Characterization of Cyanobacterial Hydrocarbon Composition and Distribution of Biosynthetic Pathways

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Abstract

Cyanobacteria possess the unique capacity to naturally produce hydrocarbons from fatty acids. Hydrocarbon compositions of thirty-two strains of cyanobacteria were characterized to reveal novel structural features and insights into hydrocarbon biosynthesis in cyanobacteria. This investigation revealed new double bond (2- and 3-heptadecene) and methyl group positions (3-, 4- and 5-methylheptadecane) for a variety of strains. Additionally, results from this study and literature reports indicate that hydrocarbon production is a universal phenomenon in cyanobacteria. All cyanobacteria possess the capacity to produce hydrocarbons from fatty acids yet not all accomplish this through the same metabolic pathway. One pathway comprises a two-step conversion of fatty acids first to fatty aldehydes and then alkanes that involves a fatty acyl reductase (FAAR) and aldehyde deformylating oxygenase (ADO). The second involves a polyketide synthase (PKS) pathway that first elongates the acyl chain followed by decarboxylation to produce a terminal alkene (olefin synthase, OLS). Sixty-one strains possessing the FAAR/ADO pathway and twelve strains possessing the OLS pathway were newly identified through bioinformatic analyses. Strains possessing the OLS pathway formed a cohesive phylogenetic clade with the exception of three Moorea strains and Leptolyngbya sp. PCC 6406 which may have acquired the OLS pathway via horizontal gene transfer. Hydrocarbon pathways were identified in one-hundred-forty-two strains of cyanobacteria over a broad phylogenetic range and there were no instances where both the FAAR/ADO and the OLS pathways were found together in the same genome, suggesting an unknown selective pressure maintains one or the other pathway, but not both.

Introduction

Cyanobacteria are a diverse group of photosynthetic bacteria that have evolved a remarkable array of adaptive traits including oxygenic photosynthesis, N₂ fixation, a wide morphological diversity, extensive secondary metabolite biosynthetic capacity, and a range of symbiotic relationships with other organisms. Cyanobacteria are estimated to contribute 30% of Earth’s annual oxygen production and play a major role in biogeochemical cycles [1]. One trait less well characterized and potentially of great societal importance is their universal ability to produce long chain hydrocarbons. First recognition of this latter trait resulted from investigations in the 1960’s [2–4] and was of importance in the context of identifying the origin of hydrocarbons found in sedimentary and oil deposits. In recent years, there has been a growing recognition of the negative environmental impacts of continued fossil fuel use, as well as an ever increasing worldwide energy demand, and these facts have combined to increase interest in developing sustainable biofuels such as hydrocarbons from cyanobacteria [5,6]. Many sources of renewable energy can be envisioned to help meet society’s demand for electrical power, however, there remains an acute need for low cost liquid fuels, and particularly gasoline, diesel and jet fuel [6].

Diesel and jet fuel quality are measured using cetane values whereas octane ratings are the principal measure of gasoline quality. Quality of the fuel is thus largely determined by the structure of the hydrocarbons in the fuel, and the type of fuel being considered. Long, straight-chain saturated hydrocarbons exhibit higher cetane ratings whereas short highly branched molecules (i.e. isooctane) exhibit superior octane ratings [7,8]. It is important to consider these structural characteristics when assessing the applicability of various biofuel molecules to different engine applications. In this context, the commonly observed hydrocarbons of cyanobacteria, including heptadecane (cetane rating: 105) and methylheptadecane (cetane rating: 66), are promising candidates for diesel fuel applications (normal cetane rating: 40–55) [5,7,8].
In a broader context, cyanobacteria are one of only a few types of organisms that are known to directly produce hydrocarbons. However, their highest reported native production of alk(a/e)nes is 0.12% of dry biomass [4], and the physiological or ecological function of alk(a/e)ne production in cyanobacteria is not yet understood. In this latter regard, various possibilities exist including prevention of grazing from herbivores, intra- or inter- species chemical signaling, prevention of desiccation, enhanced buoyancy, or membrane fluidity/stability. Unfortunately, none of these potential functions have been directly evaluated, and this remains an area in need of further investigation.

It is important to recognize that the extremely low native yields of hydrocarbons from wild type cyanobacterial strains are insufficient to cost competitive with petroleum derived fuels, and therefore, some moderation of the expression of cyanobacterial hydrocarbons pathways will be necessary if they are to be used in an industrial or societal context. Thus, a deeper understanding of these pathways is needed in order to rationally engineer and enhance this trait in modified organisms.

Fatty acid-derived hydrocarbons are produced in a variety of life forms. As described more extensively below, cyanobacteria synthesize long chain alk(a/e)nes using two different pathways, one of which involves a deformylation of fatty aldehydes and the other decarboxylation of fatty acids [2,5,9]. Other prokaryotes such as Jeotgalicoccus sp. synthesize long chain alk(a/e)nes using two different pathways, one life forms. As described more extensively below, cyanobacteria enhance this trait in modified organisms.

As described more extensively below, cyanobacteria synthesize long chain alk(a/e)nes using two different pathways, one of which involves a deformylation of fatty aldehydes and the other decarboxylation of fatty acids [2,5,9]. Other prokaryotes such as Jeotgalicoccus sp. synthesize long chain alk(a/e)nes through cytochrome P450-catalyzed decarboxylation of fatty acids to form terminal aldehydes [10]. Long-chain alkenes in Microcystis autotrophic ATCC 4698 are formed via head-to-head condensation of fatty acids [11]. Although some of the final products from these hydrocarbon pathways are identical to the alk(a/e)nes produced by cyanobacteria, the specific biosynthetic steps involved are distinctly different.

Hydrocarbons produced via fatty acid pathways have also been described in various eukaryotic organisms including the long-chain fatty acid derived alkenes from Botryococcus braunii race A [12,13], the pheromone attractant hormosirene in the diatom Gomphonema parvulum [14], and the pheromone 7-methylheptadecane found in the diatom Gomphonema parvulum [14]. The ACP-bound substrate subsequently undergoes elongation (including prevention of grazing from herbivores, intra- or inter- species chemical signaling, prevention of desiccation, enhanced buoyancy, or membrane fluidity/stability. Unfortunately, none of these potential functions have been directly evaluated, and this remains an area in need of further investigation.

