Microalgae Synthesize Hydrocarbons from Long-Chain Fatty Acids via a Light-Dependent Pathway[1][OPEN]

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Microalgae are considered a promising platform for the production of lipid-based biofuels. While oil accumulation pathways are intensively researched, the possible existence of a microalgal pathways converting fatty acids into alka(e)nes has received little attention. Here, we provide evidence that such a pathway occurs in several microalgal species from the green and the red lineages. In *Chlamydomonas reinhardtii* (Chlorophyceae), a C17 alkene, *n*-heptadecene, was detected in the cell pellet and the headspace of liquid cultures. The *Chlamydomonas* alkene was identified as 7-heptadecene, an isomer likely formed by decarboxylation of cis-vaccenic acid. Accordingly, incubation of intact *Chlamydomonas* cells with per-deuterated D_{31}-16:0 (palmitic) acid yielded D_{31}-18:0 (stearic) acid, D_{29}-18:1 (oleic and cis-vaccenic) acids, and D_{29}-heptadecene. These findings showed that loss of the carboxyl group of a C_{18} monounsaturated fatty acid lead to heptadecene formation. Amount of 7-heptadecene varied with growth phase and temperature and was strictly dependent on light but was not affected by an inhibitor of photosystem II. Cell fractionation showed that approximately 80% of the alkene is localized in the chloroplast. Heptadecane, pentadecane, as well as 7- and 8-heptadecene were detected in *Chlorella variabilis NC64A* (Trebouxiophyceae) and several *Nannochloropsis* species (Eustigmatophyceae). In contrast, *Ostreococcus tauri* (Mamiellophyceae) and the diatom *Phaeodactylum tricornutum* produced C_{21} hexaene, without detectable C_{15}-C_{19} hydrocarbons. Interestingly, no homologs of known hydrocarbon biosynthesis genes were found in the *Nannochloropsis*, *Chlorella*, or *Chlamydomonas* genomes. This work thus demonstrates that microalgae have the ability to convert C_{16} and C_{18} fatty acids into alka(e)nes by a new, light-dependent pathway.

Hydrocarbons derived from fatty acids (i.e. alkanes and alkenes) are ubiquitous in plant and insect outermost tissues where they often represent a major part of cuticular waxes and play an essential role in preventing water loss from the organisms to the dry terrestrial environment (Hadley, 1989; Kunst et al., 2005). In several insect species, select cuticular alkenes also act as sex pheromones (Wicker-Thomas and Chertemps, 2010). Occurrence of alkanes or alkenes has also been reported in various microorganisms (Ladygina et al., 2006; Wang and Lu, 2013). For example, synthesis of hydrocarbons is widespread in cyanobacteria (Coates et al., 2014), and it is thought that cyanobacterial alka(e)nes contribute significantly to the hydrocarbon cycle of the upper ocean (Lea-Smith et al., 2015).

Alka(e)nes of various chain lengths are important targets for biotechnology because they are major components of gasoline (mainly C5-C9 hydrocarbons), jet fuels (C5-C16), and diesel fuels (C12-C20). The alkane biosynthetic pathway of plants has been partly elucidated (Lee and Suh, 2013; Bernard and Joubès, 2013), but the use of plant hydrocarbons as a renewable source of liquid fuels is hampered by predominance of constituents with high carbon numbers (>C25), which entails solid state at ambient temperature (Jetter and Kunst, 2008). Therefore, there is great interest in the microbial pathways of hydrocarbon synthesis producing shorter chain compounds (C15-C19). In cyanobacteria, hydrocarbons are produced by two distinct pathways. The first one comprises the sequential action of an acyl-ACP reductase transforming a C15-C19

[1] Funding was provided by the Agence Nationale de la Rercherche (grant MUSCA no. ANR-13-JSV5-0005 to Y.L-B. and grant A*MIDEX no. ANR-11-IDEX-0001-02 to G.P.), the Commissariat à l'Energie Atomique et aux Energies Alternatives (CEA), and the région Provence-Alpes-Côte-d'Azur (PACA; PhD studentship to D.S.). Support was also provided by the HélioBiotec platform funded by the European Regional Development Fund, the région PACA, the French Ministry of Research, and the CEA.

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F.B., R.J., and Y.L.-B. conceived the original research project; F.B., D.S., B.L., S.C., G.G., and G.P. designed the experiments and analyzed the data; D.S., B.L., S.C., P.M., B.M., and G.G. performed the experiments; F.B. and D.S. wrote the article with contributions from R.J., Y.B.-L., B.L., and G.P.

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www.plantphysiol.org/cgi/doi/10.1104/pp.16.00462

fatty acyl-ACP into a fatty aldehyde and an aldehyde-deformylating oxygenase catalyzing the oxidative cleavage of the fatty aldehyde into alka(e)ne and formic acid (Schirmer et al., 2010; Li et al., 2012). The second pathway involves a type I polyketide synthase that elongates and decarboxylates fatty acids to form alkenes with a terminal double bond (Mendez-Perez et al., 2011). Additional pathways of alkene synthesis have been described in bacteria. In *Micrococcus luteus*, a three-gene cluster has been shown to control the head-to-head condensation of fatty acids to form very-long-chain alkenes with internal double bonds (Beller et al., 2010). Direct decarboxylation of a long-chain fatty acid into a terminal alkene has also been reported and is catalyzed by a cytochrome P450 in *Jeotgalicoccus* spp. (Rude et al., 2011) and by a non-heme iron oxidase in *Pseudomonas* (Rui et al., 2014).

Among microbes, microalgae would be ideally suited to harness the synthesis of hydrocarbons from fatty acid precursors because they are photosynthetic organisms combining a high biomass productivity (León-Bañares et al., 2004; Beer et al., 2009; Malcata, 2011; Wijffels et al., 2013) and a strong capacity to synthesize and accumulate fatty acids (Hu et al., 2008; Harwood and Guschina, 2009; Liu and Benning 2013). However, studies on microalgal alka(e)ne synthesis are scarce. In some diatoms and other algal species, a very-long-chain alkene, a C21 hexaene, has been found (Lee et al., 1970; Lee and Loeblich, 1971). Other very-long-chain alkenes have been described in the slow-growing colonial Chlorophycea *Botryococcus brauni*, which excretes a variety of hydrophobic compounds including C27 *n*-alkadienes (Metzger and Largeau, 2005; Jin et al., 2016). A decarbonylase activity converting a fatty *n*-aldehyde substrate to a *n*-alkane has been shown in *B. brauni* (Dennis and Kolattukudy, 1992); however, the corresponding protein has not been identified so far. Presence of shorter chain alka(e)nes in some microalga species has been reported in the context of geochemical studies (Han et al., 1968; Gelpi et al., 1970; Tornabene et al., 1980; Afi et al., 1996) but the biology of these compounds has not been investigated further.

Here, we show that alka(e)nes with C15 to C17 chains can be detected in several model microalgae and originate from fatty acid metabolism. In *Chlamydomonas reinhardtii* and *Chlorella variabilis* NC64A, 7-heptadecene is identified as the major hydrocarbon produced, and we demonstrate that its synthesis depends strictly on light and uses cis-vaccenic acid as a precursor. We also show the presence of C15 to C17 alka(e)nes in *Nannochloropsis* sp., a model microalga from the red lineage. Absence of homologs to known hydrocarbon synthesis genes in the genomes of *Chlamydomonas*, *Chlorella*, and *Nannochloropsis* indicates that a hitherto unknown type of alka(e)ne-producing pathway operates in these microalgae. The wide occurrence of microalgae in marine environments suggests that microalgal alka(e)nes contribute significantly to the ocean's hydrocarbon cycle.

