Algal Lipid Bodies: Stress Induction, Purification, and Biochemical Characterization in Wild-Type and Starchless Chlamydomonas reinhardtii

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When the unicellular green soil alga Chlamydomonas reinhardtii is deprived of nitrogen after entering stationary phase in liquid culture, the cells produce abundant cytoplasmic lipid bodies (LBs), as well as abundant starch, via a pathway that accompanies a regulated autophagy program. After 48 h of N starvation in the presence of acetate, the wild-type LB content has increased 15-fold. When starch biosynthesis is blocked in the sta6 mutant, the LB content increases 30-fold, demonstrating that genetic manipulation can enhance LB production. The use of cell wall-less strains permitted development of a rapid “popped-cell” microscopic assay to quantitate the LB content per cell and permitted gentle cell breakage and LB isolation. The highly purified LBs contain 90% triacylglycerol (TAG) and 10% free fatty acids (FFA). The fatty acids associated with the TAGs are ~50% saturated C16 and C18 fatty acids and ~50% unsaturated fatty acids, half of which are in the form of oleic acid (C18:1). The FFA are ~50% C16 and ~50% C18. The LB-derived TAG yield from a liter of sta6 cells at 107 cells/ml after starvation for 48 h is calculated to approach 400 mg. The LB fraction also contains low levels of charged glycerolipids, with the same profile as whole-cell charged glycerolipids, that presumably form LB membranes; chloroplast-specific neutral glycerolipids (galactolipids) are absent. Very low levels of protein are also present, but all matrix-assisted laser desorption ionization-identified species are apparent contaminants. Nitrogen stress-induced LB production in C. reinhardtii has the hallmarks of a discrete pathway that should be amenable to additional genetic and culture condition manipulation.

There is keen interest in the development of technologies that harvest lipids from microalgae and convert them into diesel fuel (7, 11, 24, 43). A widely adopted approach is to identify algal species that are ascertained to be oleaginous (e.g., Neochloris, Nannochloropsis, and Dunaliella species) and focus on extracting and processing their oils (43). We have adopted a complementary approach, which is to start with the unicellular green soil alga Chlamydomonas reinhardtii, widely regarded as nonoleaginous (48), and utilize the highly developed molecular and genetic tools available for this organism (22) to investigate how its oil production might be enhanced. Such information could then, in the future, be applied to any algae that may prove promising for industrial application.

Algal oil production is commonly quantitated by extracting cells with organic solvents, characterizing the lipid fraction, and expressing the lipid yield as the percent dry weight of the starting material (24, 19, 43). Many cellular components (e.g., pigments, cell walls, and starch) contribute to dry weight, and these may change in quantity and/or composition during the cell cycle and/or during fluctuations in growth conditions for a given alga; they are also likely to differ among different algal lineages. Therefore, this convention provides an ambiguous metric for assessing how much oil a given cell produces when genetic background and/or culture conditions are manipulated and may yield misleading between-species comparisons.

A related difficulty with this convention is that the extract is expected to contain lipids from numerous sources, e.g., plasma membranes, endomembranes, chloroplasts, and lipid bodies (LBs) dedicated to the storage of triacylglycerides (TAG) and fatty acids (FA). Although all of these sources are of potential value for fuel production, it is problematic to evaluate the output of a particular pathway when the outputs from multiple pathways are being pooled.

We have therefore elected to focus on the biosynthesis of LBs (also referred to as lipid droplets or oil bodies), since this process has the hallmark of a discrete and inducible pathway amenable to genetic and culture condition manipulation (37), and we have developed a simple and direct microscopic assay for quantitating LB production per cell that is independent of dry weight.

The assay was used to study two parameters. (i) Increases in extractable TAG in numerous algal species, including C. reinhardtii (65), when cells are starved for nitrogen (N) have been reported previously (3, 4, 25, 26, 33, 43, 44, 49, 53, 57, 59). We demonstrate that, in C. reinhardtii, this increase correlates with a robust increase in LB production. (ii) N starvation has also been shown to stimulate a pronounced increase in the biosynthesis of starch granules in C. reinhardtii (31, 35, 45), prompting us to ask whether LB production might be enhanced if starch production was eliminated. We report that the cw15 sta6 starch null strain (66) deleted in ADP glucose phosphorylase (an essential enzyme in the starch biosynthetic pathway) produces twice as much LB-associated TAG as its parental cw15
strain under nitrogen stress, suggesting that carbon skeletons normally converted into starch are instead able to flow into the starvation-induced TAG biosynthetic pathway when the starch option is unavailable.

We have also developed a simple LB purification procedure that has permitted analysis of LB composition. The bodies contain ~90% TAG, ~10% free FA (FFA), and minor levels of charged glycerolipids (CGLs). Protein levels are also minor, and no proteins with the expected features of “dedicated” LB proteins, like the oleosins or caleosins of plant seeds, were identified.

