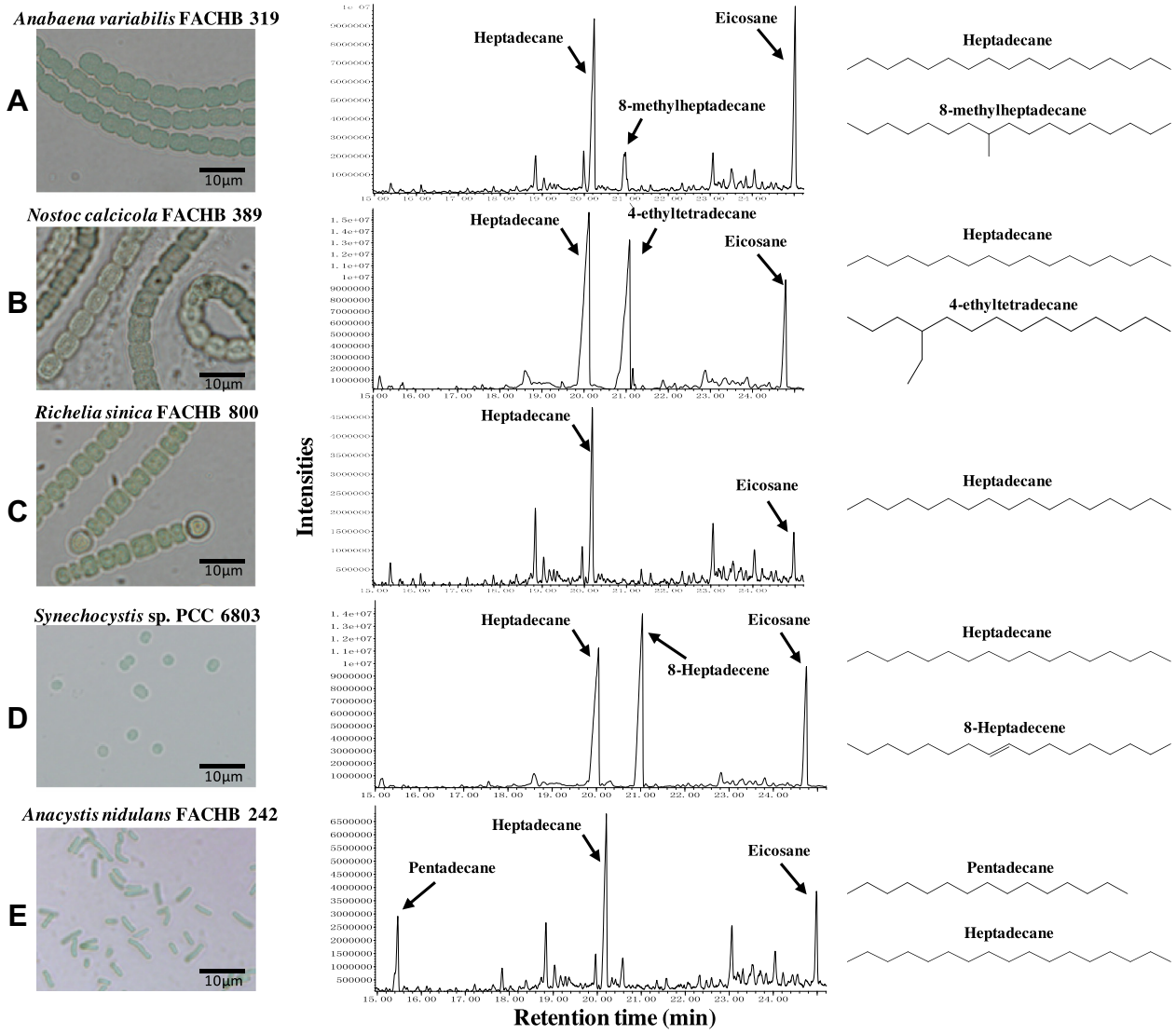

Fig. 2. Morphological identification and GC–MS analysis of hydrocarbon compositions of representative cyanobacterial strains (A) *Anabaena variabilis* FACHB 319, (B) *Nostoc cacicola* FACHB 389, (C) *Richelia sinica* FACHB 800, (D) *Synechocystis* sp. PCC6803, and (E) *Anacystis nidulans* FACHB 242. Eicosane is used as an internal standard. Hydrocarbons produced by cyanobacteria are indicated by arrows, and their chemical structures are shown on the right side.