RESULTS

C. reinhardtii and *C. variabilis* Synthesize Long-Chain Alka(e)nes

To investigate the capacity of microalgae to produce alkanes or alkenes, we first analyzed the unsaponifiable fraction of vegetative cells in two prominent green microalga models, *C. reinhardtii* (CC124 strain) and *C. variabilis NC64A*. The solvent extracts of the saponification mixtures were analyzed by gas chromatography coupled to mass spectrometry (GC-MS). The chromatograms of *C. variabilis* and *C. reinhardtii* extracts showed a peak at 14.3 min in the region of long-chain hydrocarbons (Fig. 1A). This peak displayed a mass spectrum identical to that of a heptadecene standard (Fig. 1B). In *C. variabilis*, two close peaks corresponding to heptadecene were present (Fig. 1A). In addition, *n*-heptadecane (peak at 14.6 min) and traces of *n*-pentadecane (peak at 12.3 min) were detected (Supplemental Fig. S1). The finding that all these hydrocarbons were absent from control samples generated by saponification of fresh culture medium ruled out that any hydrocarbons had been introduced as contamination from solvents, reagents, or culture medium (Fig. 1A).

In order to further exclude the possibility that the harsh conditions of the saponification may have caused an artifactual decarboxylation of fatty acids to alka(e)nes, volatile compounds present in the headspace (gas phase) of *C. reinhardtii* and *C. variabilis* cultures were analyzed by solid-phase microextraction (SPME), which does not involve any treatment of cells with chemical reagents or heat. The SPME fiber was put in the headspace of a sealed vial containing a *C. reinhardtii* liquid culture or the fresh culture medium. Analysis by GC-MS of the compounds absorbed onto the SPME fiber showed that heptadecene was indeed present in the gas phase of a *C. reinhardtii* culture but was absent when no cells were present in the culture medium (Supplemental Fig. S2). The *C. variabilis* hydrocarbons found by saponification were also detected by SPME, but no other hydrocarbon peak could be found. These results thus establish that the long-chain alka(e)nes detected in *C. reinhardtii* and *C. variabilis* cells are genuine products of microalgal metabolism.

Total alka(e)ne amounts per cell volume were similar in *C. variabilis* and *C. reinhardtii* as determined by total transmethylation of cells and GC-MS/FID (flame ionization detection) analysis of hydrocarbons and fatty acid methyl esters (FAMEs; Fig. 1C). This represented approximately 0.62 and 1.15% of total fatty acids in *C. reinhardtii* and *C. variabilis*, respectively (or 0.036 and 0.08% of biomass dry weight). Heptadecenes formed approximately 80% of hydrocarbons in *C. variabilis*. A control performed with pure oleic acid showed that the heptadecene quantified after transmethylation was not originating from spontaneous fatty acid decarboxylation (Supplemental Fig. S3).

To reveal the heptadecene double-bond positions, extracts of saponified *C. reinhardtii* or *C. variabilis* cells were derivatized with dimethyl disulfide (DMDS) and

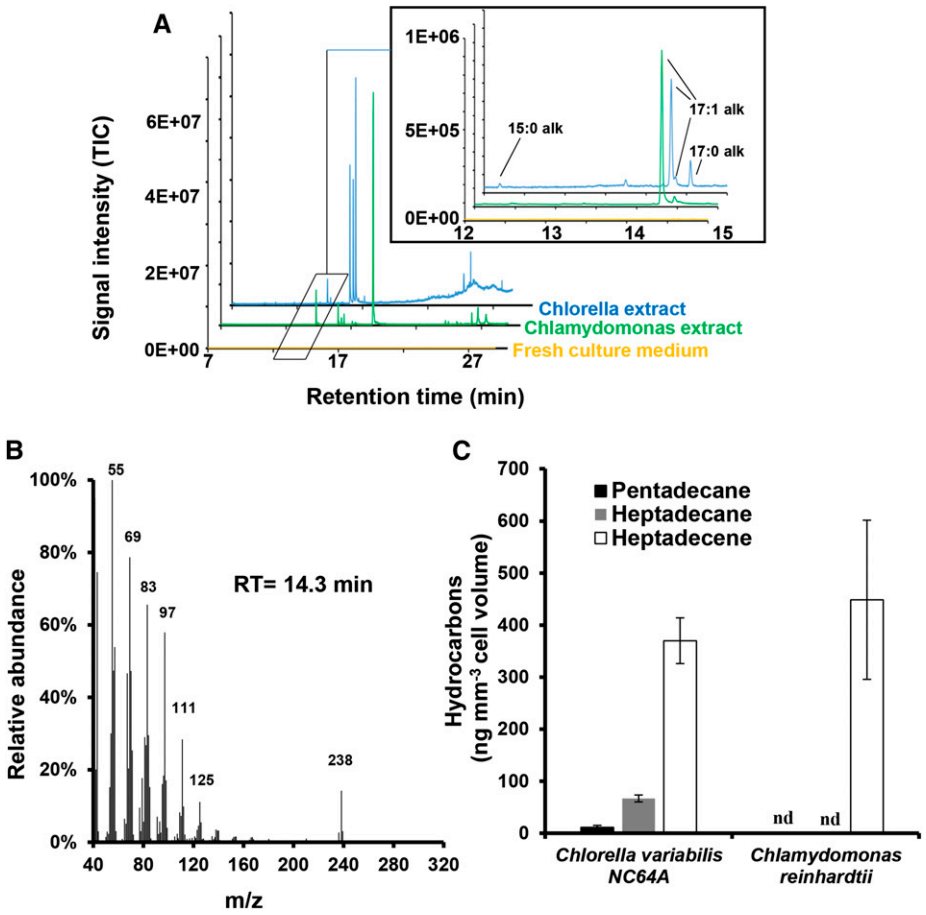

Figure 1. Long-chain alka(e)nes are synthesized by cells of *C. reinhardtii* and *C. variabilis* NC64A. A, Chromatogram of the unsaponifiable cell material analyzed by GC-MS. The long-chain hydrocarbon region is magnified (inset). 17:1-alk, *n*-heptadecenes; 17:0-alk, *n*-heptadecane; 15:0-alk, *n*-pentadecane. B, Mass spectrum of the *C. reinhardtii* peak at 14.3 min in A. This spectrum is identical to that of a commercial standard of 1-heptadecene. C, Quantification of alka(e)nes detected in *C. reinhardtii* and *C. variabilis* after transmethylation of cells and GC-MS/FID analysis. Values are mean of three biological replicates, and error bars represent SD; nd, not detected.

the reaction products were analyzed by GC-MS. In both species, a peak with a mass spectrum characteristic of the DMDS derivative of 7-heptadecene (diagnostic fragments at *m/z* 145 and 187) was observed (Fig. 2). An additional minor peak with fragments at *m/z* 159 and 173, indicative of 8-heptadecene, could be detected in *C. variabilis* but was absent in *C. reinhardtii*. Therefore, we conclude that the heptadecene produced in *C. reinhardtii* (and the major in *C. variabilis*) is 7-heptadecene. The minor isomer of *C. variabilis* is 8-heptadecene (around 20% of the major isomer).

Long-Chain Alka(e)nes Derive from a Formal Decarboxylation of Fatty Acids

One of the simplest biosynthetic routes to synthesize heptadecenes and other long-chain hydrocarbons is the formal decarboxylation of corresponding fatty acids. To determine whether *C. reinhardtii* and *C. variabilis* alka(e)nes originate from fatty acid precursors, cells were cultured for 3 d in Tris acetate phosphate (TAP) medium augmented with perdeuterated palmitic acid (D_{31}), and total fatty acid content was analyzed by GC-MS/FID following transmethylation. In the *C. reinhardtii* product mixture, D_{31}-16:0 (palmitic), D_{31}-18:0 (stearic), and D_{29}-18:1 acids were observed, indicating that the algal cells had taken up exogenous D_{31} palmitic acid and metabolized it (Fig. 3). Interestingly, D_{29}-heptadecene was identified based on MS fragmentation of the GC peak eluting at 9.2 min. Incubation of *C. variabilis* NC64A with D_{31} also resulted in the formation of D_{31}-16:0, D_{31}-18:0, and D_{29}-18:1 acids (Supplemental Fig. S4). Formation of D_{29}-heptadecene and additionally of D_{31}-heptadecane was observed for *C. variabilis* (Fig. 3). It should be noted that the retention behavior of per-deuterated fatty acids and hydrocarbons, with elution long before corresponding unlabeled compounds, was coherent with previous observations on per-deuterated alkanes (Matucha et al., 1991). Overall, labeling experiments therefore showed that *C. reinhardtii* and *C. variabilis* had enzyme(s) that catalyzed the conversion of long-chain fatty acids into alka(e)nes by a formal decarboxylation.