MATERIALS AND METHODS

Strains and culture conditions. The cw15 (330) and cw15 stab (BAFJ5) strains were sent independently to the Goedewaagen and Waffenschmidt laboratories by the laboratories of Steven Ball (Université des Sciences et Technologie de Lille, Lille, France) and David Dauvillée (CNRS, Villeneuve d’Ascq, France) within a 6-month period. Neither recipient laboratory was mindful that strain 330 was an arg7 auxotroph, and hence, each plated the shipment onto arginine-free Tris-acetate-phosphate (18) medium, thereby selecting for an arg7 revertant or suppressed clone in each shipment that grows at wild-type rates without arginine supplementation; this cw15 clone was used in all reported experiments. BAFJ5 was derived from strain 330 by insertional mutagenesis using an arg7-containing plasmid and has been shown previously to carry a deletion in the stab gene (66); both recipient laboratories confirmed the absence of starch. Both strains lack flagella.

Liquid cultures were grown on glucose-buffered high-salt medium (HS) containing 9.3 mM NH4Cl as a nitrogen source and supplemented with 0.2% K acetate (HSM + Ac).

For microscopy, cells were grown either on plates (containing HSM + Ac solidified with 1.5% agar [Fluka]) or in 250-ml Erlenmeyer flasks after inoculation of 100 ml liquid HSM + Ac with a 1-ml volume containing 1 × 106 to 5 × 106 cells from plates. Cultures were agitated by magnetic spin bars at 2,000 × g on a rotary shaker at 250. When cultures reached stationary phase (at 1 × 106 to 2 × 106 cells/ml), 1-ml aliquots were placed into 1.5-ml microcentrifuge tubes, spun at 5,900 × g for 3.5 min, resuspended in 1 ml HSM + Ac lacking NH4Cl (HSM + Ac-N), and placed on a rotor at 2,000 × g for 24 or 48 h.

For LB purification, a stock culture was grown to stationary phase in 10 ml liquid Tris-acetate-phosphate medium and inoculated into 300 ml HSM + Ac. The culture was grown on a rotary shaker at 250 lx. When cultures reached stationary phase (at 1 × 108 cells/ml), cells were pelleted at 2,000 × g for 4 min, resuspended in 300 ml HSM + Ac-N, and returned to the rotary shaker for 18 h before harvesting.

Microscopy. (i) Confocal imaging of live cells. Cells at the stages indicated in the figure legends were stained with Nile Red (1-μg/ml final concentration; Fluka). Images were acquired using a Leica TCS-SP2 confocal microscope and a 63× oil immersion (1.40 numerical aperture) objective. Nile Red signal was captured using a laser excitation line at 488 nm, and emission wavelength, 572 nm; emission wavelength, 632 nm) to visualize chlorophyll fluorescence, and if the region derived from two overlapping cells, it was not photographed. The Analyze Particles tool of ImageJ software counts and then sums up all the pixels in a chosen field that are above a selected threshold of brightness, thereby computing the total area of the above-threshold entities. A popped-cell image (magnification, ×2,470) was first delimitated with the elliptical selection tool in the ImageJ program to exclude any extraneous LBs in the field. The threshold was then adjusted by highlighting LBs; if they varied in brightness, the least-bright LB in the selected field served as the threshold baseline. The output of each Analyze Particle calculation yielded the total LB area per cell; outputs were stored and then subjected to statistical analysis using OpenOffice Calc. Measurements of area, it should be noted, underestimate spherical volume and hence yield, but a comparison of areas allows accurate assessments of relative yields from different samples. Reproducibility is documented in Table S1A in the supplemental material, where data are given for three parallel N-starved samples derived from two stationary-phase cultures, one grown at high light and one at low light.

