A High-Throughput Fatty Acid Profiling Screen Reveals Novel Variations in Fatty Acid Biosynthesis in Chlamydomonas reinhardtii and Related Algae

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Analysis of fatty acid methyl esters (FAMEs) by gas chromatography (GC) is a common technique for the quantitative and qualitative analysis of acyl lipids. Methods for FAME preparation are typically time-consuming and labor-intensive and require multiple transfers of reagents and products between reaction tubes and autosampler vials. In order to increase throughput and lower the time and materials costs required for FAME preparation prior to GC analysis, we have developed a method in which 10- to 20-mg samples of microbial biomass are transferred to standard GC autosampler vials, transesterified using an emulsion of methanolic trimethylsulfonium hydroxide and hexane, and analyzed directly by GC without further sample handling. This method gives results that are essentially identical to those obtained by the more labor- and material-intensive FAME preparation methods, such as transesterification with methanolic HCl. We applied this method to the screening of laboratory and environmental isolates of the green alga Chlamydomonas for variations in fatty acid composition. This screening method facilitated two novel discoveries. First, we identified a common laboratory strain of C. reinhardtii, CC-620, completely lacking all ω-3 fatty acids normally found in this organism and showed that this strain contains an inactivating mutation in the CrFAD7 gene, encoding the sole ω-3 desaturase activity in this organism. Second, we showed that some species of Chlamydomonas make Δ6-unsaturated polyunsaturated fatty acids (PUFA) rather than the Δ5 species normally made by the previously characterized laboratory strains of Chlamydomonas, suggesting that there is species-specific variation in the regiospecificity and substrate selectivity of front-end desaturases in this algal genus.

Gas chromatography (GC) of fatty acid methyl esters (FAMEs) is a commonly used technique for analysis of acyl lipids in biological systems (1). Numerous methods have been developed for the preparation of FAMEs from biological materials, and among the most often used are acid-catalyzed transesterifications using methanolic solutions of H$_2$SO$_4$ or HCl (2). These methods are rapid and convenient and offer nearly quantitative conversion of acyl chains to their methyl ester. However, the acid-catalyzed FAME synthesis protocols typically have multiple steps in which reagents must be added to tubes or samples must be transferred from one tube to another. For example, acidic methanolysis is usually conducted in a sealed tube at elevated temperature, after which FAMEs must be extracted from the tube by the addition of saline solution and an appropriate apolar solvent, such as hexane or heptane, followed by phase partitioning and transfer of the organic phases to vials compatible with the GC instrument to be used for analysis (see workflow scheme of Fig. 1A). For the purposes of brute-force biochemical screening of mutant collections, or screening of environmental isolates of algae for selection of strains with desirable metabolic or lipid composition properties, hundreds or thousands of individual samples may need to be processed. When FAMEs are prepared for analysis in a large-scale screen, each individual addition, incubation, or transfer step represents a major bottleneck that reduces sample throughput, especially in laboratories that lack expensive automated instruments dedicated solely to sample preparation.

In order to streamline the preparation of microbial samples for GC-based fatty acid analysis, we have developed a simple method for the one-step, in-vial transmethylation and extraction of FAMEs from very small amounts of microbial biomass. This method makes use of transesterification with methanolic trimethylsulfonium hydroxide (TMSH), which can be prepared in large quantities using readily available reagents. Using the green alga Chlamydomonas reinhardtii, we demonstrated that the one-step TMSH method gives fatty acid profile results that are essentially identical to those obtained with a methanolic HCl protocol also in use in our laboratory (2) while requiring only one sample handling step: the introduction of a small amount of cells transferred from a petri plate into a standard 2.0-ml crimp-cap autosampler vial. Using this procedure, a single worker can typically prepare 60 to 100 samples per hour, and, under appropriate chromatographic conditions, 150 to 200 samples per day can be analyzed on a standard single-injector GC–flame-ionization detection (GC–FID) system. To demonstrate the usefulness of this FA profiling method, we analyzed hundreds of algal strains, including various laboratory isolates of Chlamydomonas and offspring from genetic crosses of these strains as well as environmental isolates of Chlamydomonas. Having this high-throughput FA profiling capacity thus allowed us to identify a Chlamydomonas strain deficient in the sole ω-3 desaturase gene in this organism as well as variations

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in the complement of fatty acids synthesized by members of the genus, implying variations in regiospecificity and substrate selectivity in the front-end desaturase enzyme of the endoplasmic reticulum (ER) in this genus.