Heptadecene Content Varies with Growth Stage and Culture Conditions

We next sought to determine the effect of physiological conditions, growth stage, or life cycle on the content of hydrocarbons in a microalga. We used *C. reinhardtii* for these experiments because it is the most-studied model

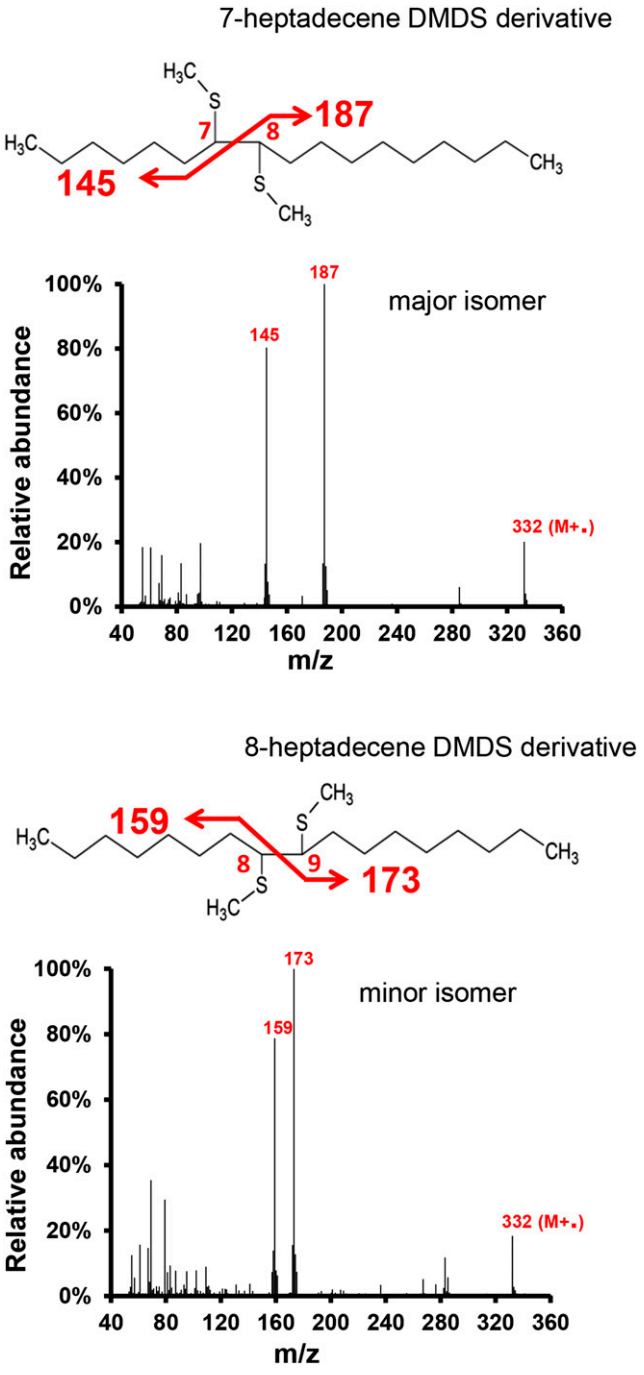

Figure 2. The heptadecene isomers are 7-heptadecene and 8-heptadecene. Cells were saponified and a solvent extract was reacted with DMDS before analysis by GC-MS. The major diagnostic ions expected for DMDS derivatives of 7-heptadecene and 8-heptadecene and the mass spectra obtained for the two *C. variabilis n*-heptadecene isomers modified by DMDS are shown.

for algal physiology, including lipid metabolism (Merchant et al., 2012; Li-Beisson et al., 2015). Initial experiments showed no significant differences in heptadecene content between the vegetative cells of the strains CC124 (mating type: minus) and CC125 (mating type: plus) (Supplemental Fig. S5). Similarly, induction of gametogenesis in the vegetative cells of the two mating types did not result in any significant decrease or increase.

Hydrocarbons were then quantified in vegetative cells of CC124 under various culture conditions. We first determined the heptadecene content as a function of algal growth under standard laboratory conditions, i.e. in flasks under constant illumination, either in photoautotrophy (minimal medium; i.e. with light as only source of energy) or in mixotrophy (TAP medium; i.e. acetate and light as energy sources). Heptadecene levels increased steadily, up to 422 ng/mm^3 cell volume at 168 h in TAP media, corresponding to 368 ng/mg biomass dry weight. The alkene amounts were at all times substantially higher under mixotrophic conditions than under photoautotrophic conditions (Fig. 4A). The increase in heptadecene concentration in the cells appeared to correlate with the increase in cell concentration in the culture (Fig. 4B). Under TAP medium, transfer to a nitrogen-deprived medium, which causes slowdown and eventually arrest of cell division (Merchant et al., 2012), resulted in an initial strong reduction of heptadecene content and an arrest of the synthesis of heptadecene (Fig. 4C). By contrast, under minimal medium conditions, nitrogen deprivation caused an initial reduction of heptadecene, but after 24 h the synthesis resumed and at 96 h reached similar values as in minimal medium (Fig. 4D). When cells were cultivated at various temperatures, heptadecene content tended to increase with temperature, with a significant difference between 35°C and 4°C (Supplemental Fig. S6).

Synthesis of Alka(e)nes Is Strictly Dependent on Light But Not on Photosynthesis

In order to gain more insights into the environmental parameters that might affect synthesis of heptadecene by *C. reinhardtii* cells, the effect of light was investigated. When *C. reinhardtii* cells were grown in flasks under a night and day cycle (12 h light/12 h dark), it was observed that heptadecene content per cell increased by about 50% during the day, decreased steeply during the first 3 h of the night, and stayed constant during the rest of the night (Fig. 5A). This suggested that heptadecene synthesis was dependent on light and prompted us to investigate the synthesis of heptadecene in complete absence of light. Cells were grown heterotrophically for five days in a flask in the dark and heptadecene was quantified. Strikingly, no alkene could be detected in dark-grown cells, in stark contrast to cells grown in the same medium under constant light (Fig. 5B). This showed that formation of *C. reinhardtii* heptadecene was strictly dependent on light. A strong light dependence of alka(e)ne synthesis was also observed in *C. variabilis* (Supplemental Fig. S7). Interestingly, when *C. reinhardtii* cells were broken and chloroplasts isolated, 80% of the cellular heptadecene was found to be present in the chloroplast fraction (Fig. 5C).