(ii) Popped-cell assay. A 100-μl sample of the cell culture was placed into a 500-μl microcentrifuge tube and mixed with 1 μl Nile Red stock solution (containing 1 mg/ml acetone) for 5 min. An 8-μl aliquot was placed onto a clean glass slide and covered with a coverslip, and the slide was allowed to dry for 30 min before being viewed with a CCMI Deltavision microscope using a green fluorescent protein filter (excitation wavelength, 470 nm; emission wavelength, 525 nm). Freshly “popped” cells are recognized having perfectly round LBs; with time, these can degenerate into pale, irregularly shaped entities (possibly because their postulated surrounding membrane is compromised, allowing the entrance of polar substances that inhibit Nile Red fluorescence), signaling the need either to move to a new region of the slide or to prepare a new slide. Once a suitable popped cell was identified and photographed, a photograph was taken of the cell closest to it and then the cell closest to that cell to avoid sampling bias. LB clusters were generally of similar sizes; for any oversized or ambiguous cluster, the field was illuminated using an M-Cherry filter (excitation wavelength, 572 nm; emission wavelength, 632 nm) to visualize chlorophyll fluorescence, and if the region derived from two overlapping cells, it was not photographed. The LB purification and size measurement. LBs were harvested from cells maintained under N-free conditions for 18 h by using slight modifications of a protocol developed to isolate intact C. reinhardtii chloroplasts (28). Cell samples (2 × 107 to 3 × 107 cells/ml in 300-ml aliquots) were spun at 2,000 × g for 4 min at 4°C and resuspended in 100 ml ice-cold digitonin solution (5 mM K phosphate buffer, pH 6.5, 6% [wt/vol] polyethylene glycol 6000, 0.004% [wt/vol] digitonin [Sigma]). Low levels of digitonin have been shown previously to bind to and destabilize plasma membrane sterols (primarily ergosterol in C. reinhardtii [2]) without causing lysis (40). Cells were then rapidly warmed to 30°C (the suspension was transferred into a metal beaker in a 48°C water bath and, when it reached 30°C, poured into a metal beaker maintained in a second 30°C water bath) and incubated for 7 min, after which they were transferred into an ice-cold flask and spun for 2 min at 4°C. The cell pellet was overlain with 10 ml cold osmotic lysis buffer (150 mM NaCl, 0.1 mM Na2EDTA, 0.01 mM MgCl2, 15% [wt/vol] sucrose), which causes the digitonin-stabilized plasma membranes to rupture (28). The samples were briefly subjected to a vortex to resuspend the pellets and immediately transferred into 10 ml tubes and centrifuged at 100,000 × g in an L7-55 ultracentrifuge (Beckman) for 45 min. LBs float to the surface and were collected with a 1-ml Eppendorf pipette with a cutoff tip to yield initial LB preparations, which were used to determine protein concentration as a quality control parameter to maximize yield. Initial and washed preparations were suspended in 10 ml osmotic lysis buffer and subjected to a final spin at 100,000 × g for 45 min. For size measurement, washed LBs in osmotic lysis buffer were stained with Nile Red, 1-μl samples were applied to SuperFrost Ultra Plus slides (VWR) that carry an adhesive coating and were sealed with nail enamel to prevent drying, images were enlarged 600-fold, and individual LBs were measured using Nikon EZ-C1 3.80 software. Analysis of TAG and FFA in purified LBs. Washed LB preparations were suspended in equal volumes of cyclohexane-acetic acid (98:2, vol/vol), subjected to a vortex for 1 min, and centrifuged at 10,000 × g for 1 min. The nonpolar upper phase containing TAG and FFA was subjected to solid-phase extraction (SPE) using a 1-g Supelco (Bellefonte, PA) for 4 min; this was followed by elution of lipid material with 1 ml hexane.

stationary-phase cultures, one grown at high light and one at low light.

GL analysis. Stationary-phase cw15 stab cells and initial LB preparations (from cw15 stab cells starved for N for 24 h) were extracted using chloroform-methanol (5:1). The extract was passed over a solid-phase silica acid cartridge
(Macherey and Nagel, Germany) and washed with chloroform (4 column volumes) to remove TAG and FFA. Neutral GLs (NGLs; galactolipids) were then eluted with 4 column volumes of acetone, after which CGLs were eluted with 4 column volumes of methanol (protocol courtesy of Jan Jaworski, Danforth Plant Science Center). Both fractions were evaporated to dryness, dissolved in a small volume of chloroform, and analyzed using high-performance thin-layer chromatography plates (Merck) according to the procedures described in reference 63. For evaluation of total GLs, plates were sprayed with concentrated sulfuric acid and incubated for 15 min at 230°C; tertiary amines were detected by spraying with freshly prepared Dragendorff reagent (http://www.cyberlipid.org/phlippt/pl2a0006.htm).

Protein, chlorophyll, and enzyme activity determinations. Protein contents of LB samples were determined by the Neuhoff assay (39). For chlorophyll, cells and LBs were extracted with chloroform and measurements of optical densities at 630 nm were made; the chlorophyll content of cells was set as 100%, and the LB chlorophyll content was calculated accordingly. Detergent-stimulated inosine diphosphatase was assayed (38) as a Golgi apparatus marker. (K-stimulated vanadate-sensitive ATPase was assayed (15) as a plasma membrane marker. Antimycin A-insensitive, NADH-dependent cytochrome c reductase activity was assayed (16) as an endoplasmic reticulum (ER) marker. To prepare endomembranes for these assays (see Table 1), cells ruptured by digitonin treatment or osmotic lysis were spun at 8,000 x g for 15 min to pellet nuclei, chloroplasts, and mitochondria. The supernatant was layered onto a linear sucrose gradient (15 to 60%) and centrifuged overnight at 35,000 rpm in an SW41 rotor (Beckman).