MATERIALS AND METHODS

Strains and culture conditions. Previously characterized *Chlamydomonas reinhardtii* strains were obtained from the *Chlamydomonas* Genetic Stock Center at the University of Minnesota. Additional isolates of strain CC-620 were obtained from the laboratories of Heriberto Cerutti (University of Nebraska—Lincoln) and Ursula Goodenough (Washington University, St. Louis, MO). Cultures were grown under cool-white fluorescent illumination (100 μE/m²/s) at 22 to 25°C under phototrophic conditions on solidified Tris-acetate-phosphate (TAP) medium.

Environmental isolates of *Chlamydomonas* were selected as follows. After rainstorms in early June 2013, standing water in and around the parking lot of the George W. Beadle Center on the University of Nebraska—Lincoln campus was sampled by dipping a centrifuge tube into the water, and 10-ml portions of puddle water were centrifuged in 15-ml conical centrifuge tubes at 1,500 g for 5 min. The pelleted material (which would include any motile, phototactic algal cells, such as those of *Chlamydomonas* and related members of the *Volvocales* algal class) was then mixed with 1.0 ml of a 50% Percoll solution in TP medium (same composition as TAP medium solidified with 2% agar), transferred to a sterile 15-ml tube, and overlaid with 2.0 ml of 25% Percoll–TP medium and then 10 ml of sterile TP medium. The bottom of the tube was then wrapped in aluminum foil with the top exposed to a fluorescent bulb to prompt any motile, phototactic organisms to swim to the top of the tube. After 1 h, 20-μl aliquots of medium were collected and plated on TP medium solidified with 2% agar. Essentially all of the sampled locations yielded green algal colonies, which were apparent within 5 to 7 days. The colonies were examined by bright-field microscopy using a 20× objective on an AMG EvoFl microscope. Large colonies containing cells that were morphologically similar to *Chlamydomonas reinhardtii* (biflagellate spherical or ovoid cells, cup-shaped plastid, and one apparent pyrenoid) were picked and purified by several rounds of single-colony isolation, and the strains were propagated as patches on solid TP medium. Additional strains of *Chlamydomonas*-like algae were isolated in a similar manner from soil collected from a cow pasture outside Mayview, MO, in October 2012.

Genetic analysis. *C. reinhardtii* crosses were carried out by standard procedures as documented in reference 4 with modifications as follows. Gametes of strains CC-620 (mating type positive [mt+]), CC-621 (mating type negative [mt-]), CC-124 (mt-), and CC2342 (mt-) were generated by suspending several loops from a lawn of cells on a TAP plate into 5 ml of ammonium chloride-free liquid TAP medium (TAP-N), followed by overnight incubation with shaking in the light. These gamete cultures of the opposite mating types were mixed and allowed to mate in the light for 4 h, and aliquots were spotted onto a plate of TAP medium solidified with 4% (wt/vol) agar. This plate was left in the light for 24 h and then wrapped with several layers of aluminum foil, and sporulation was allowed to proceed at room temperature in the dark for an additional 6 to 14 days. Plates were then unwrapped, and unmerged vegetative cells were removed by scraping the agar surface with a sterile razor blade, leaving behind the matured zygotic spores, which remained attached to the agar surface. This was followed by exposure of the plate for 30 s over a 500-ml beaker containing a small amount of chloroform and then freezing the plate at −20°C for at least 24 h. This treatment is designed to kill any remaining haploid or vegetative diploid cells, leaving the spores to germi-