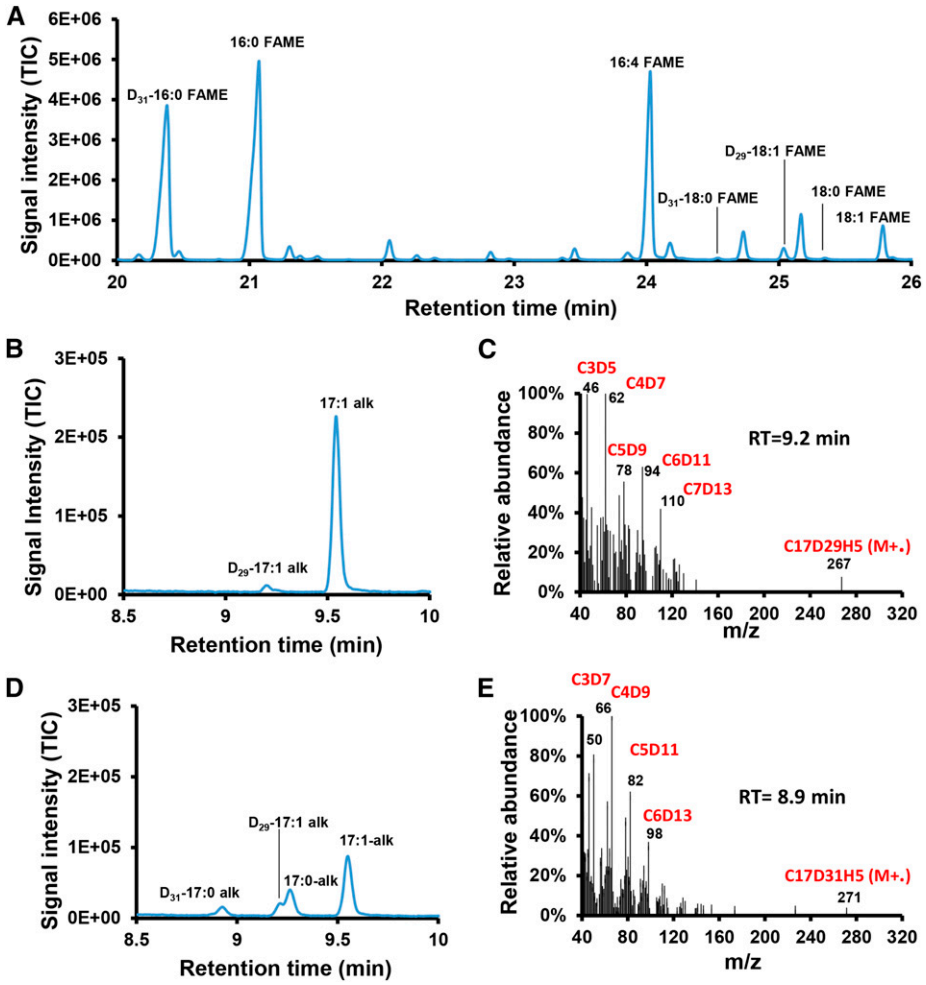

Figure 3. *C. reinhardtii* and *C. variabilis* hydrocarbons derive from fatty acids. Cells were cultivated for 5 d in presence of deuterium-labeled (D_{31}) palmitic acid, collected, and directly transmethylated. The transmethylation extract was analyzed by GC-MS/FID. A, Part of the GC-MS chromatogram showing endogenous and labeled *C. reinhardtii* FAMEs. B and D, Part of the chromatograms showing *C. reinhardtii* (B) and *C. variabilis* (D) hydrocarbons. C and E, Mass spectra of the *C. reinhardtii* peak at RT = 9.2 min (C) and the *C. variabilis* peak at RT = 8.9 min (E). An interpretation of the fragments observed is shown in red and is consistent with the compound being D_{29} *n*-heptadecene and D_{31} *n*-heptadecane, respectively.

To further investigate the dynamics of light-dependent heptadecene formation and its potential link to photosynthesis, cells were grown for 5 d in the dark and then moved to light. The levels of newly formed heptadecene reached 10 ng per mm^3 cell volume already 10 min after switching the light on, and this amount increased only slightly in the next few hours to reach approximately 15 ng per mm^3 cell volume at 6 h (Fig. 5D). Similar results were obtained in the presence or absence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU), an inhibitor of photosystem II, showing that heptadecene formation was not dependent on photosynthesis.

Finally, we sought to test the effect of light intensity on alkene synthesis. For this purpose, *C. reinhardtii* cells were grown in a photobioreactor operated as a turbidostat (i.e. at constant cell density) to ensure a constant illumination of the culture. Heptadecene content per cell volume unit at each light intensity increased in the range 125 to 300 μmol photons m^{-2} s^{-1} (Fig. 5E). Heptadecene productivity (calculated from steady state heptadecene cellular contents and dilution rates) followed the same trend as biomass productivity, increasing steeply with light intensity from 15 to 125 μmol photons m^{-2} s^{-1}, but kept increasing at high light intensities (Supplemental Fig. S8) due to the increase in the heptadecene amount per unit of cell volume (Fig. 5).

Microalgae-Producing Long-Chain Alka(e)nes Have No Homologs to Known Alka(e)ne-Forming Enzymes

The presence of long-chain hydrocarbons in *C. reinhardtii* and *C. variabilis NC64A* raised the questions whether similar compounds also occurred in other microalgae of the green lineage or in species of the red lineage and whether these compounds could be produced by enzymes homologous to the ones found in plants, cyanobacteria, or other organisms. We thus investigated the presence of hydrocarbons in some other model microalgae with sequenced genomes: the Mamiellophycea *Ostreococcus tauri* (green lineage), the diatom *Phaeodactylum tricornutum*, and the Eustigmatophycea *Nannochloropsis* sp. (red lineage). Heptadecane and heptadecene were observed in all strains of *Nannochloropsis*, and in all species except *Nannochloropsis limnetica* pentadecane was also detected

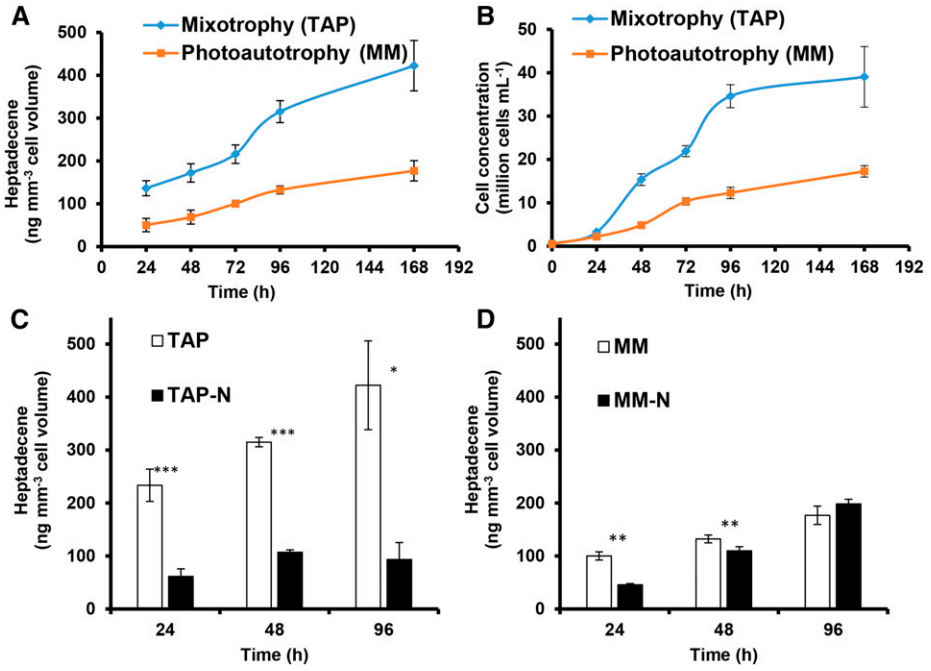

Figure 4. Heptadecene content varies with growth and culture conditions in *C. reinhardtii* after cell transmethylation products were analyzed by GC-MS/FID. A and B, Heptadecene content (A) and growth curve (B) of cells grown for 9 d in flasks under mixotrophic (TAP medium) or photoautotrophic (minimal medium) conditions. C and D, cells were grown for 2 d in TAP medium (C) or minimal medium (D) and changed to the same culture medium devoid of nitrogen (TAP-N and MM-N). Values are means of four biological replicates, and error bars represent SD. Asterisks denote significant differences at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ in a two-sided *t* test.

(Fig. 6). The total amounts of hydrocarbons varied up to 10-fold between the various *Nannochloropsis* species, with a maximum of 597 ng/mm^3 cell volume in *Nannochloropsis gaditana*. Although we could not detect any C15-C17 hydrocarbons in *P. tricornutum* and *O. tauri* under the culture conditions used, we identified a C21:6 hexaene, all-cis-3,6,9,12,15,18-heneicosahexaene, based on matching mass spectrum and retention time with the same compound previously described for several diatoms and various other algae (Lee and Loeblich, 1971), namely, the one derived from all-cis-4,7,10,13,16,19-docosahexaenoic acid (DHA; Supplemental Fig. S9). Therefore, all the model microalgal species investigated produced alka(e)nes, either in the form of C15-C17 alka(e)nes or the C21:6 hexaene.

Finally, to determine if the microalgal alka(e)nes detected here could be produced along known hydrocarbon biosynthesis pathways, the algal genomes were searched for potential orthologs to genes encoding enzymes known to be involved in hydrocarbon formation. BLASTP searches were performed in the genomes of *C. reinhardtii* (Merchant et al.; 2007), *C. variabilis* (Blanc et al., 2010), *Nannochloropsis* (Radakovits et al., 2012; Vieler et al., 2012), *P. tricornutum* (Bowler et al., 2008), and *O. tauri* (Derelle et al., 2006) using the sequences of the cyanobacterial acyl ACP-reductase and aldehyde deformylating oxygenase (Schirmer et al., 2010), the plant ECERIFERUM1 (CER1) and CER3 (Aarts et al., 1995; Rowland et al., 2007; Bernard et al., 2012), the insect cytochrome P450 CYP4G1 (Qiu et al., 2012), the bacterial cytochrome P450 CYP152/OleT (Rude et al., 2011), and the bacterial nonheme oxidase UndA (Rui et al., 2014). The genomes of *O. tauri* and *P. tricornutum* both contained one potential ortholog to the plant *CER1/3* (Table I). However, no other homologs to known hydrocarbon synthesis genes were found in the genomes of *Chlorella*, *Chlamydomonas*, and *Nannochloropsis*.