Protein composition of LBs. In our first experiments, washed LBs were dried in a SpeedVac and solubilized in 150 μL of boiling sodium dodecyl sulfate (SDS)-urea sample buffer (40 mM Tris HCl, pH 6.8, 1 mM EDTA, 20% [wt/vol] glycerol, 1 M urea, 0.4% [wt/vol] SDS, 5% 2-mercaptoethanol, 0.002% [wt/vol] bromophenol blue) for 5 to 10 min. Samples were run on a Tricine-SDS-polyacrylamide gel electrophoresis minigel system optimized for low-molecular-weight proteins (46). Two bands, migrating at molecular weights of 20,000 (20K) and 16K, were detected by Coomassie blue staining and identified by matrix-assisted laser desorption ionization as diacylglycerol N,N,N-trimethylhydroxymethylamine synthesis protein, eukaryotic translation elongation factor 1α, and an open reading frame product (CHLREDRAFT_192823) of unknown function lacking a predicted frame. The 20K protein was identified as the CHLREDRAFT_192823 protein using the Ball laboratory’s database.

RESULTS

Strains and culture conditions relevant to experimental results. The cw15 stab mutation was generated by insertional mutagenesis in a cw15 parental strain (66). The cw15 strain was originally isolated as a cell wall-less mutant (10); the strain utilized by the Ball laboratory to derive the cw15 stab strain has also lost its capacity to produce flagella. Both strains display normal growth rates in acetate-supplemented medium (mean doubling times, ~6 h at 2,000 lx and ~12 h at 250 lx) and reach stationary phase at ~2 x 10^7 cells per ml, comparable to wild-type strains (22).

Cells were grown under continuous light in HSM+Ac; ammonium was omitted from the N-free medium (HSM+Ac-N). Since cells in liquid N-free medium become increasingly prone to lysis with time and become moribund after 48 h, cells starved for N for 18 h were used for LB purification to avoid contamination and cells starved for N for 24 and 48 h were used to evaluate LB production capacity.

LBs visualized in intact C. reinhardtii cells under conditions of N starvation. Living cw15 and cw15 stab cells were stained with Nile Red, a reagent that yields brilliant yellow fluorescence in a neutral lipid environment (8, 28) and selectively stains LBs in C. reinhardtii. Figure 1 shows confocal images of parental cw15 cells and starch null cw15 stab cells after 24 h of N starvation; LBs are abundant in cw15 cells and very abundant in cw15 stab cells. Presumably, these LBs correspond to the membrane-delimited gametic vesicles and LBs seen previously in thin-sectioned N-starved cells (32, 35).

Confocal optical sectioning was used to evaluate the sizes and locations of the Nile Red-localized LBs in living cw15 and cw15 stab cells after 24 h of N-free maintenance. Figure 2A shows several representative sections (full scans are provided in Fig. SA1 in the supplemental material), and Fig. 2B shows two three-dimensional reconstructions. (i) The LBs are cytoplasmic, most being closely appressed to the large cup-shaped chloroplast. (ii) They vary in size (more size data are given below) and abundance. (iii) Each LB maintains an integral spherical shape, indicating that each is surrounded by a membrane and/or a coating material.

Individual LBs were measured in through-focal optical sections of six cw15 cells and six cw15 stab cells after 24 h of N starvation and in sections of five cw15 stab cells after 48 h of N starvation. Pooled results are shown in Fig. 3A. (i) All three samples, as well as individual cells, display a range of LB sizes (Fig. 1 and 2). (ii) In the 24-h samples, the mean total LB volume (11.6 μm^3) in cw15 and cw15 stab cells are the same but the cw15 stab cells have more abundant, smaller LBs and the cw15 cells have fewer, larger LBs. (iii) In the 24-h samples, the LB range in size from 0.3 to 2 μm, whereas they are larger (0.8 to 2.9 μm) in the 48-h cw15 stab cell sample, and the total volume in the 48-h sample (34.6 μm^3) has tripled compared to that in the 24-h samples.

Measurements of LB contents and sizes in through-focal optical sections are tedious to generate, limiting the number of cells that can be practically evaluated per sample. We therefore developed an alternative microscopic approach to evaluate lipid content that readily accommodates large sample sizes.

LBs visualized in C. reinhardtii cells popped in situ. While photographing cells for Fig. 1, we noticed that in regions of the slide that were drying out, the plasma membranes of the wall-less cw15 and cw15 stab cells had ruptured and the cells had popped, releasing their LBs in situ (Fig. 4). Confocal sectioning of the corresponding images revealed that the LBs retain their spherical shape under these conditions and that the range of sizes (0.3 to 2.9 μm) is strikingly similar to the range in intact
cells (compare Fig. 3A and B), indicating that the popping process does not compromise the integrity of LBs, although they can deteriorate with time (see Materials and Methods).