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**FIG 1** Method schematic and sample GC traces for *Chlamydomonas*. Samples were prepared and analyzed by GC/FID as described in the text. (A) Method schematic of sample preparation using a methanolic HCl protocol (2) starting with Bligh-Dyer lipid extracts (ext.) of algal biomass. (B) Outline of steps in the TMSH in-vial derivatization protocol described in this work. R.T., room temperature. (C and D) Example GC/FID trace of FAMEs from *Chlamydomonas reinhardtii* derived by the methods outlined in panels A and B, respectively, as described in the text.
nate in the absence of potentially confounding parental contaminants. We attempted to perform tetrad analysis on the progeny of individual spores; however, the CC-620 and CC-621 strains are known to produce synthetic lethal offspring (Elizabeth Harris, Duke University, personal communication), rendering it very difficult to obtain complete tetrads. Given this fact, we analyzed random haploid progeny of the crosses by plating cells isolated from microcolonies arising from spores that had grown for 48 h after the chloroform/freezing treatment. Individual F1 progeny were patched to TAP medium and the fatty acid composition was determined as described below.

Preparation of TMSH reagent. Trimethylsulfonium hydroxide (0.2 M in methanol) was prepared essentially as described by Butte (5) as follows: 4.4 g trimethylsulfonium iodide (catalog no. T80489; Sigma) was dissolved with stirring in 100 ml high-performance liquid chromatography (HPLC)-grade methanol (Fisher) with gentle warming in a 50°C water bath. Silver (I) oxide (Sigma catalog no. 221163) (5 g) was added, and the resulting slurry was stirred for 4 h at room temperature. This preparation was filtered through Whatman no. 2 filter paper and stored in a sealed bottle at 4°C for up to 6 months without a discernible loss of transmethylating activity. The filtrate of this reaction mixture contains toxic residues of silver oxide and silver iodide and should be disposed of according to institutional guidelines for the handling of hazardous waste. TMSH is a strongly basic and caustic reagent capable of producing chemical burns and should therefore be handled only in a fume hood with appropriate personal protective equipment.

FAME derivatization and extraction. A sterile toothpick was used to transfer 10 to 20 mg of algal cells to a 2-ml crimp-cap GC autosampler vial, and the microbial biomass was either deposited on the side of the vial or suspended in 50 µl of TMSH reagent that had already been introduced into the vial, followed by brief vortex mixing to generate a suspension of cells and methanolic TMSH. Hexane (a mixture of isomers; Sigma) (500 µl) was added to the vial, which was then closed with a standard crimp cap. The vial was subjected to vigorous vortex mixing and allowed to sit at room temperature for 15 to 30 min and was then subjected to vortex mixing again and loaded into autosampler racks (Agilent model 7693) for analysis. After a further 10 to 15 min of incubation at room temperature, the resulting sample vials typically contain a particulate deposit of cell debris on the bottom and sides of the vial, a thin aqueous/methanol phase on the bottom of the vial consisting of methanolic TMSH and water extracted from the cells, and an upper hexane phase containing the FAMES. Biological replicates of the samples used for TMSH transmethylated were also prepared from Bligh-Dyer total lipid extracts of *Chlamydomonas* by the methanolic HCl method of Ichihara and Fukubayashi (2). Data are presented as the means ± standard errors of the means (SEM) of the results obtained with at least three independent biological replicate experiments conducted as duplicate or triplicate technical replicates.

For analysis of fatty acids esterified to individual membrane lipid classes, lipids were extracted from cultures as previously described (6) and lipid classes were separated on glass-backed Sil-60 TLC plates (Sigma) with chloroform/methanol/water (65:25:4 [vol/vol/vol]) as the developing solvent. Bands were identified by UV illumination after the plate was sprayed with a 0.2% (wt/vol) ethanolic solution of 8-anilinonaphthalene sulfonic acid. The silica from the MGDG (monogalactosyldiacylglycerol) and DGTs (diacylglyceryl-N,N,N-trimethylhomoserine) bands was scraped into screw-cap tubes (13 mm by 100 mm), and 10 µg of pentadecanoic acid was added as an internal standard prior to transmethylolation according to the method described in reference 2. Quantification by gas chromatography and flame-ionization detection (GC-FID) analysis were performed as described below.