DISCUSSION

C15-C17 Alka(e)nes Are Produced by Various Microalgae and Are as Abundant as in Cyanobacteria

The C21 hexaene derived from DHA that is formed by diatoms has been well characterized (Lee et al., 1970; Lee and Loeblich, 1971). By contrast, the endogenous or exogenous origin of the various shorter chain alkanes reported in geochemical studies for some microalgal species has not been investigated further. Possible exogenous origin of hydrocarbons detected in minute to trace amounts in biological material is a major concern because medium- and long-chain hydrocarbons are common contaminants of organic solvents and culture media. Here, using SPME analysis of intact cells and culture medium as well as labeling with deuterated fatty acid precursors (Figs. 1 and 2; Supplemental Figs. S1–S3), we show unambiguously that C15-C17 alka(e)nes are synthesized by several model microalgae (Table I). These include the Chlorophycea *C. reinhardtii* and the Trebouxiophycea *C. variabilis NC64A*, which belong to the Archaeplastida (green lineage), and the Eustigmatophyca *Nannochloropsis* sp., which is a microalga of the red lineage closely related to the Phaeophyceae (brown algae). *Nannochloropsis* species are mostly considered as marine species but can thrive in a variety of environments and a freshwater species is known (*N. limnetica*). Interestingly, all six reported species of *Nannochloropsis* including *N. limnetica* produced C15-C17 hydrocarbons. Although *O. tauri* (green lineage) and the diatom *P. tricornutum* (red lineage) have C16-C18 fatty acid, they

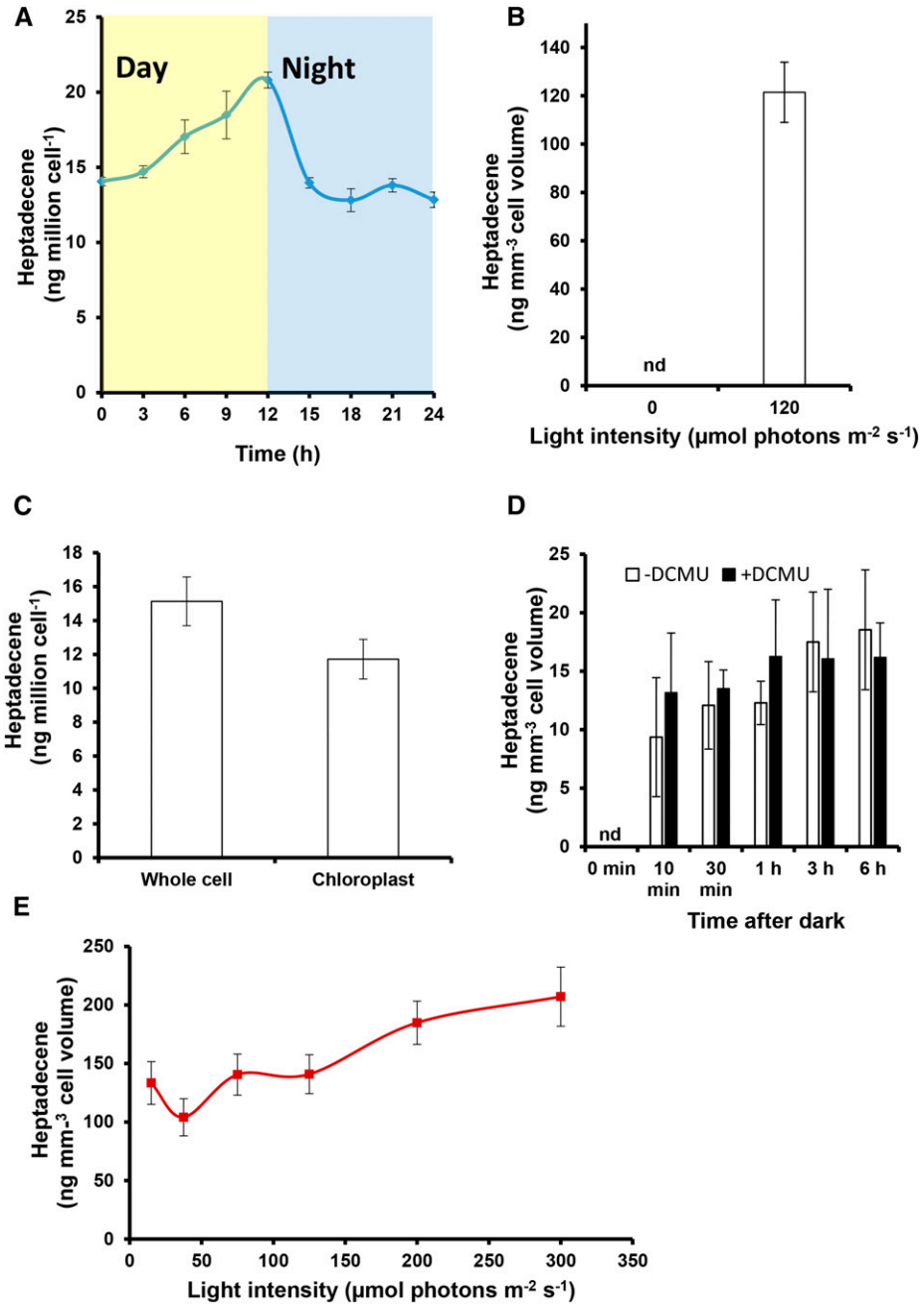

Figure 5. *C. reinhardtii* heptadecene synthesis is strictly dependent on light and increases with light intensity. A, Variation of heptadecene during day-night cycles. Cells were grown in flasks under a day-night cycle (12 h light/12 h dark) for 3 d and then diluted at the beginning of the day and samples were collected during the next 24 h. Values are means of three biological replicates, and error bars represent SD. B, Heptadecene synthesis in dark- and light-grown cultures. Cells were grown in flasks in TAP medium at 120 μmol photons m^{-2} s^{-1} and then diluted in the same medium to be grown for an extra 5 d either in the same condition or in the dark (i.e. under heterotrophic conditions). Values are means of three biological replicates, and error bars represent SD; nd, not detected. C, Quantification of heptadecene in isolated chloroplasts. Whole cells or isolated intact chloroplasts were transmethylated and heptadecene was quantified using GC-MS/FID. The proportion of cell heptadecene present in the chloroplast was calculated using the chlorophyll content of isolated chloroplasts and assuming all chlorophyll was present in chloroplasts. Values are means of three biological replicates, and error bars represent SD. D, Kinetics of heptadecene synthesis upon light exposure. Cells were grown for 5 d in the dark in TAP medium and heptadecene was quantified at various times after exposure to light (120 μmol photons m^{-2} s^{-1}) in presence or absence of the photosynthesis inhibitor DCMU. Values are means of three biological replicates, and error bars represent SD; nd, not detected. Using two-sided *t* tests, no significant differences were found at $P < 0.01$ with and without DCMU. E, Influence of light intensity on heptadecene content per cell volume. Cells were grown in minimal medium at different light intensities in a photobioreactor operated at constant cell density. Cells cultures