This serendipitous observation has been translated into the popped-cell assay, wherein such fields are photographed and the total area of fluorescent pixels released per cell is calculated using ImageJ software (see Materials and Methods for details). Figure 5 shows images of cells corresponding to the mean area for a given sample, flanked by images that correspond to areas one standard deviation (SD) above and one SD below that mean. Such area measurements are independent of individual LB sizes and are normalized with respect to differences in fluorescence intensity, thereby providing a direct measurement of total LB output per cell, and many cells in a given sample can be rapidly assessed.

Figure 6 presents the pooled results from large sample sets (>1,100 cells), with results from independent experiments given in Table SA1 in the supplemental material. At the time
of transfer from stationary-phase cultures into N-free medium, most cells have few if any LBs, yielding a median baseline value of 0.81 μm² per cell for both cw15 and cw15 sta6 cells. After 24 h of N starvation, the median value for cw15 cells increases 10-fold, to 8.9 μm², and after 48 h, it rises 15-fold (to 12.6 μm²) compared to the baseline value. In contrast, after 24 h, the value for cw15 sta6 cells has increased 18-fold (14.7 μm²), and it increases 30-fold (to 24.9 μm²) after 48 h. Thus, nitrogen starvation in liquid cultures produces a 15-fold increase in LB biogenesis in starch-producing cells, and the elimination of starch biosynthesis produces further doubling of the biogenesis level, with an increase of 30-fold.

Figure 6 demonstrates that the LB contents in cw15 sta6 cells are not normally distributed: a rightward skew is evident at 24 h and pronounced at 48 h, with some cells having at least twice the median LB content. A similar skew in the data on LB size in intact cells was noted earlier, with a smaller sample (Fig. 3A). These profiles suggest that if conditions can be found to prevent post-48-h morbidity, all the cw15 sta6 cells in a population may be capable of producing LBs at the high levels

FIG. 3. Size distributions of LBs from the following samples: through-focal optical sections of cw15 cells starved for N for 24 h and cw15 sta6 cells starved for N for 24 and 48 h (A), popped cells after 24 h of N starvation (B), and washed LBs after 18 h of N starvation (C). Frequency indicates number of LBs having a given size.
currently achieved by a subset, in which case the LB yield from cw15 sta6 cells would be markedly enhanced.

**Purification of LBs.** The observation that LBs are released when wall-less cells are subjected to drying prompted the development of a protocol, based on data from Klein et al. (28), to isolate and purify LBs. The plasma membranes of cw15 and cw15 sta6 cells starved for N for 18 h were selectively compromised by low concentrations of the sterol-binding compound digitonin (40), and the cells were then subjected to osmotic shock and high-speed centrifugation. Under these conditions, cellular organelles were pelleted while LBs float to the meniscus and are readily siphoned off for analysis. LBs from the first-obtained meniscus materials (initial LBs) were used for protein and GL analyses; LBs from pooled washes (washed LBs) were used for TAG/FFA analyses.

Figure 7A provides a survey view of a washed LB preparation; Fig. 7B shows that the vesicles visible by phase-contrast microscopy are uniformly Nile Red positive; and Table 1 documents negligible contamination of washed LBs by ER-specific, Golgi-specific, and plasma membrane-specific marker enzymes. The chlorophyll contents in initial LB preparations were 0.04 to 0.07% of whole-cell levels.

Figure 3C shows the size range of isolated LBs, allowing comparison with the ranges in intact cells (Fig. 3A) and in popped cells (Fig. 3B). The LBs clearly maintain their integrity during the isolation procedure. Note that in the results presented in Fig. 3B and C, younger cultures produced populations of LBs with diameters of >2 μm, in contrast with the restriction of LBs with these sizes to 48-h cultures in the results shown in Fig. 3A. It should be possible to obtain more meaningful assessments of LB size distributions by applying light-scattering technologies to isolated LB preparations, a project that was not undertaken in these studies.