GC conditions. For TMSH-derived samples, the upper hexane phase containing the FAMES was analyzed without further purification or concentration on an Agilent 7890A GC system equipped with a flame-ionization detector (FID) and HP-Innowax column (30 m by 0.25 mm, 0.023-mm film thickness). A 2.50-µl volume of the hexane phase was injected into the inlet (Agilent part number 5190-3164) (250°C, glass wool-plugged split/splitless inlet liner) at a 10:1 split ratio. H₂ for carrier gas and FID fuel and zero-grade air were provided by an FID gas station (Parker-Ballston). For separation of algal FAME mixtures, carrier H₂ was supplied at a constant inlet pressure of 18 lb/in², and the oven temperature profile was a linear gradient from 180 to 240°C (10°C/min), followed a 2-min hold at 240°C, for a total run time of 8 min. FID conditions were as follows: 400°C detector temperature, 300 ml/min purified air, 30 ml/min hydrogen fuel gas, and no makeup gas. Identification of fatty acid species was performed by the use of comparisons to retention times of authentic standard compounds, previously characterized algal or plant lipid extracts, or standardized oils purchased from Sigma or Nu Chek Prep. Pine nut oil was isolated by hexane extraction of crushed pine nuts (Kirkland brand; Costco) and used as a standard for the Δ5 fatty acids and pinolenic (18:3Δ5,9,12) and coniferonic (18:3Δ5,9,12,15) acids. Escherichia coli strain XL-1 Blue lipid extracts were used as a source of cis-vaccenic acid (18:2 Δ9,11), Soybean oil from plants engineered to accumulate gamma-linolenic (18:3Δ6,9,11) and stearidonic (18:3Δ6,9,12,15) acids (7) was used as a standard for these FA. GC-MS analysis was used to verify the identity of a prominent peak that was present in the TMSH extracts but not in methanolic HCl extracts, and these analyses were carried out under similar conditions of gas flow rates and temperature profiles using an Agilent 7890A GC equipped with a 30-m HP5-MS column and 5975C mass-sensitive detector.

RESULTS

Development and utility of TMSH method for determination of microbial fatty acid profiles. With the recent increase in interest in the use of oleaginous photosynthetic microbes as biofuel and chemical feedstocks (8, 9), there has been a corresponding need for improved methods to facilitate the cost-effective and high-throughput determination of the fatty acyl composition of these industrially important microorganisms. The use of methanolic TMSH as a FAME preparation reagent was initially described for the rapid determination of the fatty acid composition of food-grade fats and oils (5) and has been applied to the determination of fatty acid composition in pathogenic bacteria as a taxonomic marker (10, 11). We chose to streamline these previously described TMSH-mediated transesterification methods for the purpose of decreasing the number of hands-on sample-handling steps required for FAME preparation prior to GC analysis of microbial fatty acid composition, thus increasing sample throughput. An outline of the TMSH method is presented in Fig. 1B in a comparison to an acid-catalyzed FAME preparation method in use in our laboratory (Fig. 1A). As shown in Fig. 1C and D, the FAME profiles derived from *Chlamydomonas* cells that had been derivatized with either TMSH or methanolic HCl are essentially identical. This derivatization method also appears to reduce artifacts that manifest as a series of compounds eluting prior to methyl palmitate (16:0) which are present in the samples prepared using methanolic HCl (Fig. 1C) but not in those prepared by TMSH derivatization (Fig. 1D). This series of compounds was variable in prevalence and composition from sample to sample and likely consisted of degradation products of polyunsaturated FA arising from heating at 80°C in the presence of HCl, which is previously documented for acidic methanolysis protocols (1), as well as degradation products of phytol derived from chlorophyll decomposition. The GC-FID traces presented in Fig. 1 were quantified and are presented in Fig. 2. Apart from very slight deviations in the mole percentages calculated for the 18-carbon monoenoic species, perhaps due to minor differences between the two methods in FAME extraction efficiency, the fatty acid profiles generated by the methanolic HCl and TMSH methods are essentially identical. Also

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of note, a species eluting after 18:4 was present in extracts derived from the TMSH method but not in MeOH/HCl-derived FAMEs and was subsequently determined by GC/MS analysis to be phytol, presumably resulting from TMSH-catalyzed release of this isoprenoid moiety from chlorophyll (data not shown).