morphology. This mixed morphology group contains 5 filamentous strains and 3 unicellular strains, among which is *Synechococcus* sp. PCC 7002, which lacks a regular *ado–aar* two-gene pathway but has an olefin biosynthesis pathway. To further examine the molecular evolution of key enzymes involved in hydrocarbon biosynthesis, the deduced amino acid sequences were used to construct phylogenetic trees by neighbor-joining and maximum likelihood methods. The AAR phylogeny shows a similar pattern to the 16S rDNA analyses in that the sequences of cyanobacterial strains with types 2, 4 and 5 hydrocarbon profiles form a small monophyletic clade (Fig. 4B and Fig. S1B). For strains possessing a type 1 or 3 hydrocarbon profile, there was a relatively close relationship but without the formation of an obvious subclade. However, in the ADO phylogenetic analysis, the distribution of hydrocarbon profile types follows no discernible pattern that can be used for the examination of a phylogenetic relationship (Fig. 4C and Fig. S1C). These analyses showed that a 16S rDNA tree has a topology similar to that of AAR but not to that of ADO, which indicates that AAR enzymes most likely have an earlier origin than those of ADO.

4. Discussion

The hydrocarbon production profiles of the 19 cyanobacterial strains in this study were classified into 5 major types with a novel distribution pattern. The branched-chain hydrocarbons appeared to be present predominantly in filamentous strains because no side-chain hydrocarbons were found in all the single-celled strains used in this study (Fig. 1). However, previous reports revealed that several unicellular cyanobacterial species also produced branched hydrocarbons. For example, methylheptadecane could be detected in the unicellular *Anacystis nidulans*, *Anacystis cyanea* and *Chroococcus turgidus* in the 1970s [1,10,16] (Table 1). However, the amounts of these branched alkanes were small and not much larger than the error of the instrument. Moreover, methylheptadecane was not found in *Anacystis nidulans* FACHB 242 in our study. Further investigations are required to provide a solid conclusion for a branched-chain distribution pattern. The species studied in this work are mainly distributed in subsections I, III and IV of the five traditional subsections of cyanobacteria. Although the baeocystous type (subsection II) and the ramified type (subsection V) exhibit

Table 1
Hydrocarbon production profiles in cyanobacterial strains previously reported. (Numbers before the colon indicate numbers of carbon atoms; Numbers after the colon indicate numbers of double bonds).

Strains	C15:0	C17:0	C17:1	C17Br[a]	C19:n[b]	References
Unicellular cyanobacteria						
Agmenellum quadruplicatum					+	[8]
Anacystis cyanea		+				[16]
Anacystis montana[c]			+	+		[48]
Anacystis Montana	+	+	+			[49]
Anacystis nidulans[c]	+	+	+	+		[8,10,16,46,50,51]
Coccochloris elabens					+	[8]
Cyanothece sp. ATCC 51142	+					[1]
Cyanothece sp. PCC 7425		+				[1]
Gloeobacter violaceus PCC 7421		+				[1]
Microcystis aeruginosa	+	+				[38]
Prochlorococcus marinus CCMP 1986	+					[1]
Synechococcus bacillaris	+	+	+			[52]
Synechococcus elongatus PCC 6301		+				[1]
Synechococcus elongatus PCC 7942	+	+				[1]
Synechococcus sp.					+	[53]
Synechococcus sp. PCC 7002					+	[28]
Synechocystis sp. PCC 6803		+				[1]
Synechocystis sp. UTEX 2470		+	+			[54]
Filamentous cyanobacteria						
Anabaena cylindria		+	+	+		[49]
Anabaena variabilis ATCC 29413		+		+		[1]
Anabaena variabilis		+		+		[37,50]
Calothriax scopulorum		+		+		[55]
Calothrix sp.		+				[51]
Chlorogloea fritschii		+		+		[16]
Limnothrix redekei		+	+	+		[56]
Lyngbya aestuarii	+	+		+		[16]
Lyngbya lagerhaimii		+		+		[8]
Microcoleus chthonoplastes	+	+				[8]
Microcoleus lyngbyaceus	+	+	+			[51]
Microcoleus lyngbyaceus	+	+				[16]
Microcoleus vaginatus		+	+	+		[57,58]
Microcoleus vaginatus		+		+		[49]
Nostoc commune	+	+	+	+		[51]
Nostoc endophytum		+		+		[49]
Nostoc muscorum		+		+		[8,45]
Nostoc punctiforme PCC 73102		+				[1]
Nostoc sp.		+		+		[10,59]
Nostoc sp. PCC 7120		+		+		[1]
Oscillatoria f. *granulate*		+				[60]
Oscillatoria williamsii	+	+				[8]
Osillatoria woronichinii	+	+				[61]
Phormidium luridum		+		+		[50,52]
Planktothrix agardhii		+	+	+		[56]
Planktothrix mougeotii		+	+	+		[56]
Planktothrix suspense		+	+	+		[56]
Planktothrix rubescens		+	+	+		[56]
Plectonema terebrans	+	+	+			[8]
Prochloron sp.	+	+				[8]
Pseudanabaena sp.	+	+				[62]
Scytonema sp.[d]		+	+			[18]
Spirulina platensis		+	+			[51]
Spirulina platensis	+	+	+			[16,51]
Trichodesmium erythraeum		+				[8]