**Lipid profiles of LBs.** Analyses of lipids in washed LB preparations (Fig. 8, 9, and 10) demonstrate the following. (i) The LBs are 90% TAG and 10% FFA, with minor levels of CGLs. (ii) The TAG-derived FA profile (Fig. 8) is diverse, with about 50% saturated and about 50% unsaturated species. There is no evidence of the long-chain (C20 and C22) polyunsaturated species that are abundant in *Nannochloropsis* (47) and *Arabidopsis* (50) or of other unusual lipids or β-carotenes observed in various other algal species (21, 62). (iii) The FFA (Fig. 9) are predominantly saturated species. (iv) The TAG-derived FA and FFA profiles are equivalent for cw15 and cw15 sta6 cells (Fig. 8 and 9), indicating that substrates for FA synthesis flow through the same pathway whether or not starch biosynthesis diverts a portion of the flow. (v) The thylakoid-membrane-localized NGLs monogalactosyldiacylglycerol and digalactosyldiacylglycerol are absent from initial LB preparations (Fig. 10), again attesting to the purity of the preparation (the bulk of the putative contaminating membranes in *C. reinhardtii* would be chloroplastic). (vi) The CGL profile of initial LB preparations is indistinguishable from the whole-cell CGL profile (63) (Fig. 10), consistent with the postulate that LB assembly is ER associated and not chloroplast associated (see reference 37).

**Protein composition of LBs.** As detailed in Materials and Methods, the protein content of twice-washed LB preparations is very low (35 μg protein in LBs harvested from 3 × 10⁶ cells), and repeated efforts to identify specific protein(s) associated with the preparations produced negative results.

**TAG yield from LBs.** Two methods—dry weight analysis and a glycerol assay—were used to calculate the per-cell yield of TAG purified from washed LB preparations obtained from cells starved for N for 18 h. The mean values from five independent experiments are shown in Fig. 11, where SDs are low. By averaging and rounding the means from the two assays, the cw15 strain was found to produce ~10 ng TAG/10⁴ cells while the cw15 sta6 strain was found to produce ~17 ng TAG/10⁴ cells after 18 h of N starvation, again demonstrating the differential outputs of the two strains. These numbers translate into 100 and 170 mg TGA per liter of culture at 10⁷ cells/ml. Since the cw15 sta6 strain is expected to at least double its yield between 18 and 48 h (Fig. 6), the data indicate that it should be possible to derive at least 340 mg of LB-derived TAG per liter of cw15 sta6 stationary-phase culture following 48 h of N starvation. Since the LBs also include 10% FFA, which are not measured in the TAG assays, and LB recovery is presumably incomplete, the predicted LB oil yield from the cw15 sta6 strain approaches 400 mg per liter at 10⁷ cells/ml.

**DISCUSSION**

In an influential 1998 report summarizing the U.S. Department of Energy's Aquatic Species Program (48), it is stated that "*Chlamydomonas* does not accumulate lipids, and thus was not considered for use in the [Aquatic Species Program]." We show here that *C. reinhardtii* indeed accumulates very few LBs during its growth phase (Fig. 6; see also Table SA1 in the supplemental material), but when starved for nitrogen in stationary phase in the presence of 0.2% (33 mM) exogenous acetate, wild-type cells undergo a 15-fold increase in LB production within 48 h. When starch synthesis is blocked by mutation, moreover, LB production increases 30-fold, to double the wild-type levels. Purified LB preparations are ~90% TAG and ~10% FFA, and their FA profiles show a promising mix of saturated and monounsaturated species.

We discuss these findings in terms of LB biogenesis, the N starvation "trigger" and its relationship to autophagy, the influence of starch, and the potential applications of these observations to biodiesel research.
LB characteristics. LBs have been the subject of several recent reviews (14, 37, 41, 56, 64) that document considerable diversity in their TAG, GL, and protein profiles depending on the organism, cell type, and physiological status.

By using various strains and growth conditions, the smallest LBs detectable in *C. reinhardtii* via Nile Red fluorescence were ~0.2 μm and the largest were ~3 μm, with all intervening diameters amply represented (Fig. 3); similar size ranges (0.5 to 2 μm [60] and 0.1 to 3 μm [23]) in angiosperm preparations have been reported previously. Two processes could generate this pattern (41): small LBs, presumably smaller than our 0.2-μm detection limit, may continuously accumulate TAG/FFA until they reach the 3-μm maximum; alternatively, or in addition, LBs may (occasionally) fuse with one another until they reach the maximum size. While LBs may well be capable of fusing in vivo under the aegis of cytoplasmic factors, they are remarkably stable in vitro, lying adjacent to one another on a glass surface without fusing together.

This stability is presumably conferred by a surrounding GL monolayer membrane (37, 55), evident in electron micrographs of *C. reinhardtii* LBs (31, 35). In *Chlamydomonas*, the composition and relative proportions of LB acidic GLs are...
indistinguishable from those of the whole-cell acidic GL fraction (Fig. 10) and no chloroplast-specific NGLs (galactolipids) are present, indicating a likely ER localization for LB assembly, as is the case for LB assembly in most other systems (31).