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Within just the last few years, two separate pathways have been described in cyanobacteria that produce hydrocarbons from fatty acid substrates, with each pathway involving a quite different mechanism for removing the terminal oxidized carbon atom [5,9]. One pathway involves the conversion of fatty acyl-ACP to fatty acyl-aldehydes by a fatty acyl-ACP reductase (FAAR), followed by a unique oxygen dependent conversion of the aldehyde via an aldehyde-deformylating oxygenase (ADO) to produce an odd-chain length saturated alkane (or alkene if the fatty acid possesses preexisting unsaturation) (Figure 1) [5,17]. FAARs have been found in a wide range of organisms including plants, eukaryotic algae, bacteria and humans [18]. This reaction is known to require NADPH that likely provides a hydride that attacks the carbonyl of the fatty acid thioester. Nevertheless, the precise mechanism of this FAAR enzyme has not been conclusively verified [5] and there are no published crystal structures of a FAAR enzyme from any source. The next step involving the ADO enzyme is unique to cyanobacteria [17]. This enzyme was first described by [5] as an aldehyde decarbonylase after verifying its function through mutagenesis, heterologous expression, and a re-investigation of a previously published crystal structure of the ADO enzyme from Prochlorococcus marinus MIT9313. Carbon monoxide was initially considered to be the oxidized byproduct of this reaction [5,19]. However, after some debate, it was shown by Li et al. to produce an alkane and formate from a unique oxygenation reaction thus justifying an alternative name of aldehydes deformylating oxygenase [17].

In 2010, Ridley et al. [20] and subsequently in 2011, Mendez-Perez et al. [9] and Donia et al. [21] published reports on terminal olefin biosynthetic pathways in Synechococcus sp. PCC 7002 and Prochloron didemnum. Mendez-Perez verified the function of the OLS pathway in Synechococcus sp. PCC 7002, tested a variety of potential substrates, and enhanced productivity using an alternative promoter [9]. Recently, Gehret-McCarthy et al. compared the structure and function of the activating sulfotransferases (STs) from the CurM pathway in M. producens 3L and the OLS pathway in Synechococcus sp. PCC 7002, and found that both STs accepted substrates leading to production of long-chain hydrocarbons [22]. This second described pathway in cyanobacteria thus uses fatty acid substrates and distinctively produces odd-numbered carbon chains with terminal olefinic bonds. In 2011, Donia et al. and Mendez-Perez et al. identified a large (~10 Kb) multi-domain PKS pathway that proceeds via an elongation/decarboxylation mechanism (Figure 1) [21,9]. The pathway was initially located by searching for homologs of the well characterized CurM domain of the curacin A biosynthetic pathway in Moorea producens 3L (formerly Lyngbya majuscula 3L) [9,21,23,24]. Curacin A is a secondary metabolite with promising anticancer activity [25]. CurM is known to produce the terminal alkene functionality in curacin A via an elongation-decarboxylation mechanism and has recently been shown to accept fatty acid substrates to produce terminal alkenes [22,25]. Mendez-Perez et al. proposed that the olefin-producing pathway (the OLS pathway) includes an ATP-consuming fatty acyl ACP ligase (FAAL) domain that transfers a fatty acid or fatty acyl-ACP to the OLS acyl carrier protein (ACP) [9]. The ACP-bound substrate subsequently undergoes elongation by an extension module [ketosynthase (KS), acyltransferase (AT), and ketoreductase (KR)] to add two carbons from malonyl-CoA and reduce the β-keto group to a β-hydroxy functionality. Gehret-McCarthy et al. characterized the final step of the OLS pathway to involve a sulfotransferase (ST) and thioesterase (TE) that transfers sulfonate from the donor 3′-phosphoadenosine-5′-phosphosulfate (PAPS) to the β-hydroxyacyl substrate [22]. This activates the intermediate for subsequent TE-catalyzed hydrolysis from the enzyme surface, decarboxylation and desulfation to form the terminal alkene product [22]. The inherent formation of a terminal double bond in the product is a unique structural signature of the OLS pathway compared to the FAAR/ADO pathway (Figure 1).

Although these two cyanobacterial hydrocarbon biosynthetic pathways were only recently identified and characterized, knowledge of the diagnostic structural features of the resulting alk(a/e)nes and their respective gene sequences can be used along with previous reports of cyanobacterial hydrocarbon compositions to better understand the taxonomic distribution of these two pathways and the structural diversity found in cyanobacterial hydrocarbons (Table S1). However, these previous investigations have focused on a small subset of cyanobacteria, mostly comprised of strains from subdivision I (Unicellular), subdivision III (Filamentous) and subdivision IV (Heterocystous); only a very limited number of investigations on the hydrocarbons of subdivisions II (Bacocystous) and subdivision V (Ramified) have been conducted (Table S1) [26]. In this context, the older morphology-
based taxonomic perspective of cyanobacteria is severely limited due to the complex evolution of multicellularity in cyanobacteria. Indeed, 16S rRNA-based phylogenetic trees of the cyanobacteria reveal that the cyanobacterial subdivisions are polyphyletic [27] (Figure 2, Table S2). Nonetheless, the limited sampling to date of cyanobacterial diversity for hydrocarbons suggested that there may be as yet uncharacterized structural diversity. Additionally, previous reports of cyanobacterial hydrocarbons have rarely verified chain length, double bond positions or methyl group positions with authentic standards or other rigorous methods of structural analysis, but rather, have relied heavily upon mass spectral library searches (Table S1) [28,29]. This latter approach introduces ambiguity concerning these key structural features, and therefore, could contribute to misunderstandings concerning the nature and distribution of hydrocarbon biosynthetic pathways and their evolution. For example, a number of the reported hydrocarbons are inconsistent with either of the known mechanisms of hydrocarbon production, such as naturally derived even chain-length hydrocarbons [30–33] despite the expected production of only odd-chain alk(a/e)nes from either the FAAR/ADO or OLS pathways following predicted and the experimentally verified pathway mechanisms [5,9,22]. This observation could be explained by either the existence of yet another pathway for alk(a/e)ne biosynthesis, or that the reported even chain alk(a/e)nes have been mis-identified.