[a] Methylheptadecane.
[b] C19:1 and C19:2.
[c] Trace amount of methylheptadecane were detected in these unicellular strains [10,16,48].
[d] There was also 1-heptadecyne in these strains.

unicellular and filamentous phenotypes, respectively [6], further study of these subsections is necessary for a thorough understanding of the hydrocarbon distribution in cyanobacteria on the phylum level. *Nostoc spongiaeforme* FACHB 130, which showed a greater hydrocarbon production than all wild type strains previously reported [18,29], was identified as a prospective strain for the production of hydrocarbons with a biomass productivity of 1.3 g/L DCW and a hydrocarbon yield of 1.8 mg/g DCW (Table 2). It is well known that hydrocarbons can constitute up to 75% of the DCW of *Botryococcus braunii*, an eukaryotic microalga belonging to the green algae division of chlorophyta [35]. However, it grows slowly relative to most cyanobacteria. The hydrocarbons produced in *Botryococcus braunii* usually have long aliphatic chains (from C25 to C40 among different species), which require further pyrolysis or catalytic cracking for use in internal combustion engines.

C17 hydrocarbon is widely distributed in most cyanobacterial strains. Although the qualitative characteristics of major fatty acids in cyanobacteria were not significantly affected by the culture conditions, the relative quantities are easily influenced by growth