Unexpectedly, we found no evidence for a stable population of polypeptides associated with the LBs, despite numerous attempts using a variety of solubilization strategies (see Materials and Methods), including a protocol that successfully releases oleosins from purified Arabidopsis tapetosomes (23). In land plants, LB-associated transmembrane proteins (oleosins and caleosins) have been implicated in LB generation (13), size maintenance (50), and degradation of stored lipid (42); algal genomes, including C. reinhardtii genomes, lack genes encoding oleosin homologues but encode caleosin homologues, whose expression patterns have yet to be characterized. By definition, we cannot rule out the presence of LB proteins that have resisted the solubilization procedures employed, and they may be identified in future studies; if they are, they will presumably have quite different properties from the readily extractable oleosins and caleosins. Alternatively, it may be the case that in C. reinhardtii (and perhaps other algae), LB biogenesis under N starvation conditions, while doubtless mediated by protein-based pathways, does not entail insertion of stably associated proteins into the final LB product.

The nitrogen starvation trigger and the autophagy connection. Any algal biodiesel production system needs to optimize two input parameters: (i) the amount of lipid produced per cell and (ii) the number of cells per unit of culture per unit of time.

FIG. 6. Pooled distributions of LB contents in popped cw15 and cw15 sta6 cells after 0, 24, and 48 h of N starvation. Arrows indicate median values. Data for individual experiments are given in Table SA1 in the supplemental material. Frequency indicates number of cells having a given lipid area.
The work described in this report does not directly address the second parameter, but it has an impact on the second parameter in an important respect: we show that, at least with *C. reinhardtii*, one can grow the cells to maximum density (stationary phase) and then induce the cells to produce LBs via N starvation. The imperative to maximize cell density remains, but it is not necessary to introduce a growth-compromising procedure like N starvation until after maximum cell density is achieved.

A variety of stresses have been reported to enhance algal lipid production (20, 48, 58), and in future studies, the popped-cell assay will be used to assess their relative levels of stimulation. Our initial focus on the N starvation stimulus derives from familiarity with the system.

In a pioneering paper, Sager and Granick (45) reported that N starvation elicits two responses in *C. reinhardtii*: the cells differentiate into gametes (17), and they accumulate copious amounts of starch. Work in our laboratory confirmed these observations (31) and went on to demonstrate a third response to N starvation, namely, the execution of a highly sophisticated autophagy program. Within the first 12 h of N starvation, *C. reinhardtii* cells selectively destroy the majority of their cytoplasmic and chloroplastic ribosomes (32); they also degrade the bulk of their chloroplast membranes, and CO₂ fixation rates plummet (31). Additional studies document that N-starved cells selectively degrade the cytochrome b₆-cytochrome f complex of the photosynthetic apparatus (6) and upregulate expression of two proteasome subunits and a valosin-containing protein (1). Recently, microRNA profiles have been shown to be altered in *C. reinhardtii* cells starved for N for 18 to 21 h (34, 67), possibly contributing to the autophagy program. It will be of interest to ascertain whether stress-induced autophagy is operant in other algal lineages. A close inverse relationship between LB formation and autophagy in mammalian systems has recently been reported (51).

A fourth feature of the N starvation program may relate to the fact that *C. reinhardtii* is a soil organism. Whereas cells become moribund following 48 h in liquid N-free medium, if they are instead allowed to deplete the medium of nitrogen on 1.5% agar plates, they undergo autophagy and then shift into a G₀ resting stage that allows them to survive for months and often years on the agar surface (22, 31). In preliminary experiments, we have examined such N-depleted cells maintained on agar for 2 weeks and found them to be as fully engorged with LBs as cells starved for 48 h in liquid medium. Possibly such a protocol will permit all the *cw15 sta6* cells in a population to achieve the maximal LB content that only a subset achieve in liquid (Fig. 6).

These observations indicate the following. (i) LB accumulation fails to occur without N starvation, indicating the existence of a nitrogen trigger or, perhaps more generally, a stress trigger. (ii) N starvation is accompanied by autophagy, meaning that the nitrogen trigger may act directly to stimulate downstream events and/or it may act indirectly by stimulating the formation of stimulatory products generated via autophagy. Future studies will investigate the role played by exogenous

![FIG. 7. Washed LB preparation. (A) Survey view. (B) Phase contrast versus Nile Red fluorescence.](image)

| Table 1. Levels of ER-specific, Golgi apparatus-specific, and plasma membrane-specific marker enzymes in washed LB preparations |
|-----------------|-----------------|-----------------|
| Fraction        | CCR (μkat/mg protein) | IDPase (nkat/mg protein) | ATPase (nkat/mg protein) |
| Endomembrane    | 10.97            | 6.9              | 10.4             |
| LBs             | 0.043            | Below detection limit | Below detection limit |

*CCR, cytochrome c reductase; IDPase, inosine diphosphatase.*
acetate and the effects of increasing CO₂ levels and varying light intensity.