Considerations and caveats of the one-step TMSH FAME preparation method. Given the apparent advantages of streamlined sample preparation and reduction of artifacts, the one-step TMSH protocol will be useful for studies that necessitate the high-throughput profiling of fatty-acyl species in diverse biological systems. However, it is necessary to highlight several caveats that must be considered in the use of this method. First, we find that free fatty acids are poorly, if at all, esterified under the conditions we outline (data not shown). In cases where a substantial fraction of acyl chains are present as free fatty acids or salts thereof, the acid-catalyzed esterification protocols remain preferable. This finding also makes it necessary to use an esterified form of the odd-chain fatty acids typically employed as internal standards for quantitative analysis, e.g., tripentadecanoin, triheptadecanoin, or odd-chain-containing phosphatidylcholine species.

With regard to instrumentation, we recommend the use of glass-wool-plugged GC inlet liners (e.g., Agilent part number 5190-3164) since the glass wool offers a protective barrier to block inadvertent introduction of particulate material into the inlet or the column. We also found that in our initial development of this method, repeated injection of the hexane phase derived from algal samples may lead to the buildup of a sticky residue that coats the plunger and barrel of the injection syringe. After several hundred samples, this can cause the syringe to seize during a washing or injection step and can result in “Z-folding” of the syringe piston. This residue is not removed by extensive washing of the syringe with hexane but can be removed by washing the syringe with methanol after injection. Therefore, we include two methanol washes after injection, followed by three washes with hexane, and an additional three hexane washes prior to injection of the next sample. Inclusion of the methanol washes after sample injection completely eliminated this issue with the injection syringe. Our laboratory has developed these protocols for our Agilent 7890A system, and method files are available upon request. Additionally, this method for FAME preparation from microbial biomass has successfully been applied to the yeasts *Candida albicans* and *Saccharomyces cerevisiae* and to the alga *Chlorella sorokiniana* strain UTEX 1230 (Riekhof laboratory, unpublished results), demonstrating the broad applicability of the method to microbes with cell wall chemistries distinctly different from those of *Chlamydomonas* spp.

ω-3 fatty acids are completely absent from strain CC-620. Given the diversity of wild isolates of *C. reinhardtii*, as well as strain-to-strain differences in the laboratory isolates of this organism arising from decades of serial subculturing (12, 13), we applied the TMSH profiling method for detailed analysis of the fatty acid composition of a number of different strains to assess whether there are population-level variations in this aspect of lipid metabolism and membrane homeostasis. As shown in Fig. 3, the most striking difference we noted in our collection of laboratory *Chlamydomonas* isolates was the complete absence of the follow-

![FIG 2](image-url) **FIG 2** Chlamydomonas fatty acid composition determined by TMSH versus MeOH/HCl. Samples prepared as described in the text and represented in Fig. 1 were quantified and peak areas integrated using ChemStation software (Agilent). Data represent the mean (± SEM) moles percent (mol%) of each fatty acid determined from three independent biological replicates conducted as triplicate technical replicates.

![FIG 3](image-url) **FIG 3** *C. reinhardtii* strain CC-620 lacks ω-3 fatty acid species. GC-FID analysis of trimethylsulfonium hydroxide (TMSH)–derived fatty acid methyl esters (FAME) in strains CC-124 (top) and CC-620 (bottom), demonstrating the apparent absence of all ω-3 fatty acid species (16:3Δ4,7,10, 16:4Δ4,7,10,13, 18:3Δ9,12,15, and 18:4Δ5,9,12,15) in CC-620.
ing fatty acid species from strain CC-620: 16:3Δ7,10,13, 16:4Δ4,7,10,13, 18:3Δ9,12,15, and 18:4Δ5,8,12,15. These specific fatty acids make up the C. reinhardtii ω-3 class, defined as bearing a double bond between the third and fourth carbon atoms from the methyl terminus of the fatty acyl chain. All of the other strains displayed the full complement of fatty acids expected for C. reinhardtii, in relative quantities similar to those reported by other investigators (14, 15).