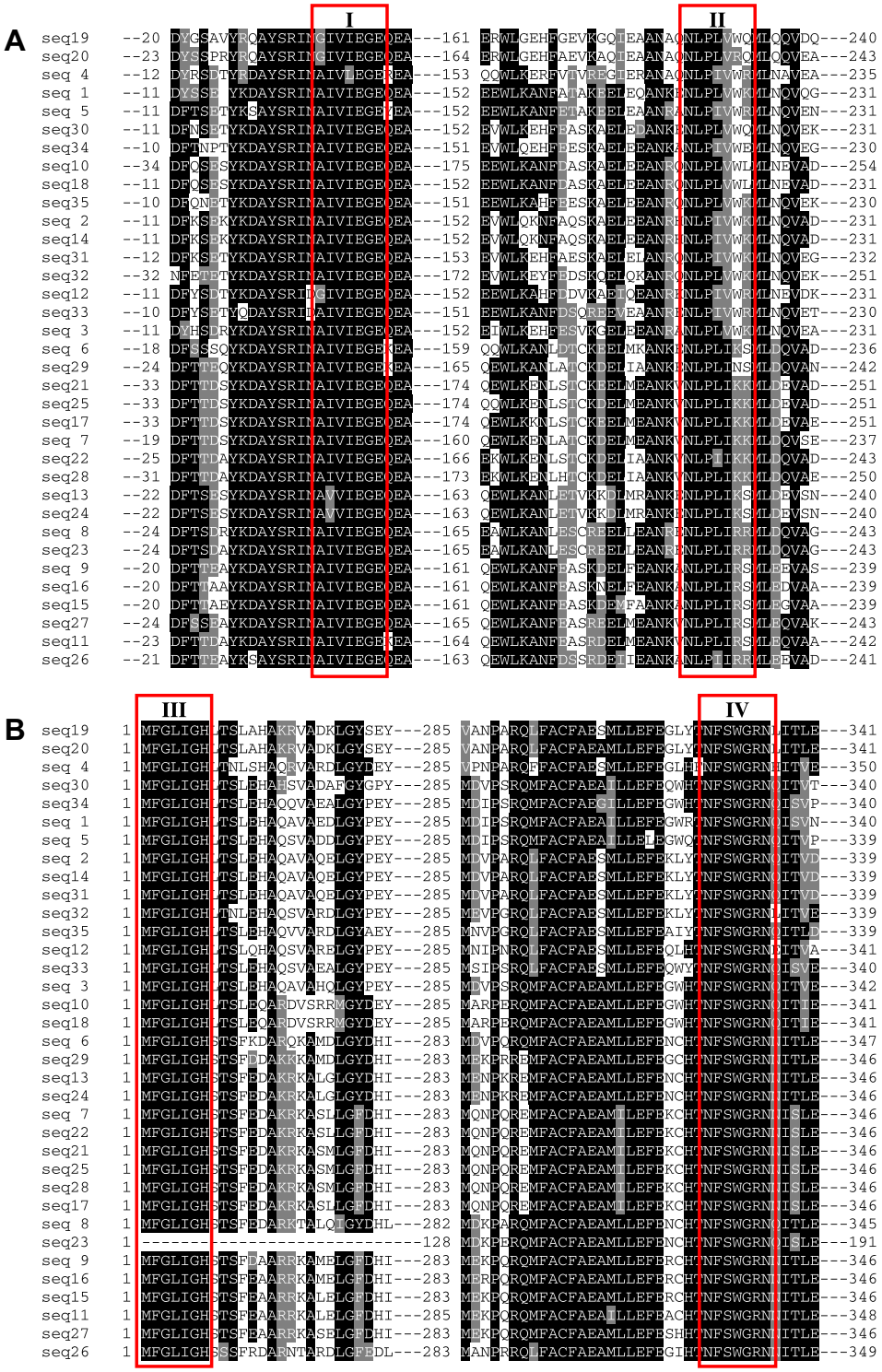

Fig. 3. Allignment of partial deduced amino-acid sequences of ADO (A) and AAR (B) orthologs retrieved from previously known cyanobacterial genomes from Cyanobase. Red boxed I, II, III and IV indicate conserved regions for designing degenerated oligonucleotides of *ado*-1, *ado*-2, *aar*-1 and *aar*-2, respectively. (seq1) *Synechocystis* sp. PCC 6803, (seq2) *Anabaena* sp. PCC 7120, (seq3) *Thermosynechococcus elongatus* BP-1, (seq4) *Gloeobacter violaceus* PCC 7421, (seq5) *Microcystis aeruginosa* NIES-843, (seq6) *Prochlorococcus marinus* SS120, (seq7) *Prochlorococcus marinus* MED4, (seq8) *Prochlorococcus marinus* MIT9313, (seq9) *Synechococcus* sp. WH8102, (seq10) *Synechococcus elongatus* PCC 6301, (seq11) *Synechococcus* sp. CC9311, (seq12) *Acaryochloris marina* MBIC11017, (seq13) *Prochlorococcus marinus* str. NATL2A, (seq14) *Anabaena variabilis* ATCC 29413, (seq15) *Synechococcus* sp. CC9902, (seq16) *Synechococcus* sp. CC9605, (seq17) *Prochlorococcus marinus* str. MIT 9312, (seq18) *Synechococcus elongatus* PCC 7942, (seq19) *Synechococcus* sp. JA-2-3B'a(2-13), (seq20) *Synechococcus* sp. JA-3-3Ab, (seq21) *Prochlorococcus marinus* str. AS9601, (seq22) *Prochlorococcus marinus* str. MIT 9515, (seq23) *Prochlorococcus marinus* str. MIT 9303, (seq24) *Prochlorococcus marinus* str. NATL1A, (seq25) *Prochlorococcus marinus* str. MIT 9301, (seq26) *Synechococcus* sp. RCC307, (seq27) *Synechococcus* sp. WH 7803, (seq28) *Prochlorococcus marinus* str. MIT 9215, (seq29) *Prochlorococcus marinus* str. MIT 9211, (seq30) *Cyanothece* sp. ATCC 51142, (seq31) *Nostoc punctiforme* ATCC 29133, (seq32) *Trichodesmium erythraeum* IMS101, (seq33) *Cyanothece* sp. PCC 7425, (seq34) *Cyanothece* sp. PCC 8801, (seq35) *Arthrospira platensis* NIES-39.