The current study thus aimed to expand the characterization of structural diversity of cyanobacterial hydrocarbons through rigorous structural identification methods and across a wide range of cyanobacterial diversity. We hypothesized that this expanded structural diversity may reveal new insights concerning cyanobacterial biosynthetic pathways. Additionally, through a comparison of the hydrocarbon pathways found in the currently available sequenced cyanobacterial genomes, we were able to develop a deeper understanding of the relationships and evolution of these biosynthetic pathways in cyanobacteria.

**Materials and Methods**

**Strain Selection**

Strains of cyanobacteria were selected from a wide phylogenetic distribution ensuring that every major clade was sampled (Figure 2). Strains of cyanobacteria with available genome sequences and established genetic techniques were given preference. Strains that required extremely low or high temperature or unusual media requirements for growth were excluded. *Anaabaena (Nostoc) sp. PCC 7120* was provided by James Golden at UCSD. *Synechococcus elongatus PCC 7942* and *Leptolyngbya* BL0902 was provided by Susan Golden at UCSD. *M. producens* 3L, *Moorea bouillonii PNG5-198* and *Moorea producens* JHB, *Leptolyngbya sp. PAC10-3*, *Moorea sp. PNG 4/22/06-1*, *Moorea sp. PAL8/15/08-1*, *Okeania comitata 3LOSC*, cf. *Okeania hisuta*, PAB 10-Feb-10-1, *Okeania hisuta PAB 21-Jun-10-1*, cf. *Phormidium sp. ISB 3/Nov/94-8*, *Okeania sp. PAC-18-Feb-10-1.1* were isolated from field collections and are maintained among the Gerwick Laboratory

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**Figure 1. Hydrocarbon biosynthetic pathways in cyanobacteria.** A) The Fatty Acyl-ACP Reductase (FAAR)/Aldehyde Deformylating Oxygenase (ADO) involves first a reduction of a fatty acyl substrate to a fatty aldehyde followed by an oxidative conversion to an alkane with the release of formate [17]. The OLS (olefin producing) pathway involves a polyketide synthase that first elongates a fatty acyl-CoA by two carbons from malonyl-CoA via ketosynthase (KS) and acyl transferase (AT) domains followed by reduction to the β-hydroxyacid by a ketoreductase (KR). The ST activates the β-hydroxy group via sulfonation, and then the thioesterase (TE) acts on this substrate to catalyze decarboxylation and loss of sulfate to form the terminal alkene.

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culture collection as described in [24,34,35]. *Planktothrix agardhii*
NIVA-CYA 168 was provided by Rainer Kurmayer at the Austrian Academy of Sciences Institute for Limnology. *Hapalosiphon* *westiichii* IC-52-3 and *Westella miniata* HT-29-1 were provided by Thomas Hemscheidt of University of Hawaii via Melinda Micallef and Michelle Moffitt. The remaining strains were purchased from the Pasteur Culture Collection of Cyanobacteria (PCC) or the American Type Culture Collection (ATCC) as identified in the text.

Culture Conditions
Cyanobacterial strains were grown in 2.8 L Fernbach flasks under 16:8 day:night light cycle between 20–60 μE/m²/sec at a constant temperature at 20°C, 25°C, or 28°C in BG-11, SWBG-11, or ASNH. Cultures were shaken continuously (80 rpm) and grown statically between 14 and 35 days depending upon strain growth rates. Cultures (1L) were harvested via centrifugation at 4000 rpm for 15 min in 500 mL conical containers and combined (using 0.5 M ammonium formate to remove salts for marine strains and deionized water for freshwater strains) into 50 mL Falcon tubes and subsequently centrifuged again to yield a packed pellet that was frozen and dried for extraction and analysis. For filamentous strains that were not amenable to centrifugation, filaments were removed from the media using forceps (rinsed using 0.5 M ammonium formate to remove salts for marine strains), and frozen for drying, extraction and analysis. All biomass was lyophilized for at least 24 h.

Extraction and Structural Analysis
Dried biomass was used as a mortar and pestle and weighed. Biomass was extracted using 5 mL of 100% hexanes or 2:1 dichloromethane:methanol (DCM:MeOH) followed by 20 sec of sonication. The extract was filtered using Whatman GF/F and the residual biomass re-extracted two additional times using the same method followed by a 10 mL wash with hexanes or 2:1 DCM:MeOH. Octadecane (Fluka-74691) was added to each extract after initial solvent addition as an internal standard for quantitation. Octadecane was added at approximately 0.1% of the initial dry biomass. DCM:MeOH (2:1) crude extracts were fractionated using a normal phase 500 mg Bonna-Agela Clearert silica SPE column with collection of the first fraction which eluted with 100% hexanes. Extracts were dried under N₂ gas. A comparison between hexane and DCM:MeOH extraction methods for three strains (*Anabaena* (Nostoc) PCC 7120, *S. elongatus* PCC 7942, *Synechococcus* sp. PCC7002) found that yields and composition of hydrocarbons were not significantly different (data not shown).

To characterize potential fatty acid substrates for hydrocarbon biosynthesis, a fatty acid analysis was performed on crude extracts (2:1 DCM:MeOH) of *Anabaena (Nostoc)* sp. PCC7120, *M. producens* 3L, and *Synechococcus* sp. PCC 7002. Fatty acid methyl esters (FAMEs) were produced by transesterification by adding 3 mL of 4% H₂SO₄ (in MeOH) to at least 0.1 mg of a crude extract. Samples were then stirred and incubated at 110°C for 1 h. Four mL of H₂O and 3 mL of hexanes were then added to the sample. After vortexing for 30 sec and centrifugation at 2500 rpm for 5 min the hexanes layer (top) was removed and dried in a pre-weighted vial for GC-MS analysis. FAME preparation was followed by dimethyl disulfide (DMDS) derivitization for determination of double bond positions [36].

