Animals and fungi produce cholesterol and ergosterol, respectively, while plants produce the phytosterols stigmasterol, campesterol, and β-sitosterol in various combinations. The recent sequencing of many algal genomes allows the detailed reconstruction of the sterol metabolic pathways. Here, we characterized sterol synthesis in two sequenced Chlorella spp., the free-living C. sorokiniana, and symbiotic C. variabilis NC64A. Chlamydomonas reinhardtii was included as an internal control and Coccomyxa subellipsoidea as a plant-like outlier. We found that ergosterol was the major sterol produced by Chlorella spp. and C. reinhardtii, while C. subellipsoidea produced the three phytosterols found in plants. In silico analysis of the C. variabilis NC64A, C. sorokiniana, and C. subellipsoidea genomes identified 22 homologs of sterol biosynthetic genes from Arabidopsis thaliana, Saccharomyces cerevisiae, and C. reinhardtii. The presence of CAS1, CPI1, and HYD1 in the four algal genomes suggests the higher plant cytochrome branch for sterol biosynthesis, confirming that algae and fungi use different pathways for ergosterol synthesis. Phylogenetic analysis for 40 oxidosqualene cyclases (OSCs) showed that the nine algal OSCs clustered with the cycloartenol cyclases, rather than the lanosterol cyclases, with the OSC for C. subellipsoidea positioned in between the higher plants and the eight other algae. With regard to why C. subellipsoidea produced phytosterols instead of ergosterol, we identified 22 differentially conserved positions where C. subellipsoidea CAS and A. thaliana CAS1 have one amino acid while the three ergosterol producing algae have another. Together, these results emphasize the position of the unicellular algae as an evolutionary transition point for sterols.

Key index words: algal sterol composition; Chlorella sorokiniana; Chlorella variabilis NC64A; Clotrimazole; Ketoconazole; oxidosqualene cyclase; terbinafine

Abbreviations: BBM, Bold’s basal medium; CAS, cytochrome branch synthase; ER, endoplasmic reticulum; % GC, percent guanine cytosine; GC/MS, gas chromatography mass spectroscopy; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IPP, isopentenyl pyrophosphate; LAS, lanosterol synthase; MBBM, modified Bold’s basal medium; MEP,
Sterols are a type of lipid found within the membrane of animals, fungi, and plants and contribute to membrane stability and other important cellular functions (He et al. 2003). Sterols are found in many forms: free sterols, sterol esters, sterol alkyl ethers, sterol sulfates, or linked to a glycoside moiety (Benveniste 2004). As integral components of the cell membrane, free sterols are crucial for the integrity, fluidity, and permeability of the lipid bilayer (Benveniste 2004). In addition to their importance for cell membrane stability, they affect membrane-bound protein composition and influence the functionality of enzymes, receptors, and channels (Porsbring et al. 2009) and play an important role in host defense during viral infection (Blanc et al. 2011).

Interestingly, despite their consistent presence, sterols are found in distinctly different compositions between the kingdoms. Whereas cholesterol is the primary zoosterol, ergosterol is the principal mycossterol, and separately, a handful of phytosterols such as stigmasterol, campesterol, and sitosterol, and separately, a handful of phytosterols such as stigmasterol, campesterol, and sitosterol dominate the plant membrane. In all cases, sterol biosynthesis occurs in the endoplasmic reticulum (ER) through a functional complex of enzymes that display specific protein-protein interactions. In fungi, this complex of enzymes has been termed the “ergosome” (Mo and Bard 2005). In comparison, the sterol biosynthetic pathway present in plants differs from fungi and animals because plants produce a wide variety of phytosterols and intermediates in the phytosterol pathway. While the sterol composition of most algae is well established, the recent availability of full genomes allows a detailed reconstruction of the sterol metabolic pathways.