In order to verify the complete absence of ω-3 FA from this common laboratory strain, we obtained and analyzed additional isolates of CC-620 from the laboratories of Heriberto Cerutti (University of Nebraska—Lincoln) and Ursula Goodenough (Washington University, St. Louis, MO). As with the strain in use in our laboratory (obtained in 2009 from the Chlamydomonas culture collection at the University of Minnesota), the CC-620 isolates from the Cerutti and Goodenough laboratories displayed a complete lack of ω-3 FA species. This demonstrates that the Cerutti laboratory CC-620 strain, which was obtained from the Chlamydomonas culture collection in 1999 (H. Cerutti, personal communication), contained the mutation leading to this defect prior to that date, indicating that the desaturase defect in CC-620 did not arise during recent routine subculturing of the strain in our laboratory. The Goodenough laboratory isolate also bore this defect, indicating that the ω-3 deficiency was likely present at the time the strain was isolated by U. Goodenough in the early 1970s.

**Compartment-specific ω-3 species are missing from membrane lipid classes specific to the ER and chloroplast.** Previous work by Giroud et al. (15, 16) on the acyl-chain composition and lipid-linked desaturation of membrane lipids in C. reinhardtii strongly suggested that two separately localized ω-3 desaturase activities are operative in this organism: a chloroplast-localized enzyme which acts principally on the acyl moieties of monogalactosydialglycerol (MGDG) and an ER-localized enzyme which prefers the betaine lipid diacylglyceryl-N,N,N-trimethylhomoserine (DGTS). We isolated MGDG and DGTS from strains CC-620 and CC-124 by thin-layer chromatography and analyzed the acyl chain composition of these lipids by transmethylation and quantification by GC-FID. As expected from the analysis of FAMEs derived from whole-cell extracts, Fig. 4 shows that the compartment-specific ω-3 species are missing from their representative polar lipid classes. In CC-124, used as the wild-type reference strain for these experiments, MGDG (Fig. 4A) is dominated by a mixture of 16:0, 16:4Δ4,7,10,13, and 18:3Δ9,12,15 acyl chains. In the CC-620 background, the ω-3 polyunsaturated fatty acid (PUFA)
species are quantitatively replaced with the presumed precursor species, 16:3Δ4,7,10 and 18:2Δ9,12, demonstrating a loss of the ω-3 desaturase activity required for generation of the chloroplast-specific ω-3 FA 16:4Δ4,7,10,13.

Likewise for the cytoplasmic compartment (Fig. 4B), CC-124 shows a typical complement of FA species esterified to DGTS, consisting predominantly of 16:0, 18:2Δ9,12, 18:3Δ5,9,12, 18:3Δ5,9,12,15, and 18:4Δ5,9,12,15. In CC-620, DGTS lacks the Δ15 (ω-3) species and accumulates the corresponding Δ12 (ω-6) series, demonstrating the loss of the ω-3 desaturase activity responsible for generating 18:4Δ5,9,12,15, the ω-3 FA specific to the extrachloroplastic compartments. Taken together, these data are consistent with two possibilities: (i) the presence of multiple genetic defects in strain CC-620 that could lead to loss of multiple compartment-specific ω-3 desaturase activities and (ii) a loss-of-function mutation in a single gene encoding a desaturase that has access to lipids in both the ER and chloroplast compartments. The latter hypothesis is consistent with a recent report (17) describing a Chlamydomonas strain with a transposon insertion in the promoter of the CrFAD7 gene, as discussed below.