Each extract was resuspended to a concentration of 100 μg/mL in hexanes and 1 μL was analyzed by gas chromatography mass spectrometry (GC-MS) using a Thermo Trace GC-DSQ instrument equipped with an Agilent DB-5ms column (30 m, ID: 0.25, Film: 0.25 μm). Helium (constant flow 1 mL/min) was used as the carrier gas. The inlet temperature was 240°C and the following temperature program was applied: 40°C for 1 min with an increase of 4.5°C/min to 250°C for 10 min. Data were acquired and processed with the Thermo Xcaliber software. Hydrocarbons were determined using a combination of mass fragmention patterns, retention time and comparison to authentic standards when available [heptadecane (Fluka-51578), 1-heptadecene (TCI-90347), 7-methylheptadecane (kindly provided by Dieter Enders and Wolfgang Betray, RWTH, Aachen University)], or published mass spectra and the NIST mass spectral library for Xcaliber (2005) when not available. Double bond positions were confirmed for all alkenes and unsaturated fatty acids using the DMDS method [36].

GC-MS detector response factors for heptadecane, 1-heptadecene, and 7-methylheptadecane were determined in comparison with the octadecane standard by creation of standard curves. Standard curves for hydrocarbons with authentic standards were verified using the low mass common to all hydrocarbons analyzed via GC-MS (57 m/z). Hydrocarbon concentration was calculated using hydrocarbon peak area compared to the internal standard (octadecane) peak area using 57 m/z. Percent dry weight was calculated as an average of three biological replicates. Statistical analysis of the hydrocarbon yields between the OLS and FAAR/ADO pathway were completed using a Mann-Whitney-Wilcoxon Test.

Genome Sequencing and Bioinformatic Analysis
The sequenced genomes of *M. bouilonii* PNg3-198, *M. producens* JHB, and cf. *Phormidium* sp. ISB 3/Nov/94-8 were generated at either the Genomic Center at The Scripps Research Institute or at the University of Michigan using Illumina technology [37]. Genomes were corrected with Quake and assembled using SPAdes 2.4 [38,39]. Contigs were binned by GC content to remove non-cyanobacterial DNA sequences. Identification of cyanobacterial hydrocarbon pathways was accomplished using blastn searches against newly sequenced genomes and blastp searches using representative genes from each pathway (FAAR/ADO and OLS) against publicly available cyanobacterial genomes (131 total) from GenBank [40] and the Joint Genome Institute [JGI] Integrated Microbial Genomes (IMG) database (Version 4, [41]). Draft genome sequences for *M. bouilonii* PNg3-198*, M. producens* JHB, cf. *Phormidium* sp. ISB 3/Nov/94-8, *P. agardhii* NIVA-CYA 126/8 [provided by Rainer Kurmayer, bio project accession number: PRJNA163669], *H.
Cyanobacterial Hydrocarbons

The fatty acids of Anabaena (Nostoc) sp. PCC 7120, M. producens 3L, and Synechococcus sp. PCC 7002 were analyzed to evaluate the available substrates for alkane or alkene biosynthesis (Figure 5). Hexadecanoic acid was the most abundant fatty acid in all three strains followed by various unsaturated C16 and C18 fatty acids. Anabaena (Nostoc) sp. PCC 7120 produced 9-hexadecenoic acid, 9- and 11-octadecenoic acid and octadecenoic acid in descending order of abundance. M. producens 3L also possessed 11-octadecenoic acid but in significantly lower abundance compared to 9- or 11-hexadecenoic acid, the two most abundant fatty acids after hexadecanoic acid. M. producens 3L also possessed tetradecanoic acid as well as 9-tetradecanoic acid. Synechococcus sp. PCC 7002 possessed 9-hexadecenoic acid in addition to 12-octadecenoic acid, 9, 12-octadecadienoic acid, octadecanoic acid and tetradecanoic acid in descending order of abundance. There was no evidence of odd chain length fatty acids in any of the strains investigated.

Taxonomic Pathway Distribution

The recent addition of genome sequences from the CyanogeBA project has more than doubled the amount of genomic information available for cyanobacteria and dramatically improved the distribution of genomic information across the phylogenetic diversity of cyanobacteria [43]. Prior to the availability of these sequences, only five cyanobacteria were known to possess the OLS pathway [Synechococcus sp. PCC 7002, M. producens 3L, Cyanothece sp. PCC 7822, Cyanothece sp. PCC 7424, and uncultured Prochloron symbiont]. The newly available CyanogeBA data and newly sequenced genomes of M. producens JHB, M. baillonii PNG5-198, cf. Phormidium sp. ISB 3/Nov/94-8, P. agardii NIVA-CYA 126/8, H. volcanis IC-52-3, W. intricata HT-29-1 and Leptolyngbya sp. BL0902 (see experimental for sources), twelve additional strains were identified as possessing the OLS pathway, bringing the total to 17 strains (Figure 2). Nevertheless, the vast majority of genome-sequenced cyanobacteria possess the FAAR/ADO pathway (122 of 139). Interestingly, some of these latter cyanobacteria possess two homologs of the ADO gene (Cyanothece sp. PCC 7425, Cyanobium gracile PCC 6307, Leptolyngbya sp. PCC 7375, Synechococcus sp. CB0101, Synechococcus sp. RS9917). Strains that possess the OLS pathway are largely (14 of 17) within a single clade (Clade A), mostly comprised of strains from subdivisions II (formerly Pleurocapsales), subdivision III (formerly Oscillatoriales) and subdivision 1 (formerly Chroococcales). In addition, two strains in this study that fall into this clade (D. incrassata PCC 7326 and Pleurocapsa sp. PCC 7516) are not yet sequenced but produce terminal alkenes indicating that they likely also possess the OLS pathway. The two clades that are exceptions to this OLS pathway phylogenetic clustering are the clade
containing *M. producens* 3L, *M. producens* JHB and *M. bouillonii* PNG5-198 as well as the more distant clade containing *Leptolyngbya* sp. The clade containing the *Moorea* strains cannot be conclusively distinguished from the main OLS containing clade due to low bootstrap and posterior probability support, but *Leptolyngbya* sp. PCC 6406 is clearly in a distinct lineage. By 16S rRNA sequence, this freshwater filamentous strain clades with other *Leptolyngbya* strains as well as unicellular marine Synechococcus strains. Thus, *Leptolyngbya* sp. PCC 6406 is from a distinct evolutionary lineage from other OLS-containing cyanobacteria yet appears to possess this same metabolic pathway.