Our aim was to study sterol biosynthesis in four unicellular, freshwater green algae. Our approach took advantage of well-characterized inhibitors which block sterol production in fungi, leading either to an accumulation of sterol precursors or to a different suite of sterols. Here, we report the sterol composition of four green algae: *Chlorella variabilis* NC64A, *Chlorella sorokiniana*, *Chlamydomonas reinhardtii*, and *Coccomyxa subellipsoidea*. A major difference among these algae was that ergosterol was the major sterol in both the *Chlorella* spp. and *C. reinhardtii*, whereas *C. subellipsoidea* did not have ergosterol but contained three phytosterols instead. Using the sterol biosynthetic pathways identified in *Arabidopsis thaliana* (Benveniste 2004), *Saccharomyces cerevisiae* (Parks and Casey 1995, Mo and Bard 2005), and *C. reinhardtii* (Brumfield et al. 2017) as plant, fungal, and algal models, respectively, we describe an ergosterol biosynthetic pathway present in both *Chlorella* spp. This pathway fits with the modified sterols and sterol precursors observed following treatment with the antibiotics ketoconazole and clotrimazole. Finally, we tackled the plant-like sterol composition of *C. subellipsoidea* by multiple sequence alignment of relevant oxidosqualene cyclase proteins. We identified 22 residues where the 3 ergosterol-containing algae used one amino acid while *C. subellipsoidea* and the *A. thaliana* CAS used another.

MATERIALS AND METHODS

**Cell cultures and growth conditions.** All species were grown under normal growth conditions as follows. *Chlorella variabilis* NC64A and *Coccomyxa subellipsoidea* cultures were grown in modified Bold’s basal medium (MBBM; Van Etten et al. 1983) shaken at 100 RPM, 22°C, and a light intensity of 30 μE. *Chlorella sorokiniana* UTEX 1290 was obtained from the University of Texas Culture Collection and grown in liquid Bold’s Basal Medium (BBM; Nichols and Bold 1965), shaken at 115 RPM, 25°C, and a light intensity of 58 μE. *Chlamydomonas reinhardtii* CC124, obtained from Dr. Donald Weeks, was grown in Tris-Acetate-Phosphate medium (Gorman and Levine 1965), shaken at 100 RPM, 22°C, and a light intensity of 30 μE. Cell abundance was determined using a Coulter Multisizer II instrument (Beckman Coulter, Fullerton, CA, USA).

**Sterol standards and inhibitors.** A plant sterols kit (cat. #1123, Matreya, Pleasant Gap, PA, USA) was used for standards. Cholesterol (1 mg · mL⁻¹) was used as a sterol internal standard for GC/MS analysis. Obtusifoliol was a gift from Prof. David Nes, Texas Tech University. Antifungal inhibitors Atorvastatin (A7658), Terbinafine (T1672), Clotrimazole (C4657), Ketoconazole (K1676), and Fluconazole (F4682) were purchased from LKT Laboratories (St. Paul, MN, USA). Thiolutin was obtained from Tocris Bioscience (Bristol, UK).