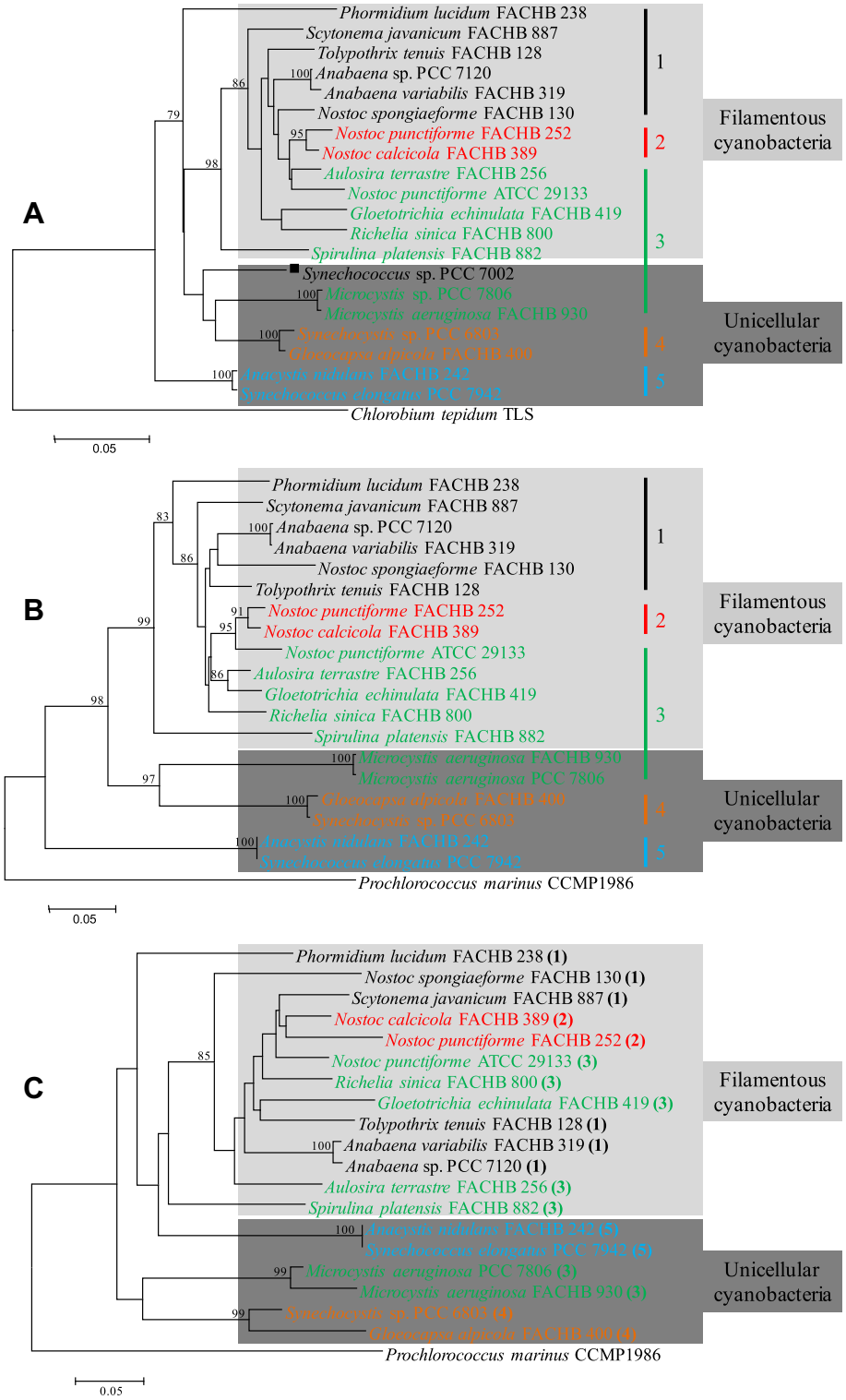

Fig. 4. Phylogenetic relationships of cyanobacteria inferred from partial sequences of 16S rDNA (A) and deduced amino sequences of AAR (B) and ADO (C). Trees were constructed by the neighbor joining method. Hydrocarbon profile subgroups 1–5 of Fig. 1 are denoted to the right of the vertical bar (A and B) and within brackets (C) in different colors. The numbers at each branch point are the bootstrap values for the percentages of 1000 replicate trees; only values >70% are shown. The black square indicates the position of *Synechococcus* sp. PCC 7002 that lacks a regular *aar–ado* two-gene pathway (A). *Chlorobium tepidum* TLS and *Prochlorococcus marinus* CCMP 1986 are used as outgroups of 16S rDNA and AAR/ADO, respectively.

and environmental conditions [36]. The hydrocarbon content and composition of cyanobacterial strains were also affected by the growth state and stress conditions. Under normal culture conditions, the relative amount of 8-methylheptadecane in *Anabaena variabilis* varies from 22% in young cultures to 84% in cultures grown for 9 days, but the total hydrocarbon percentage of the DCW remained at approximately 0.09% [37]. The abnormal component of the hydrocarbons, heptadecyne, had been found in UVB irradiated *Scytonema* sp. (1-heptadecyne) and iron/light stressed *Microcystis aeroginosa* (5-hexadecyne), and the amount of other

Table 2
Times required for reaching the stationary phase, biomass concentration and hydrocarbon productivity of 19 cyanobacterial strains studied in this work (The data of biomass and hydrocarbon yield represent means ± standard deviations of the values from 3 replicates).

Cyanobacterial strains	Time (days)	Biomass (g/L)	Production of hydrocarbons (mg/g DCW)