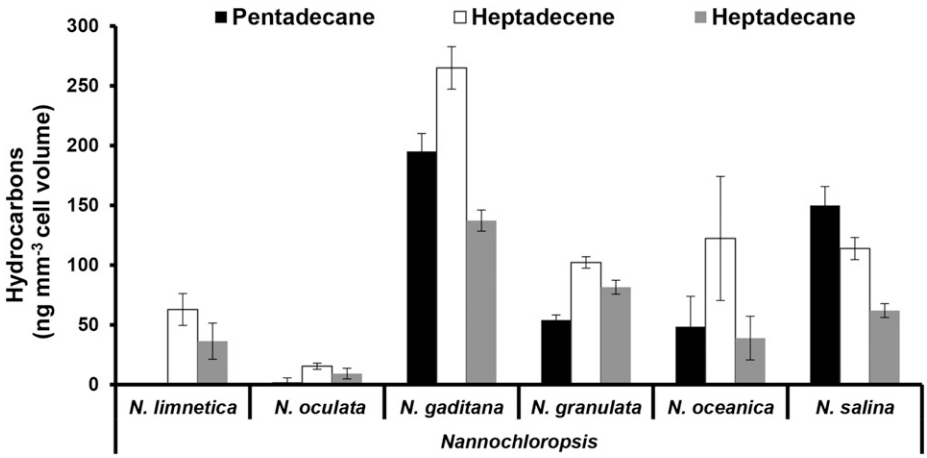

Figure 6. Long-chain hydrocarbons are synthesized by *Nannochloropsis* sp. Strains were cultivated for 5 d, and hydrocarbons were analyzed by transmethylation of whole cells and GC-MS/FID. Values are means of three biological replicates, and error bars represent SD.

did not produce detectable amounts of C15-C17 hydrocarbons under the cultivation conditions used. However, they formed a C21 hexaene (Supplemental Fig. S9). Together, these data suggest that hydrocarbons synthesized from fatty acids are likely to be present in a wide array of microalgal groups and that in microalgal species devoid of very-long-chain fatty acids (>C20), C15-C17 alka(e)nes may be synthesized, preferentially from cis-vaccenic acid.

In *C. reinhardtii* and *C. variabilis*, C15-C17 alka(e)nes represent 0.04 to 0.1% dry weight. This is in the same range as strains for two abundant marine cyanobacteria, *Prochlorococcus* and *Synechococcus*, which produce between 0.02 and 0.4% dry weight of such compounds (Lea-Smith et al., 2015). In the same work on cyanobacterial hydrocarbons, it has been suggested that cyanobacterial hydrocarbons may play a significant role in the hydrocarbon cycle of upper oceans. Given the relatively high abundance of eukaryotic phytoplankton compared to total prokaryotes in sunlit oceans (de Vargas et al., 2015), our data suggest that microalgal hydrocarbons may also contribute significantly to this cycle.

Microalgal Alka(e)ne Synthesis Requires Light

Our data show that light is required for hydrocarbon synthesis in *C. reinhardtii* and *C. variabilis* (Fig. 5B; Supplemental Fig. S7). Accordingly, when *C. reinhardtii* cells are cultivated under day and night cycles (Fig. 5A; Supplemental Fig. S10), alkenes are produced during the day (quantity of alkenes per cell increases, while cell number is constant), and at the beginning of the night the alkene amount per cell decreases in the same proportion as the cell number increases. Thus, decrease of the cellular alkene content during the dark phase seems due to cell division in absence of alkene synthesis. Upon illumination, synthesis of heptadecene occurs within a few minutes (Fig. 5D). Taken together, these data show that the microalgal synthesis of heptadecene is strongly dependent on light and occurs only during the day. Presence in the chloroplast of 80% of *C. reinhardtii* heptadecene suggests a chloroplastic location of the synthesis (Fig. 5C). Interestingly, the fact that alkene production is not affected by the photosystem II inhibitor DCMU shows that the alkene biosynthetic pathway is not coupled to photosynthetic electron transfer reactions either in a direct or indirect manner. It seems therefore clear that microalgal synthesis of C15-C17 hydrocarbons is linked to the presence of light but is not related to the photosynthetic activity, in contrast to the role suggested for alkanes in cyanobacteria (Berla et al., 2015). To our knowledge, a strict light dependency of alka(e)ne synthesis in photosynthetic cells has never been reported. In cabbage leaves, light has been shown to increase the synthesis of some cuticular wax compounds, including alkanes (Macey, 1970). In *Euphorbia* suspension cells, light has been shown to affect the chain length distribution of the alkanes produced but alkane synthesis can occur in the dark (Carrière et al., 1990). In Arabidopsis (*Arabidopsis thaliana*), the total wax loads from stems and leaves were ~20% lower in plants grown in dark conditions for 6 d compared with those grown under long-day conditions (Go et al., 2014). An AP2/ERF-type transcription factor that is preferentially expressed in the epidermis and induced by darkness has been show to down-regulate the expression of alkane-forming enzymes. In *C. reinhardtii*, the complete absence of

Figure 5. (*Continued.*)
were stabilized for 3 d at a constant light intensity (starting at 125 μmol photons m^{-2} s^{-1}) before switching to the next higher intensity (up to 300 μmol photons m^{-2} s^{-1}). Cells aliquots were harvested from stabilized cultures at each intensity to measure heptadecene content. Values are means of three biological replicates, and error bars represent SD.

Table I. *Summary of hydrocarbons detected in the species investigated and potential orthologs to proteins known to be involved in hydrocarbon synthesis*

Potential orthologs were identified by BLASTP reciprocal best hits in complete genomes. The five microalgal target genomes were first searched with BLASTP 2.3.1 (NCBI website, default parameters) using as queries the following proteins (UniProt accession number in parentheses): AtCER1 (F4HVY0), AtCER3 (Q8H1Z0), CYP152/OleT (E9NSU2), CYP4G1 (Q9V3S0), UndA (Q4K8M0), AAR (Q54765), and ADO (Q54764). Possible orthologs were only found for AtCER1 and AtCER3 (in *O. tauri* and *P. tricornutum*). These single hits were then used as a query to search with BLASTP the genome of Arabidopsis.

	C. reinhardtii	*Nannochloropsis* sp.	*C. variabilis NC64A*	*O. tauri*	*P. tricornutum*
Alka(e)nes detected	7-Heptadecene	Pentadecane, heptadecane, 7-heptadecene	Pentadecane, heptadecane, 7-heptadecene, 8-heptadecene	21:6-Alkene	21:6-Alkene
Potential orthologs to hydrocarbon synthesis genes	None	None	None	One hit (to AtCER1/3, A0A090MA0, score 478, e value 3e-163, 40% identity)	One hit (to AtCER1/3, B7G477, score 102, e value 2e-22, 27% identity)

heptadecene in the dark and the rapid induction of heptadecene synthesis by light (Fig. 5, B and D) seems to point to a direct positive regulation by light of alkane-forming enzymes or genes rather than a negative regulation induced by dark. It should be noted that light may not be the only environmental factor affecting heptadecene. Transfer of cells to a nitrogen-deprived medium (Fig. 4) clearly caused an arrest of its synthesis at least for 24 h, and the initial drop in heptadecene content after transfer suggests the alkene is degraded or released. Also, heptadecene increases with cultivation temperature (Supplemental Fig. S6), which might indicate a role in membrane fluidity.

The Major Long-Chain Alka(e)ne Produced Derives from cis-Vaccenic Acid

Labeling experiments with per-deuterated palmitic acid indicate that heptadecenes result from the loss of the carboxyl group in a C18 monounsaturated fatty acid (Fig. 3). The two major possible origins of heptadecenes are oleic acid (double bond in Δ9 position) and its positional isomer cis-vaccenic acid (Δ11), a bacterial-type fatty acid fairly abundant in some microalgae, including *C. reinhardtii* (Giroud et al., 1988). Loss of the carboxyl group in oleic and cis-vaccenic acids would yield 8-heptadecene and 7-heptadecene, respectively (Supplemental Fig. S11). Analysis of *C. reinhardtii* or