### Pathway Evolution

An alignment of DNA sequences for all known OLS pathways was constructed with annotations for open reading frames (Figure S4). This alignment also includes the CurM domain of the curacin A biosynthetic pathway, and as expected, shows that CurM does not contain the fatty acyl ACP ligase (FAAL) domain or the first acyl carrier protein (ACP) found in the OLS pathway (Figure S4). An alignment of the amino acid sequences of all 17 of the known OLS pathways and CurM shows that all of the OLS pathways contain the same domains and domain architecture (Figure S5). A phylogenetic tree of all OLS pathways was created to investigate their evolutionary relationships (Figure S6). This phylogenetic tree shows a similar topology with that produced from the corresponding 16S rRNA sequences (Figure 2) with the exception of *Lepolyngbya* sp. PCC 6406. In this latter case, the OLS sequence clades with those from the *Moorea* strains and CurM. A phylogenetic tree was generated for all available ADO genes as well; however, evolutionary relationships and topological comparisons to the 16S phylogenetic tree were prohibited due to poor bootstrap support (data not shown). Significantly, none of the genome sequenced cyanobacteria appear to contain both the FAAR/ADO pathway as well as the OLS pathway. Additionally, no pseudogenes with homology to either of the genes in the FAAR/ADO pathway were detected in OLS-containing cyanobacteria, and similarly, no pseudogenes were detected for the OLS pathway in strains containing the FAAR/ADO pathway.

### Discussion

A striking general conclusion that emerges from review of previous studies along with the results of this study is that hydrocarbon production is a universal phenomenon among cyanobacteria (Figure 2–4 and Table S1). However, hydrocarbon production in cyanobacteria appears to be derived from at least two very different pathways. Moreover, the specific structural features of the hydrocarbons reflect which pathway is present; saturated alkanes are found in strains with the FAAR/ADO pathway.
pathway and terminal alkene containing-hydrocarbons are found only in OLS containing strains. As reported previously, heptadecane is the most commonly observed hydrocarbon in cyanobacteria followed by heptadecene, pentadecane and 7-methylheptadecane (Table S1) [2,5,44]. This observation is consistent with octadecanoic acid (FAAR/ADO) or hexadecanoic acid (OLS) precursor fatty acids, and these along with a variety of unsaturated derivatives are the most common fatty acids in cyanobacteria [45].

Hydrocarbon Composition

Most of the strains investigated in this study had never been previously characterized for their hydrocarbon composition (22 of 32). This investigation found a wide variation in the content of hydrocarbons between these cyanobacterial strains with a range of only 0.024±0.01% in Cyanothecae sp. PCC 7425 to 0.262±0.01% in Pleurocapsa sp. PCC 7320 (Figure 3). These results expand the previously reported range of natural cyanobacterial hydrocarbon yields that was between 0.025–0.12% dry weight [2,4]. Strains possessing the OLS pathway appear to have significantly higher hydrocarbon yields (0.173±0.032) than strains with the FAAR/ADO pathway (0.070±0.008) [p-value = 0.0002].

Branched alkanes have been used as a biomarker for cyanobacteria, and consistent with this, they appear to be widely distributed across cyanobacterial phylogeny [Figure 2, Table S1] [2,44]. Branched alkanes have been observed mostly in filamentous cyanobacteria but there are a few reports of branched hydrocarbons in unicellular strains (Anacystis nidulans, Anacystis cyanea, and Chroococcus turgidus) [2,30,33]. Expanding this distribution, we show for the first time that the unicellular cyanobacterium G. violaceus PCC 7421 also produces 7-methylheptadecane. This study also expanded the known structural diversity of cyanobacterial hydrocarbons to include additional double bond positions (2- and 3-heptadecenes) and methyl group positions (3-, 4- and 5-methylheptadecanes). Overall, branched alkanes were observed in 7 of the 32 strains examined in this investigation. Branched alkane biosynthesis was previously investigated in cyanobacteria and the pendant methyl group found to be derived from S-adenosylmethionine (SAM) through a methyltransferase reaction [46,47]. However, the methyltransferase involved in this pathway has yet to be identified and characterized. Han et al. [2] and Fehler & Light [46] used radiolabeled substrates to verify that vaccenic acid (11-octadecenoic acid) is the likely precursor to 7- or 8-methylheptadecane in Nostoc muscorum. We also observed 11-octadecenoic acid in our fatty acid analysis of Anabaena (Nostoc) sp. PCC 7120, thus confirming the possibility that this fatty acid is the precursor to these branched hydrocarbons in this strain (Figure 5).

All three Moorea strains as well as D. incrassata PCC 7326 produced the unique alkenes 2- and 3-heptadecene. Two possible pathways may be responsible for the production of these observed alkenes. First, a desaturase could act upon a fatty acid substrate to produce double bonds at the ω-2 or ω-3 position. The unsaturated

Figure 4. Hydrocarbon composition expressed as a percentage of total hydrocarbons for 32 strains of cyanobacteria displayed by phylogenetic relationship and pathway distribution. Percentages are displayed as mean percentage between three replicates except for those indicated with an asterisk for strains that were characterized using a single sample. Blue strain names indicate strains possessing the FAAR/ADO pathway and red indicates those with the OLS pathway. Purple strain names indicate a strain that does not have a genome sequence and therefore the pathway type is unknown. To the left of the figure, a 16S rRNA phylogenetic tree (Maximum Likelihood, see Figure S2 for complete tree) is presented for all 32 displayed strains. Branch tips are aligned to the corresponding strain names. Branches corresponding to W. intricata HT-29-1, cf. Phormidium sp. ISB 3/Nov/94-8 and Pleurocapsa sp. PCC 7320 are not shown because 16s rRNA sequences are not available for these strains. Vertical connection lengths were modified to accommodate the location of these three strains in the table.