Unicellular cyanobacteria			
Syneochcystis sp. PCC 6803	12	1.2 ± 0.08	1.3 ± 0.11
Gloeocapsa alpicola FACHB 400	14	0.8 ± 0.04	1.1 ± 0.07
Anacystis nidulans FACHB 242	12	1.2 ± 0.05	0.4 ± 0.04
Synechococcus elongatus PCC7942	12	0.8 ± 0.1	0.5 ± 0.02
Microcystis aeruginosa FACHB 930	16	1.0 ± 0.13	0.4 ± 0.05
Microcystis aeruginosa PCC 7806	16	1.0 ± 0.03	0.3 ± 0.01
Filamentous cyanobacteria			
Nostoc spongiaeforme FACHB 130	16	1.3 ± 0.09	1.8 ± 0.21
Anabaena sp. PCC 7120	12	1.1 ± 0.04	1.6 ± 0.14
Phormidium lucidum FACHB 238	12	0.9 ± 0.21	1.5 ± 0.21
Nostoc calcicola FACHB 389	14	0.8 ± 0.2	1.6 ± 0.14
Spirulina platensis FACHB 882	16	1.0 ± 0.05	1.2 ± 0.13
Nostoc punctiforme ATCC 29133	12	1.0 ± 0.05	1.1 ± 0.17
Scytonema javanicum FACHB 887	16	0.8 ± 0.08	1.3 ± 0.22
Gloetotrichia echinulata FACHB 419	14	1.2 ± 0.02	0.8 ± 0.12
Aulosira terrastre FACHB 256	16	1.2 ± 0.03	0.8 ± 0.18
Anabaena variabilis FACHB 319	12	1.0 ± 0.02	0.7 ± 0.13
Richelia sinica FACHB 800	16	1.1 ± 0.02	0.4 ± 0.21
Nostoc punctiforme FACHB 252	12	0.7 ± 0.14	0.6 ± 0.23
Tolypothrix tenuis FACHB 128	14	1.2 ± 0.11	0.2 ± 0.13

hydrocarbons increased after stress in both cyanobacterial species [38,39]. In this study, 4-ethyltetradecane was found in *Nostoc calcicola* FACHB 389 and *Nostoc punctiforme* FACHB 252. This is the first time that a high content (>10%) of ethyltetradecane from a cyanobacterial species has been identified. In *Nostoc calcicola* FACHB 252, the amount of ethyltetradecane ranged from approximately 20% (Fig. 1) to nearly 50% of the total hydrocarbons (Fig. 2B), which varied with the culture time. In general, the C17 family of heptadecane, heptadecene and 8-methylheptadecane comprise the main hydrocarbons found in most cyanobacteria studied to date.