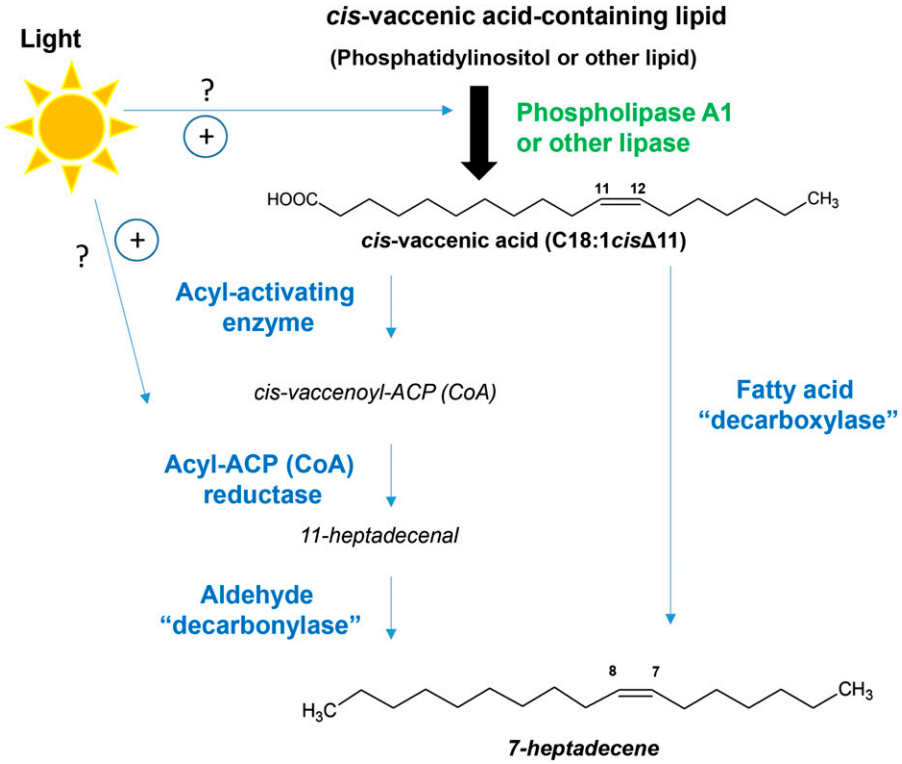

Figure 7. Possible pathways for 7-heptadecene synthesis in *C. reinhardtii*. Two major possibilities are presented based on findings of this work and reactions known in other organisms. The specificity for cis-vaccenic acid would be brought by a phospholipase A1 or another type of lipase depending on the lipid used as substrate. Left side: cyanobacterial- or plant-type pathway with acyl reduction and formal decarbonylation of aldehyde intermediate. Chloroplast localization would involve acyl-acyl carrier protein (acyl-ACP) substrate, while other subcellular localizations would involve acyl-coenzyme A (acyl-CoA) substrate. Right side: bacterium-type pathway with decarboxylation of a free fatty acid (but without formation of terminal double bond). Decarbonylase and decarboxylase are indicated with quotes because decarbonylation and decarboxylation might be only formal and not actual reactions. Regulation by light may occur at the step of free fatty acid generation and/or at its conversion to heptadecene.

C. variabilis hydrocarbons with DMDS revealed that the heptadecene produced in *C. reinhardtii* (and the major heptadecene in *C. variabilis* and *Nannochloropsis*) was 7-heptadecene (Fig. 2). The 8-heptadecene is a minor isomer in *C. variabilis*. The major hydrocarbon of *C. variabilis*, *C. reinhardtii*, and *Nannochloropsis* is therefore derived from cis-vaccenic acid and not from oleic acid. This indicates a high substrate specificity of at least one enzyme in the biosynthetic pathway. A simple explanation to account for this specificity could be the involvement of a phospholipase A1 (Fig. 7). Indeed, in *C. reinhardtii* the lipid class most enriched in cis-vaccenic acid is phosphatidylinositol and this fatty acid is found almost only in the *sn*-1 position (Giroud et al., 1988). The free fatty acid released by might be converted to an alkene via a fatty aldehyde intermediate (cyanobacterial- or plant-type pathway involving an acyl-ACP or acyl-CoA reductase). Alternatively, the alkene might be directly produced from the fatty acid by a bacterium-type pathway (Rude et al., 2011; Rui et al., 2014) involving an actual or formal decarboxylation.

Microalgae Harbor New Alka(e)ne-Forming Enzymes

Regardless of whether the microalgal synthesis of long-chain alka(e)nes involves an aldehyde intermediate, the presence of C15-C17 alka(e)nes in *C. reinhardtii*, *C. variabilis*, and *Nannochloropsis* and the absence in these species of proteins homologous to alkane-forming enzymes from plants, cyanobacteria, bacteria, or insects clearly indicate that some hitherto unidentified alka(e)ne-forming enzyme(s) are present in these three microalgae (Table I). This conclusion is consistent with the unique strong light dependency of heptadecene synthesis observed in *C. reinhardtii* and *C. variabilis*. The C21 hexaene found in the Mamiellophycea *O. tauri* and in the diatom *P. tricornutum* might be synthesized by the plant CER1/CER3 homolog found in these two species only. Microalgal homologs of CER1/CER3 might be thus more specific of very-long-chain fatty acids, but another biochemical role cannot be excluded. Clearly, further genetic or biochemical work will be needed to identify the enzyme(s) responsible for long-chain alka(e)ne synthesis in microalgae. This will be an important step toward unraveling the function of alka(e)nes within the microalgal cell as well as elucidating its regulation in connection with light and other environmental factors. Possible roles of hydrocarbons in chloroplasts of microalgae include cell signaling or the regulation of the fluidity of photosynthetic membranes in response to changes in light intensity or temperature. Given the high hydrophobicity of hydrocarbons, a role in regulating membrane properties seems more likely. From a biotechnological perspective, the discovery of the microalgal alkane synthase(s) would also expand the repertoire of enzymes available to harness hydrocarbon synthesis in microbes (André et al., 2013; Choi and Lee, 2013; Liu et al., 2014; Chen et al., 2015). The presence of hydrocarbons of various chain length in *C. variabilis* cells shows that microalgal hydrocarbon-forming enzymes may in fact have a broad substrate specificity. Therefore, they could be used to generate alkanes and alkenes from a variety of cellular fatty acids in a bacterial or microalgal host. Combination of this alkane-forming system with a medium-chain specific thioesterase would allow to produce volatile alka(e)nes and circumvent the need for intracellular alkane storage (Choi and Lee, 2013). Identification of the enzymes forming C15-C17 hydrocarbons in green microalgae is therefore an exciting task from the point of view of microalgal physiology but also from a biotechnological perspective.

MATERIALS AND METHODS

Strains and Culture Conditions

Chlamydomonas reinhardtii wild-type strains CC124 (nit1 nit2; mt^-) and CC125 (nit1 nit2; mt^+) were used. *Nannochloropsis* species were from the CCMP culture collection: *Nannochloropsis oceanica* CCMP 531 and 1779; *Nannochloropsis gaditana* CCMP 527; *Nannochloropsis granulata* CCMP 529; *Nannochloropsis limnetica* CCMP 505; *Nannochloropsis salina* CCMP 537 and 538; and *Nannochloropsis oculata* CCMP 525. *Chlorella variabilis* NC64A was from the laboratory of J.L. Van Etten (University of Nebraska). Other strains were from the Roscoff Culture Collection: *Ostreococcus tauri* RCC745 (strain OTTH0595-genome); *Phaeodactylum tricornutum* RCC 2967 (strain Pt1_8.6). All strains except *O. tauri* were grown routinely in conical flasks in incubation shakers at 25°C (Infors HT) under air enriched with 2% CO_2, with agitation at 140 rpm and light intensity at 120 μmol photons m^{-2} s^{-1} (70 for *C. variabilis*). *O. tauri* was grown in a culture chamber at 25°C in flasks without agitation and using a light intensity of 80 μmol photons m^{-2} s^{-1}. Regarding culture media, *C. reinhardtii* and *C. variabilis* were cultivated in TAP medium and minimal medium (Harris, 1989) containing 20 mM MOPS. Other microalgae were grown in artificial sea water added with various nutrients: Conway's, Guillard's F/2 (Lananan et al., 2013) and Keller's (Keller et al., 1987) media were used for *Nannochloropsis*, *P. tricornutum*, and *O. tauri*, respectively. Cells were routinely counted using a Multisizer 3 (Coulter). To produce gametes, culture medium of vegetative cells in exponential growth phase (10 million cells mL^{-1}) was changed from TAP medium to TAP nitrogen depleted medium (TAP-N). To be sure to remove all nitrogen, cells were washed three times with TAP-N medium. Cells were kept in TAP-N for 16 h before analysis.