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fatty acid could then undergo elongation and decarboxylation via the OLS pathway followed by a single round of reduction to remove the newly introduced terminal double bond. Alternatively, an isomerase might act on 1-heptadecene to produce these 2- and 3-heptadecenes. Fatty acid analysis of *M. producens* 3L did not reveal any unsaturated fatty acids with double bonds at these ω-2 or ω-3 positions, but instead, hexadecanoic acid, 11-hexadecenoic acid, 9-hexadecenoic acid, and 11-octadecenoic were observed in order of decreasing abundance (Figure 5). The lack of ω-2 or ω-3 unsaturated fatty acids in this cyanobacterium suggests that an isomerase is likely involved in 2- and 3-heptadecene biosynthesis. Consistent with this hypothesis, the required 16:0 fatty acid is the most abundant fatty acid in *M. producens* 3L.

**Figure 5. Fatty acid analysis of three cyanobacteria.** Fatty acid analysis from single samples of *Anabaena (Nostoc) sp. PCC 7120*, *M. producens* 3L, and *Synechococcus sp. PCC 7002*. All three strains exhibit similar proportions of hexadecanoic acid and 9-hexadecenoic acid; however, *Anabaena (Nostoc) sp. PCC 7120* exhibits a higher proportion of 9-octadecenoic acid and 11-octadecenoic acid and tetradecanoic acid is absent. *M. producens* 3L contains 11-hexadecenoic acid and no octadecanoic acid. *Synechococcus sp. PCC 7002* exhibited a similar composition to *Anabaena (Nostoc) sp. PCC 7120* yet has a higher proportion of hexadecanoic acid. doi:10.1371/journal.pone.0085140.g005

**Phylogenetic Distribution**

The phylogenetic distribution of the two known hydrocarbon biosynthetic pathways among cyanobacteria is revealing of their evolutionary history. The FAAR/ADO pathway is the most widely distributed pathway taxonomically (122 of 139 strains) and is therefore most likely to be the ancestral hydrocarbon pathway in cyanobacteria (Figure 2). Alternatively the OLS pathway is only found in a small number of cyanobacteria (17 of 139) and likely evolved later than the FAAR/ADO pathway (Figure 2). A striking finding revealed by this genomic investigation is the absence of the alternative hydrocarbon pathway in a strain when either the OLS or the FAAR/ADO pathway is present. More specifically, none of the cyanobacteria with the OLS pathway possess the FAAR/ADO pathway or any pseudogenes derived from it. In reciprocal fashion, none of the organisms that possess the FAAR/ADO pathway also possess the OLS pathway or any derived pseudogenes. Because the OLS pathway contains many of the same enzymatic domains found in PKS and fatty acid biosynthesis pathways (ACP, KS, AT, KR, TE), homologs for each domain or even groupings of domains can be found in all cyanobacteria. Thus, the distinctive domains of this pathway are the fatty acyl ACP ligase (FAAL), sulfotransferase (ST) and thioesterase (TE). These domains impart the specificity of the pathway to first utilize fatty acid substrates, activate the ω-hydroxy fatty acid via sulfonation and then catalyze hydrolysis and decarboxylation to produce terminal alkenes. While homologous genes for each of these latter domains can be found throughout cyanobacterial phylogeny, the unique domain architecture that makes up the OLS pathway is found only in OLS containing cyanobacteria.

**Hydrocarbon Pathway Evolution**

Most of the strains that possess the OLS pathway are found within a single clade on the 16S rRNA phylogenetic tree (Clade A, Figure 2). Cyanobacterial phylogenies using multi-locus sequence analyses appear to exhibit the same placement of clade A within the tree topology [48]. However, this clade also includes many strains that possess the FAAR/ADO pathway, suggesting a unique evolutionary history that may have involved multiple horizontal gene transfer events. Thus, the evolutionary history of the OLS pathway in clade A cannot be definitively attributed to horizontal
gene transfer without additional information including further genome sequences of OLS-containing cyanobacteria and phylogenomic comparisons.

The clade containing the *Moorea* strains (Clade B) as well as the separate clade containing *Leptolyngbya* sp. PCC 6406 (Clade C) are phylogenetically outside of this major clade of OLS containing cyanobacteria. The *Moorea* strains, however, cannot be definitively characterized as separate given the rather low bootstrap/posterior probability support values. Nevertheless, the topological placement of this group of filamentous tropical marine cyanobacteria in figure 2 is consistent with previous phylogenetic analyses [24]. Additionally, the OLS pathway found in the three genome sequenced strains of *Moorea* and in *Leptolyngbya* sp. PCC 6406 is consistently separated into two open reading frames with 9 to 17 bp intervening between the two ORFs (Figure S4). This distinctive pathway architecture is not found in any of the other 13 OLS-containing strains, and thus indicates a potentially distinct evolutionary history for these two groups of OLS pathways. Additionally, for all three *Moorea* strains and *Leptolyngbya* sp. PCC 6406, the full AA sequences from the OLS pathway, as well as the KS sequence considered separately, clade together and thus suggest a common evolutionary history (Figure S5 and S6). Despite the distinct pathway architectures and distinctive evolutionary history, the resulting hydrocarbon products do not appear to be fundamentally different as indicated by the production of 1-heptadecene in *Cyanothec* sp. PCC 7822 and the three *Moorea* strains. *Leptolyngbya* sp. PCC 6406 contains the OLS pathway, but according to its 16S rRNA phylogeny, is very distantly not found to be related to the other strains containing the OLS pathway (Figure 2). This latter discrepancy between 16S rRNA and OLS gene tree topologies suggests that the OLS pathway in *Leptolyngbya* sp. PCC 6406 may have been obtained by horizontal gene transfer. Supporting this conclusion, the GC content of the *Leptolyngbya* sp. PCC 6406 OLS pathway (64%) is higher than that of the entire genome (55%); this contrasts with the OLS pathways in all other cyanobacteria which exhibit similar GC contents to their respective genomes (Table S3).

Thus, horizontal gene transfer appears to have played a role in the evolutionary history of the OLS pathway in cyanobacteria; however, the extent of this is unclear at this point. The competitive exclusion of the OLS and the FAAR/ADO pathway is intriguing however, the extent of this is unclear at this point. The competitive exclusion pressure.