The structural determination of cyanobacterial hydrocarbons in this work was mainly based on the comparison of mass fragmentation patterns to the NIST mass spectral library. GC–MS cannot be used to assign the accurate position of a carbon–carbon double bond or a branched chain in hydrocarbons. The hydrocarbon composition of *Synechocystis* sp. PCC 6803 is consistent with the previous reports of Tan et al. [29] and Guan et al. [40]. However, these results contrast with a recent report that this strain produces 9-heptadecene [18]. Moreover, the position of the methyl group in the aliphatic chain was confirmed using the NIST mass spectral library and the analysis of the Cn-1 hydrocarbon biosynthesis mechanism. The structure and distribution of ethyl-branched alkanes in ancient sediments and oils have been discussed [41–44], and the origin of these compounds were considered to be either algae in fresh water or terrestrial based on the identification of monoethylalkanes (MEA) in the extract from massive Eocene algal deposits [41,44]. However, there were a few reports on the high yield of ethyl-branched alkanes in a pure culture of cyanobacterial strains. Our finding that 4-ethyltetradecane is abundant in *Nostoc calcicola* FACHB 389 and *Nostoc punctiforme* FACHB 252 provides more direct evidence for the origin of MEA in ancient sediments and oils. Although the unusual branching positions are not readily explained by known biosynthetic mechanisms, the common *ado–aar* hydrocarbon biosynthesis pathway exists in these two filamentous strains. Further study of the possible precursors and biosynthetic pathways for this molecule is required.

Because of the higher value of the octane number, branched hydrocarbons are usually superior to straight-chain hydrocarbons in terms of fuel properties. Initial *in vitro* investigations of methylheptadecane biosynthesis in *Nostoc muscorum* and *Anabaena variabilis* were performed based on isotope experiments [8,37,45]. It was found that there was likely a methylase that catalyzed the incorporation of the methyl group from S-adenosylmethionine in the double bond of cis-vaccenic acid, which forms equal amounts of 7- and 8-methylheptadecane after decarboxylation [37,46]. However, a detailed *in vivo* mechanism for the introduction of branched groups (methyl, ethyl, etc.) in cyanobacteria remains unclear. Considering the dominant distribution of branched hydrocarbons in filamentous cyanobacterial species, we postulate that the differences between filamentous species and unicellular species in the precursors of fatty acid metabolites and/or the substrate specificity of enzymes responsible for the introduction of branched groups might be associated with the distribution pattern of branched hydrocarbons in cyanobacteria. The carbon–carbon double bond in the unsaturated carbon chain is likely to be the methyl reaction site. *Anabaena* sp. PCC 7120 is a filamentous model strain which synthesizes methylheptadecane, and *Synechocystis* sp. PCC 6803 is a unicellular strain which lacks branched alkanes. For these strains, the available genomic information and facile manipulation using a genetic method makes it possible to identify the key genes responsible for the methylation process using the comparative genomic approach. The conservation of the methylated pathway remains to be studied.

Using the appropriate degenerate primers to amplify genes coding for AAR and ADO in cyanobacterial strains provides not only abundant gene resources for any future microbial engineering research but also a molecular basis for understanding the correlation between cyanobacterial evolution and hydrocarbon biosynthesis through cluster-based analysis. *Synechococcus* sp. PCC 7002 and *Cyanothece* sp. PCC 7424, strains belonging to subsection I of the traditional taxonomy, are the only cyanobacterial strains with complete genome information that lack regular two-gene hydrocarbon-producing pathways. Recently, the C19:1 hydrocarbon and different hydrocarbon-producing pathways have been identified in *Synechococcus* sp. PCC 7002 [28]. We used the basic local alignment search tool (BLAST) from NCBI to search for homologs to the Ols protein (SYNPCC7002_A1173) involved in α-olefin biosynthesis in *Synechococcus* sp. PCC 7002, and dozens of possible Ols proteins were identified in cyanobacteria (Table S2, only queries covering >75% and amino acid sequence identities >50% are listed). *Cyanothece* sp. PCC 7424, which lacks the regular *ado–aar* pathway but contains a homolog of the Ols protein, might produce α-olefin in the same manner as *Synechococcus* sp. PCC 7002. Similar to the correlation between the fatty acid distribution patterns and phylogenetic relationships in microalgae [11,12,47], phylogenetic analysis based on 16S rDNA and AAR displayed general congruence (Fig. 4A, B and Fig. S1A, B). This finding indicated that AAR is a phylogenetically ancient protein in most cyanobacteria. The 16S rDNA sequence of *Synechococcus* sp. PCC 7002 was grouped into cluster 3 (Fig. 4A and Fig. S1A, black square), which consisted of both unicellular and filamentous strains, indicating that possible olefin-producing strains (Table S2) might have a strong phylogenetic relationship with cyanobacteria producing straight-chain hydrocarbons. As reported by Shih et al., bioinformatic analyses show no signature proteins specific to any of the 5 traditional morphologies [6]. If regulatory elements play more important roles in establishing morphological transitions than genes, which have been previously proposed to underlie morphological attributes, a hydrocarbon biosynthesis pathway that might be involved in several signal transductions should also make a contribution. Although a hydrocarbon profile as a taxonomic tool is not enough to discriminate cyanobacterial strains in detail, it provides interest-