Cultures in Photobioreactors

C. reinhardtii CC124 (*mt- nit1 nit2*) cells were cultured in minimal medium (Harris, 1989) in 1-liter photobioreactors (Biostat Aplus; Sartorius Stedim Biotech) operated as turbidostats. A_{880} was measured continuously using a biomass probe (Excell probe; Exner), and cultures were maintained at constant A_{880} by injection of fresh medium. The pH was maintained at a constant value of 7 by injection of KOH (0.2 N) or HCl (0.2 N). The cultures were stirred using a metal propeller (250 rpm). The gas flow rate was adjusted to 0.5 L min^{-1}. Air enriched with 2% CO_2 was generated using mass flow meters (EL flow; Bronkhorst). Light was supplied by eight fluorescent tubes (Osram Dulux L 18 W) placed radially around the photobioreactor. Cells were cultivated for 3 d with the same light intensity before collecting cells. Light intensity was increased gradually from 15 to 300 μmol photons m^{-2} s^{-1}.

Cell Labeling with Deuterated Palmitic Acid

One hundred million cells of *C. reinhardtii* 137c and 200 million cells of *N. gaditana* CCMP 527 and *C. variabilis NC64A* were incubated for 24, 48, or 72 h in sealed tubes with 5 mL of TAP medium containing 100 μM perdeuterated (D_{31}) palmitic acid (from a 10 mM stock solution in dimethyl sulfoxide). Total lipids and alka(e)nes were transmethylated and analyzed by GC-MS/FID as described below.

Saponification

Cell pellets (100 million cells for *C. reinhardtii*; 200 million cells for other microalgae) were added with 4 mL of an aqueous solution of 5% (w/v) KOH and heated for 90 min at 85°C in sealed vials. After cooling down, 4 mL of 0.9% (w/v) NaCl was added, and unsaponifiable compounds were extracted three times with 4 mL methyl-*tert*-butyl-ether (MTBE). Samples were vortexed for 10 min and the organic phases were recovered by centrifugation at 3,000*g* for 2 min and pooled. MTBE was evaporated under a gentle stream of nitrogen gas and unsaponifiables were resuspended in 500 μL hexane and 1 μL was injected in the GC-MS.

Transmethylation

To quantify hydrocarbons together with fatty acids, transmethylation of whole cells was used. Briefly, cell pellets (100 million cells for *C. reinhardtii*; 200 million cells for the other microalgae) were added with 2 mL of a solution containing methanol with 5% (v/v) sulfuric acid and 25 μg of triheptadecanoate (from a stock solution 2.5 mg mL^{-1} in chloroform) and 5 μg of 16:0-alkane (stock solution 1 mg mL^{-1} in chloroform) were included as internal standards. Samples were incubated at 85°C for 90 min in sealed glass tubes. After cooling down, FAMEs and hydrocarbons were extracted by adding 500 μL hexane and 500 μL 0.9% (w/v) NaCl. Samples were mixed for 10 min and the organic phase was separated from the aqueous phase by centrifugation at 3,000*g* for 2 min. The hexane phase was recovered and 1 μL was injected in the GC-MS/FID.

Solid-Phase Microextraction

One microgram of 16:0-alkane internal standard (0.1 mg mL^{-1} in chloroform) was added to a 250-mL flask and the flask was left at room temperature under a hood for 2 min to evaporate the chloroform. Microalgal cell culture (200 mL at 15 millions cells per mL) or 200 mL of culture medium was then added to the flask. An SPME fiber (DVB-PDMS fused silica, 65 μm double-polar; Supelco) was mounted on a holder and inserted and incubated for 16 h at room temperature in the headspace of the sealed flask. After incubation, the fiber was immediately inserted into the injector of the GC-MS and analytes were desorbed at 250°C (first 2 min of the GC program). GC-MS analysis was then carried out as described below.

Determination of Double Bond Position in Alkenes

After saponification of cell pellets, MTBE extracts containing alkenes were evaporated in a vial under a gentle stream of nitrogen gas. One hundred microliters of DMDS containing 1.3 mg of iodine was then added, the vial was sealed, and the mixture was incubated for 30 min at 35°C. After cooling down the mixture, 0.9 mL of a mixture diethylether:hexane (1/9 [v/v]) was added. The whole sample was then loaded onto a mini-column containing silica (Shibahara et al., 2008). The column was washed five times with 1 mL of the diethylether:hexane mixture. The entire eluent (5 mL) was evaporated under a gentle stream of nitrogen gas. The residue was resuspended in 500 μL of hexane and 1 μL was injected in the GC-MS.

GC-MS Analyses

GC-MS analyses, which were performed after experiments of DMDS adducts, saponification, and SPME, were carried out using the following setup. A Thermo-Fischer gas chromatographer Focus series coupled to a Thermo-Fischer DSQII mass spectrometer (simple quadrupole) was used with a DB-5HT (Agilent) apolar capillary column (length 30 m, internal diameter 0.25 mm, and film thickness 0.1μm). Helium carrier gas was at 1 mL min^{-1}. Oven temperature was programmed with an initial 2 min hold time at 50°C, then a ramp from 50 to 300°C at 10°C min^{-1}, and a final 3-min hold time at 300°C. Samples were injected in splitless mode (2 min) at 250°C. The MS was run in full scan over 40 to 500 amu (electron impact ionization, 70 eV), and peaks were quantified based on total ion current using the internal standards.

GC-MS/FID Analyses

GC-MS/FID analyses were performed only after transmethylation reactions in order to quantify fatty acids and hydrocarbons. Analyses were carried out on an Agilent 7890A gas chromatographer coupled to an Agilent 5975C mass spectrometer (simple quadrupole). A Zebron 7HG-G007-11 (Phenomenex) polar capillary column (length 30 m, internal diameter 0.25 mm, and film thickness 0.25 μm) was used. Hydrogen carrier gas was at 1 mL min^{-1}. Oven temperature was programmed with an initial 2-min hold time at 60°C, a first ramp from 60 to 150°C at 20°C·min^{-1}, then a second ramp from 150 to 240°C at 6°C·min^{-1} and a final 3-min hold time at 240°C. Samples were injected in splitless mode (1 min) at 250°C. The MS was run in full scan over 40 to 350 amu (electron impact ionization at 70 eV), and peaks were quantified based on the FID signal using the internal standards.

Chloroplast Isolation

The *C. reinhardtii* cell wall-deficient strain CW15 was used to isolate chloroplasts. Culture conditions and procedure for chloroplast purification were identical to those previously published (Mason et al., 2006). Chlorophyll of whole cells and purified chloroplasts was extracted with 80% (v/v) acetone and quantified by spectrophotometry. Alkenes were quantified by transmethylation of whole cells or chloroplasts.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Heptadecane and pentadecane are detected in *Chlorella* cultures.

Supplemental Figure S2. Detection of hydrocarbons in the headspace of liquid cultures.

Supplemental Figure S3. Heptadecene does not result from artifactual decarboxylation of oleic acid during transmethylation of cells.

Supplemental Figure S4. Deuterium-labeled (D31) palmitic acid is metabolized by *Chlorella* cells.

Supplemental Figure S5. Heptadecene content in opposite mating types of vegetative cells and gametes.

Supplemental Figure S6. Chlamydomonas heptadecene content varies with growth temperature.

Supplemental Figure S7. Alka(e)ne synthesis is strongly dependent on light in *Chlorella variabilis* NC64A.

Supplemental Figure S8. Biomass and heptadecene productivities increase with light intensity.

Supplemental Figure S9. Mass spectrum of the C21:6 hexaene found in *Phaeodactylum*.

Supplemental Figure S10. Variation of *Chlamydomonas* cell concentration during day/night cycle.

Supplemental Figure S11. Heptadecene isomers that would result from formaldehyde carboxylation of oleic and cis-vaccenic acids.

ACKNOWLEDGMENTS

We thank Patrick Carrier for help with GC maintenance and Audrey Beyly-Adriano, Xenie Johnson, Jean Alric, Claire Sahut, and Frédéric Carrière for helpful discussions. Technical help of Jérémy Monlyade and gift of the *C. variabilis* NC64A strain by the J.L. Van Etten laboratory are also acknowledged.

Received March 24, 2016; accepted June 7, 2016; published June 10, 2016.

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