Supporting Information

Figure S1 Cyanobacterial 16S rRNA phylogeny and hydrocarbon pathway distribution for the compressed tree displayed in Figure 3. The 16S rRNA phylogeny is displayed for the 20 cyanobacteria quantitatively characterized for their hydrocarbon composition. Blue strain names indicate strains possessing the FAAR/ADO pathway and red strain names indicate those with the OLS pathway. Purple strain names indicate a strain that does not have a genome sequence and therefore the pathway is unknown. Cyanobacterial subdivisions are labeled using colored branches following the key in the upper left: Subdivision I. Unicellular (Formerly Chroococcales), Subdivision II. Barocystous (Formerly Pleurocapsales), Subdivision III. Filamentous (Formerly Oscillatoriales), Subdivision IV. Heterocystous (Formerly Nostocales), Subdivision V. Ramified or True Branching (Formerly Stigonematales).

Figure S2 Cyanobacterial 16S rRNA phylogeny and hydrocarbon pathway distribution for compressed tree displayed in Figure 4. The 16S rRNA phylogeny is displayed for the 32 cyanobacteria characterized for their hydrocarbon composition as an overall percentage. Blue strain names indicate strains possessing the FAAR/ADO pathway and red strain names indicate those with the OLS pathway. Purple strain names indicate a strain that does not have a genome sequence and therefore the pathway is unknown. Cyanobacterial subdivisions are labeled using colored branches following the key in the upper left: Subdivision I. Unicellular (Formerly Chroococcales), Subdivision II. Barocystous (Formerly Pleurocapsales), Subdivision III. Filamentous (Formerly Oscillatoriales), Subdivision IV. Heterocystous (Formerly Nostocales), Subdivision V. Ramified or True Branching (Formerly Stigonematales).

Figure S3 Mass spectra of branched hydrocarbons observed in *Fischerella sp.* PCC 7414. The fragmentation patterns were used to propose the locations of methyl group substitutions on a heptadecane parent structure. In addition to the fragment losses depicted, neutral ion losses were also present.

Figure S4 DNA alignment of all 17 known OLS pathways and CurM with annotated ORFs. Four of the strains have the OLS pathway split into two ORFs. In these four strains the fatty acyl ACP ligase is a separate ORF from the PKS portion of the OLS pathway. Panel A shows all 17 aligned sequences with a red square highlighting the break between ORFs for the four pathways with two ORFs. Panel B shows the expanded red highlighted region from panel A. CurM is a part of the curacin A biosynthetic pathway which does not involve a FAAL [23].

Figure S5 Amino acid alignment and phylogenetic tree of all 17 OLS pathways and the CurM domain. All 17 of the OLS pathways contain the same domain architecture. CurM does not contain the FAAL and ACP1 domains. A maximum likelihood tree is displayed on the left of the alignment to depict the phylogenetic relationships between these pathways.

Figure S6 Phylogeny of the KS domain for all 17 cyanobacterial OLS pathways. The KS domains from *Leptolyngbya* sp. PCC 6406 and the three *Moorea* strains clade together, suggesting a common evolutionary history.

Table S1 Summary of literature reports of cyanobacterial hydrocarbon composition. Strains are organized by subdivision on the left-hand side, and a ‘+’ symbol indicates that a particular hydrocarbon was reported for this strain. The relevant reference is listed in the right-hand column with full references below. Findings that were validated using authentic standards or established using derivitization techniques to verify hydrocarbon structure are denoted with superscript 1.

Table S2 Sequence and Pathway Information. Table of genes used in this investigation (16S, OLS, FAAR, ADO) with Genbank accession numbers and JGI IMG ID numbers. NA = Not Applicable, UA = Unavailable, UNK = Unknown.
Table S3: Genome and OLS gene percent GC for all 17 OLS pathway containing cyanobacteria. Genome status is indicated (P-Permanent Draft, F-Finished, D-Draft). *L. poly青苔 ind. sp.* RCC 6406 exhibits a higher GC content in its OLS pathway compared to its genome, as well as compared to other OLS pathways and their genomes.

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Author Contributions

Conceived and designed the experiments: RCC SP LG WHG. Performed the experiments: RCC SP AL LG. Analyzed the data: RCC SP AL LG WHG. Contributed reagents/materials/analysis tools: SP AL PP DHL EEA LG. Wrote the paper: RCC SP WHG.