The desaturase defect in CC-620 segregates in a manner consistent with loss of a single gene. Given the unexpected loss of all ω-3 fatty acids and the presumed loss of the associated desaturase activities in both the ER and chloroplast compartments, we tested whether the desaturase defect in CC-620 was due to one, two, or more genes. Specifically, two alternative explanations are possible for the observed loss of desaturase activities giving rise to multiple compartment-specific ω-3 FA species: (i) a single gene encodes the sole ω-3 desaturase, which is either localized to both the chloroplast and ER or is present in a compartment which gives it access to lipids in both compartments, or (ii) two genes encode different compartment-specific desaturase isoforms, both of which have been lost in strain CC-620. In the case of a single lesion leading to loss of all ω-3 desaturase activities, the individual progeny of a CC-620 × wild-type (WT) cross would either possess all ω-3 species or lack all four of the ω-3 species. In the second case (assuming that the two or more genes are not tightly linked), we would expect the individual loci to segregate independently and give rise to progeny that lack individual, compartment-specific ω-3 FA classes.

To differentiate these possibilities, we analyzed random haploid progeny from crosses between two strains (CC-124 and CC-621) whose mating type is opposite that of CC-620. In all F1 strains analyzed with the TMSH method described above (n = >200), the individual progeny contained either all four ω-3 species or no ω-3 species, with no ambiguity, and the parental phenotypes were segregated in an approximately 1:1 ratio, as expected for loss of function at a single genetic locus. The simplest explanation for these observations is that strain CC-620 bears a null mutation in a single gene that leads to the inactivation of the sole ω-3 desaturase activity of Chlamydomonas cells and that this desaturase is oriented such that it has access to glycerolipids in both chloroplast and extrachloroplastic membranes.

The FAD7 gene contains a putative inactivating missense mutation in strain CC-620. Given the previous annotation of predicted FA desaturase-encoding genes, we focused on the most likely candidate, encoded by gene Cre01-g038600 (genome version v5.3.1), which had been given the name CrFAD7 (18, 19) due to its apparent chloroplast-targeting sequence and high similarity to the Arabidopsis chloroplast ω-3 desaturase, AtFAD7 (20).

Function of CrFAD7 has also recently been reported in a paper from a study by Nguyen et al. (17) in which a mutant Chlamydomonas strain was characterized and shown to bear a TOC1 transposon insertion in the promoter of the gene, which led to an ~90% reduction in transcript abundance and a corresponding >65% reduction in ω-3 FA content. We examined an expressed-sequence-tag (EST) resource from a Roche 454-based transcrip-}

![FIG 5 CC-FAD7 creates a missense mutation affecting an absolutely conserved residue in strain CC-620. Data represent the results of a CLUSTAL Omega multiple-sequence alignment of the CrFAD7 protein sequence with comparison to previously characterized desaturases from Arabidopsis thaliana (AtFAD7), Synecocystis PCC-6803 (SynDesA and SynDesB), and Neurospora crassa (Nc12FAD and Nc1215FAD). The position of mutation T286N in the CrFAD7 sequence of strain CC-620 is highlighted. Asterisks represent absolutely conserved residues; single dots represent conserved positions with lower degrees of similarity; double dots represent conserved positions with higher degrees of similarity.](ec.asm.org)
present a FA profile essentially identical to the FA profiles determined for the laboratory strains (Fig. 6C), strains which contain the Δ6 unsaturated PUFA γ-linolenic acid and stearidonic acid (Fig. 6D) or only stearidonic acid (Fig. 6B), and several strains which accumulated an unidentified FA with a retention time intermediate between those of γ-linolenic and α-linolenic acids (Fig. 6E).

**DISCUSSION**

With the reemergence of algae as organisms of industrial and aquacultural interest regarding production of biofuels and other bioproducts, a need has arisen for methods that facilitate the high-throughput analysis of FA composition for the purposes of genetic screening and “bioprospecting” for strains with novel metabolic phenotypes. To this end, we have developed the method of FAME preparation and GC-FID analysis reported in detail in this work. During the course of method development, using a small collection of laboratory isolates of the model alga *Chlamydomonas*, the CC-620 strain, often used for production of gamete autolysin and studies on the mating reaction (23), was determined to lack ω-3 FA, and this defect was correlated with a polymorphism in the CrFAD7 gene. This gene had previously been identified bioinformatically (18) and has recently been proposed to serve as the sole ω-3 desaturase in the *Chlamydomonas* cell (17). The mutant strain deficient in CrFAD7 reported in that work was not a null allele but rather resulted from insertion of a TOC1 transposable element into the promoter, which resulted in an ~90% decrement in mRNA abundance and an ~65% reduction in ω-3 FA content. The CC-620 fad7 variant, however, appears to be a true null allele, as even when FAMEs derived from purified MGDG (containing >80 mol% of ω-3 FA in the wild type) were overloaded in a GC-FID analysis (Fig. 4A), no traces of ω-3 FA were detected. That is, within the limit of detection of our instrumentation, there are no ω-3 FA being synthesized in this strain. The identification of this null allele of CrFAD7 thus provides a more suitable genetic background for further studies on the functions of ω-3 FA in *Chlamydomonas*.