The starch connection. The accumulation of starch granules in *C. reinhardtii* is evident within 2 h after N withdrawal and reaches its maximum extent by 5 h (31), whereas LBs (presumably the gametic vesicles [31]) accumulate more slowly. In a paper describing the properties of the *sta7* strain, a second starch null mutant of *C. reinhardtii*, Mouille et al. (35) include electron micrographs of a wild-type cell and a *sta7* cell deprived of N in the presence of acetate for an unstated period; the wild type is engorged with starch and has a single cytoplasmic vesicle labeled LB, and the mutant cytoplasm is filled with vesicles labeled LBs. Lipid analyses were not performed to confirm these labeling assignments (Steven Ball, personal communication); the present report provides such confirmation.

The key step in FA biosynthesis is the irreversible conversion of acetyl coenzyme A (acetyl-CoA) to malonyl-CoA, catalyzed by the complex enzyme acetyl-CoA carboxylase. Acetyl-CoA is generated via a number of biochemical pathways, one of which utilizes the primary product of CO₂ fixation, 3-phosphoglycerate (3-PG); 3-PG is also the precursor for the glyco-
ern backbone of TAG. Since 3-PG feeds directly into the starch biosynthesis pathway, blocking this pathway would, in theory, free up more carbon skeletons to flow into both acetyl-CoA production and TAG biosynthesis. We demonstrate that this is the case: the cw15 sta6 mutant strain, with a deletion of the gene encoding the ADP glucose pyrophosphorylase enzyme of the starch biosynthesis pathway, makes twice as much LB-associated TAG in 48 h as the starch-producing parental cw15 strain (Fig. 6). Possibly this factor could be increased by selecting for oleaginous clones in the population and/or by identifying growth conditions, such as agar plates, under which the cells remain viable for longer than 48 h.

Of interest is the finding that in the starch-producing cw15 strain, LB levels at 48 h after N withdrawal are roughly the same as they are at 24 h (Fig. 6), suggesting that LB production reaches an early plateau when starch is also being produced.

These findings may be of interest to those exploring the use of other algal species as biodiesel producers: if the candidate algae are known to produce starch, then it is straightforward to screen for starch null mutants by using KI staining. Interestingly, *Nannochloropsis oculata*, a small yellow-green heterokont alga that is being developed as a biodiesel producer, synthesizes no starch (36), as is generally true for algae in the heterokont group (9).

**Applications.** Although the cw15 and cw15 sta6 strains described in this report lack both cell walls and flagella and the cw15 sta6 strain fails to produce starch, both strains grow as well as the wild type in both liquid and agar-based media and both have important features as model organisms for biodiesel research: they are suited to the popped-cell assay and to LB purification, and they are readily transformed by exogenous genes to test the effects of such manipulations on LB production and FA composition. We plan, for example, to test the effects of acetyl-CoA carboxylase overexpression on the LB-derived TAG yield and to test whether overexpression of stearoyl-acyl carrier protein-Δ9 desaturase augments the proportion of oleic acid derivatives in the LBs. The relevant constructs can, if needed, be put behind promoters that are expressed only under conditions of N starvation (30), thereby bypassing any deleterious effects of their expression on mitotic growth.

If N starvation proves to be key in stimulating LB formation in *C. reinhardtii*, this finding will introduce the awkward and energy-consuming process of centrifugation and resuspension in N-free medium. In theory, input levels of NH₄ in liquid medium could be adjusted so that NH₄ is depleted at the time cells reach stationary phase. Also promising is the finding, noted earlier, that cells on agar plates deplete medium of nitrogen (31) and produce copious LBs without a centrifugation step. Hence, there may be ways to work around this constraint.

The cw15 sta6 cells starved for 48 h appear to be so stuffed with LBs (Fig. 1 and 2) that any increases in yield per cell obtained by strain selection or engineering will presumably at some point reach a cell size-based upper limit. This anticipated constraint suggests yet another line of experimentation, namely, to manipulate cell size. *C. reinhardtii* cells can range from 2 μm in diameter for the mat3 mutant (61) to 12 μm in diameter for the haploid wild type to twice this size for diploid strains (12, 29). Possibly, cells selected for larger size and/or additional ploidy will have improved oil yields.

As noted above, the TAG yield per cell is one of two parameters to manipulate in algal biodiesel development; the second is the total cell number per unit of culture per unit of time. The time factor is determined by the mitotic doubling rate; the number of cells per unit of culture is determined by the density at which the cells shift into stationary phase. Nothing is known as to why algae go into stationary phase at species-specific cell densities; this pattern may be due to the depletion of nutrients, the buildup of toxic components in the medium, quorum effects (52), and/or other genetic constraints. Research on this parameter is very much needed.

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