References

1. Sharma N, Tiwari S, Tripathi K, Rai A (2010) Sustainability and cyanobacteria (bluegreen algae): facts and challenges. J Appl Phycol 23:1–23.
2. Han J, McCarthy ED, Van Hoven W, Calvin M, Bradley WH (1968) Organic geochemical studies. II. A preliminary report on the distribution of aliphatic hydrocarbons in algae, in bacteria, and in a recent lake sediment. PNAS 59: 29–33.
3. Han J, Chan HWS, Calvin M (1969) Biosynthesis of alkanes in *Nostoc muscorum*. J Am Chem Soc 91: 5136–5139.
4. Winters K, Parker PL, Van Baalen C (1969) Hydrocarbons of blue-green algae: geochemical significance. Science 163: 467–468.
5. Schirmer A, Rude MA, Li X, Popova E, del Cardayre S (2010) Microbial biosynthesis of alkynes. Science 329: 559–562.
6. Peralta-Villegas LF, Blain R, del Cardayre SB, Keasling JD (2012) Microbial engineering for the production of advanced biofuels. Nat Reviews 408: 329–339.
7. Balaban AT (1983) Topological indices based on topological distances in molecular graphs. Pure Appl Chem 55: 199–206.
8. Croteau R, Darveau J, de Bruijn T, Touhout H (2000) Prediction of the cetane number of diesel compounds using the quantitative structure property relationship. Energy Fuels 14: 5396–5403.
9. Mendez-Perez D, Begemann MB, Pfleger BF (2011) Modular synthase-encoding gene involved in ω-alkane biosynthesis in *Synechococcus sp.* strain PCC 7002. Appl Environ Microbiol 77: 4264–4267.
10. Rude MA, Baross JS, Brubaker S, Alabha M, del Cardayre SB, et al. (2011) Terminal olefin (1-alkene) biosynthesis by a novel ω-P450 fatty acid decarbonylase from *Synechococcus sp.* Appl Environ Microbiol 77: 1710–1727.
11. Keller HR, Goh EB, Keasling JD (2010) Genes involved in long-chain alkene biosynthesis in *Microcystis aeruginosa*. Appl Environ Microbiol 76: 1212–1223.
12. Dennis MW, Kolattukudy PE (1991) Alkanes biosynthesis by decarbonylation of aldehyde catalyzed by a microsomal preparation from *Botryococcus braunii*. Arch Biochem Biophys 287: 263–273.
13. Metzger P, Largeau C (2005) *Botryococcus braunii*: a rich source for hydrocarbons and related ether lipids. Appl Microbiol Biotechnol 66: 488–496.
14. Pohner G, Boland W (2002) The oxyphil chemistry of attraction and defense in brown algae and diatoms. Nat Prod Rep 19: 108–122.
15. Blomquist GJ, Bagneres AG, editors (2010) *Insect hydrocarbons: biology, biochemistry, and chemical ecology*. New York: Cambridge University Press.
16. Eser BE, Das D, Han J, McCarthy ED, Van Hoeven W, Calvin M, Bradley WH (1968) Organic geochemical studies, III. A preliminary report on the distribution of aliphatic hydrocarbons in algae, in bacteria, and in a recent lake sediment. PNAS 59: 29–33.
17. Winters K, Parker PL, Van Baalen C (1969) Hydrocarbons of blue-green algae: geochemical significance. Science 163: 467–468.
18. Doan TTP, Carlsson AS, Hamberg M, Bułow L, Stymne S, et al. (2009) A rich source for hydrocarbons and other volatile compounds from cultured blue-green algae. Metab Eng 13: 169–176.
19. Eser BE, Das D, Han J, McCarthy ED, Van Hoeven W, Calvin M, Bradley WH (1968) Organic geochemical studies, III. A preliminary report on the distribution of aliphatic hydrocarbons in algae, in bacteria, and in a recent lake sediment. PNAS 59: 29–33.
20. Ridley CP, Reppas NP (2010) Biosynthesis of 1-alkenes in engineered *Botryococcus* and related ether lipids. Appl Microbiol Biotechnol 66: 486–496.
21. Donia MS, Fricke WF, Ravel J, Schmidt EW (2011) Variation in tropical reef microorganisms. US Patent Appl 12/821,107.
22. Pohnert G, Boland W (2002) The oxylipin chemistry of attraction and defense in brown algae and diatoms. Nat Prod Rep 19: 108–122.
23. Gu L, Wang B, Kulkarni A, Gehret JJ, Lloyd KR, et al. (2009) Polyketide biosynthesis in *Scytonema sp.* strain PCC 7002. Appl Environ Microbiol 75: 1212–1223.
24. Engene N, Paul VJ, Byrum T, Gerwick WH, Thor A, et al. (2013) Five chemically rich species of tropical marine cyanobacteria of the genus *Okeania* gen. nov. (Oscillatoriales, Cyanoprokaryota). J Phycol 49: 1211–1215.
25. Vincent M, Guglielmetti G, Cassani G, Tonini C (1967) Determination of double-bond position in derivatives of dimethyl disulfide derivatives. Anal Chem 59: 694–699.
26. Bennett S (2004) *Solexa Ltd.* Pharmacogenomics 5: 433–438.
27. Kelley DR, Schatz MC, Salzberg SL (2010) Q Freak: quality-aware detection and correction of sequencing errors. Genome Biol 11: R116. doi: 10.1186/gb-2010-11-11-r116.
28. Bankevich A, Nurk D, Antipov AA, Gurevich M, Dvorkin AS, et al. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19: 455–477.
29. Eger J, Zitzl G, Wyslouch I, Hitzler M, Blüthgen N, et al. (2012) Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. J Plant Physiol 166: 787–796.
30. Eger J, Zitzl G, Wyslouch I, Hitzler M, Blüthgen N, et al. (2012) Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. J Plant Physiol 166: 787–796.
31. Paoletti C, Pushparaj B, Capella P, Larkin G (1976) Biosynthesis of aldehydes catalyzed by a microsomal preparation from *Botryococcus braunii*. Arch Biochem Biophys 208: 267–273.
32. Dembitsky VM, Shkrobb I, Dor I (1999). Separation and identification of aldehydes and other volatile compounds from cultured blue-green algae. Metab Eng 1: 7908–7916.
33. Gu L, Wang B, Kulkarni A, Gehret JJ, Lloyd KR, et al. (2009) Polyketide biosynthesis. J Am Chem Soc 131: 16033–16035.
34. Eger J, Zitzl G, Wyslouch I, Hitzler M, Blüthgen N, et al. (2012) Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. J Plant Physiol 166: 787–796.
35. Bankevich A, Nurk D, Antipov AA, Gurevich M, Dvorkin AS, et al. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19: 455–477.
36. Eger J, Zitzl G, Wyslouch I, Hitzler M, Blüthgen N, et al. (2012) Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. J Plant Physiol 166: 787–796.
37. Bennett S (2004) *Solexa Ltd.* Pharmacogenomics 5: 433–438.
38. Kelley DR, Schatz MC, Salzberg SL (2010) Q Freak: quality-aware detection and correction of sequencing errors. Genome Biol 11: R116. doi: 10.1186/gb-2010-11-11-r116.
39. Bankevich A, Nurk D, Antipov AA, Gurevich M, Dvorkin AS, et al. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19: 455–477.
40. Eger J, Zitzl G, Wyslouch I, Hitzler M, Blüthgen N, et al. (2012) Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. J Plant Physiol 166: 787–796.
41. Bankevich A, Nurk D, Antipov AA, Gurevich M, Dvorkin AS, et al. (2012) SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol 19: 455–477.
46. Fehler WG, Light RJ (1970) Biosynthesis of hydrocarbons in *Anabaena variabilis*. Incorporation of [methyl-14C] - and [methyl-3H3] methionine into 7- and 8-methylheptadecanes. Biochemistry 9: 418–422.

47. Fehler WG, Light RJ (1972) Biosynthesis of methylheptadecanes in *Anabaena variabilis*. In Vitro incorporation of S- [methyl-14C] adenosylmethionine. Biochemistry 11: 2411–2416.

48. Shih PM, Wua D, Latifid A, Axena SD, Fewere DP, et al. (2013) Improving the coverage of the cyanobacterial phylum using diversity-driven genome sequencing. PNAS 110: 1053–1058.