We further applied the TMSH method to the investigation of variations in fatty acid desaturase activities in strains of *Chlamydomonas* isolated from nature. We identified a number of alterations in fatty acid composition indicative of shifts in the substrate specificity and regioselectivity of the *Chlamydomonas* front-end desaturase, which acts on Δ9-unsaturated acyl chains esterified to the extrachloroplastic lipids DGTs and phosphatidylethanolamine (PtdEtn) (24). Specifically, the variability in FA composition can likely be attributed to changes in the substrate preference of the enzyme and the placement of the double bond as follows (outlined in schemes presented in Fig. 6F and G). In “wild-type” laboratory isolates of *Chlamydomonas*, linoleic and α-linolenic acids esterified to DGTs or PtdEtn are subject to desaturation by a front-end desaturase localized outside the chloroplast (24). In contrast, the *Δ5*-3 FA content.

We further applied the TMSH method to the investigation of variations in fatty acid desaturase activities in strains of *Chlamydomonas* isolated from nature. We identified a number of alterations in fatty acid composition indicative of shifts in the substrate specificity and regioselectivity of the *Chlamydomonas* front-end desaturase, which acts on Δ9-unsaturated acyl chains esterified to the extrachloroplastic lipids DGTs and phosphatidylethanolamine (PtdEtn) (24). Specifically, the variability in FA composition can likely be attributed to changes in the substrate preference of the enzyme and the placement of the double bond as follows (outlined in schemes presented in Fig. 6F and G). In “wild-type” laboratory isolates of *Chlamydomonas*, linoleic and α-linolenic acids esterified to DGTs or PtdEtn are subject to desaturation by a Δ5 front-end desaturase (24), but in strains accumulating the Δ6 PUFA γ-linolenic and stearidonic acids, we propose that the specificity of the front-end desaturase has shifted from Δ5 to Δ6. Furthermore, in strains accumulating stearidonic acid but no γ-linolenic acid, the Δ6 specific front-end desaturase is likely to prefer α-linolenic acid as a substrate and to be restricted from the use of linoleic acid. This hypothesis regarding shifts in substrate preference and double-bond placement is further supported by the observations that the Δ6 FA are esterified to DGTs and excluded from MGDG, indicating that the Δ6 desaturase is localized outside

**FIG 6** Environmental isolates of *Chlamydomonas*-like algae accumulate γ-linolenic acid (18:3Δ5,9,12) and stearidonic acid (18:4Δ6,9,12,15). (A) GC-FID trace of C18 region of chromatogram for wild-type strain CC-124. (B to E) Example traces of environmental isolates exhibiting variations in C18 PUFA composition. Abbreviations: LA, linoleic acid; PA, pinolenic acid; LNA, α-linolenic acid; CA, coniferonic acid; GLA, γ-linolenic acid; SDA, stearidonic acid; Unk, unknown. (F and G) Schemes for desaturation at the Δ5 or Δ6 position of LA or LNA to generate the complements of FA observed in chromatograms. Positions of cis-double bonds in these species are indicated in parentheses.
the chloroplast (data not shown) and that the Δ5 and Δ6 FA do not co-occur in any of the strains we analyzed, suggesting the functional equivalence of these desaturase activities in the different strains. The identification of these apparent variations in front-end desaturase activity thus provides a new model system to dissect the structural basis of front-end desaturase action in a series of closely related organisms, and studies on the molecular identification and characterization of genes encoding Δ6-specific front-end desaturases are under way.

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