Table 3
Primers used in this study.

Primer	Sequence (5′–3′)
ado-1	GCSATYGTVATTGAAGGBGA
ado-2	AGCATTHKCCAVACHADGGG
aar-1	ATGTTTGGTCTTDTHGGWCA
aar-2	TTSCGBCCCCARGARAARTT
CYA106F	CGGACGGGTGAGTAACGCGTGA
CYA781R(a)[a]	GACTACTGGGGTATCTAATCCCATT
CYA781R(b)[a]	GACTACAGGGGTATCTAATCCCTTT

[a] Reverse primer CYA781R of 16S rDNA was an equimolar mixture of CYA781R(a) and CYA781R(b).

ing information. The results based on hydrocarbon profiles and phylogenetic analysis indicated that hydrocarbon metabolites of different cyanobacterial shapes share some common features but nevertheless display an obvious divergence, specifically in the biosynthesis of branched-chain alkanes.

Cyanobacterial ADOs are small proteins of 220–250 amino acids, which catalyze the last step of alka(e)ne biosynthesis. These enzymes, which were initially named aldehyde decarbonylase (ADC), given their function of converting Cn fatty aldehydes to formate and the corresponding Cn-1 alka(e)nes [1,19,22], were finally redesignated as aldehyde-deformylating oxygenases (ADOs) because of the oxygenative nature of the reaction they catalyzed [20,21,23]. Dendograms of ADO did not correspond to a classical cyanobacterial phylogeny when compared with the phylogenetic trees of 16S rDNA or AAR (Fig. 4C and Fig. S1C), indicating that ADO gene duplication might have occurred early in the evolution of cyanobacteria, whereas AAR most likely has an even earlier origin.

5. Conclusions

Most past research on hydrocarbon production by cyanobacteria was focused on their potential geochemical and taxonomic significance before the *aar–ado* pathway was identified. Compared to the former studies, this investigation focuses on hydrocarbon biosynthetic processes and phylogenetic analyses at the molecular level. We combined new data on the hydrocarbon compositions of 19 freshwater cyanobacterial species with the existing published data of more than 50 additional strains of cyanobacteria and observed that branched alka(e)nes occur predominantly in filamentous strains but rarely in unicellular strains. *Nostoc spongiaeforme* FACHB 130 was identified as a prospective strain for hydrocarbon production because its production is greater than all previously reported wild type cyanobacterial strains. From sequence alignments and phylogenetic trees, we found that AAR sequences showed interrelationships similar to those in 16S rDNA genes but the ADO sequences did not. These results not only provide diversified genetic resources for future molecular design strategies to produce diversified hydrocarbon fuel products but also display a novel evolutionary perspective with which to study the cellular physiological functions of hydrocarbons in cyanobacteria.

Acknowledgements

This work was supported by the National Basic Research Program of China (973: 2011CBA00907), the National Natural Science Foundation of China (Grant 31200001), the Knowledge Innovation Program of the Chinese Academy of Sciences (Grant KSCX2-EW-G-1-4), the Joint Research Laboratory for Sustainable Aviation Biofuels (QIBEBT-Boeing), and the "100-Talent Program of the Chinese Academy of Sciences" foundation (Grant O91001110A).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apenergy.2013.05.008.

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