**Sterol extraction and analysis by gas chromatography-mass spectrometry (GC/MS).** Sterols were extracted in triplicate by chloroform-methanol, purified by silica-solid phase extraction, and analyzed by GC/MS. One mg/ml of cholesterol standard (Matreya, Pleasant Gap, PA, USA) was added to freeze-dried algal pellets followed by extraction three times with chloroform: MeOH (1:1, v/v). Samples were dried in a stream of nitrogen, dissolved in chloroform, and loaded onto silica SPE columns. A purified sterol fraction was eluted from the column with 30% 2-propanol in hexane. Purified sterol extracts were dried under nitrogen and then converted to trimethylsilyl ether (TMS-ether) derivatives using bis (trimethylsilyl) trifluoracetamide (BSTFA-TMCS 99:1; Sigma, St. Louis, MO, USA). Dried sterol samples were suspended in 100 μl hexane for GC/MS analysis. Initial gas chromatographic analysis was carried out using the Agilent 6890 Series Gas Chromatograph System equipped with a DB-5ms capillary column (30.0 m x 250.00 μm, 0.25 μm, J&W 122-5532, J&W Scientific, Inc., Folsom, CA, USA). Helium was the carrier gas at a linear velocity of 48 cm/sec and constant flow of 1.5 mL · min⁻¹. A dual ramp temperature program was used with the oven heated from 250 to 270°C for 30 min and then from 270 to 280°C for 3.5 min. The detector temperature was 270°C. Sterols were initially identified using the NIST98 library (Scientific Instrument Services, Inc., Ringoes, NJ, USA) followed by comparisons to published spectra based on their mass fragmentation patterns and retention times (see Table 1). Peak areas of identified sterols were quantified relative to the cholestanol standard. Values reported are the average of triplicate experiments.
**Growth curves and MIC values.** The inhibitory concentrations of terbinafine and theazole drugs were determined using methanol (MeOH) solutions of the drug ranging from 0–50 mM. A culture of *Chlorella sorokiniana* was diluted with fresh BBM to a final cell density of 1x10^6 cells·mL^-1. The culture was partitioned into 5 mL volumes and treated with 5 μL of a given stock solution, which resulted in a 1:1000 dilution of the stock drug solution. Other samples were treated with 5 μL of MeOH as solvent controls. Trials were run in duplicate. After six days, the absorbance at 750 nm was measured via a Biotek Synergy H1 Hybrid reader (Winooski, VT, USA). Inhibitory concentrations were determined by graphing absorbance vs. drug concentration and identifying the lowest concentrations at which growth was inhibited. Growth curves were similar in design except that absorbance was read every day. To determine if a given drug was algicidal (killing the algal cultures) or algastatic (merely arresting cell growth), cultures that had been inhibited for 2-3 d were centrifuged at 4200 RPM for three min and then transferred into 5 mL of fresh BBM media to allow growth.

**Sterol biosynthesis inhibition.** Algal cultures were grown to mid-log phase (ca. 1x10^6 cells·mL^-1). The sterol inhibitors were added to each culture at final concentrations of 100 μM atorvastatin, 4 μM terbinafine, 30 μM ketoconazole, and 5 μM clotrimazole. These concentrations were chosen based on the MIC values and growth inhibition we observed for *C. sorokiniana*. For terbinafine, ketoconazole, and clotrimazole, which had been inhibited for 2-3 d were centrifuged at 4200 RPM for three min and then transferred into 5 mL of fresh BBM media to allow growth.

**Phylogenetic analysis.** The 2,3-oxidosqualene cyclase (OSC) sequences used for the phylogenetic analyses were taken from the results of a BLASTp search (version 2.2.30) against the nonredundant protein database using default settings (Altschul et al. 1990). Sequences from lanosterol-producing vertebrates and fungi as well as cycloartenol-producing plants, diatoms, and algae were chosen to represent a broad representation of each lineage. The squalene-hopane cyclase from *Acetobacter tropicalis* was included as an out-group to derive the root of the tree, but not shown in the final phylogeny to maintain readability due to its divergence from the eukaryotic OSC genes. The protein sequences were globally aligned using version 7.402 of MAFFT (Katoh et al. 2019). The maximum likelihood phylogenetic tree was produced with version 20120412 of PhyML with 1000 bootstrap pseudoreplicates (Guindon et al. 2010).

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**Table 1. Retention times of sterol standards.**

| Sterol standard | Rf | m/z (TMS-ether) | PubChem CID | NIST |
|-----------------|----|----------------|-------------|------|
| Ergostatetraenol| 1.14 | 466 | 23724485 | 17866 |
| Ergosterol      | 1.15 | 468 | 444679 | 25726 |
| Campesterol     | 1.17 | 472 | 173183 | 351835 |
| 14β-Methylergosta-8,24(28)-dienol | 1.18 | 484 | 129703316 | |
| 14α-Methylergosta-8-enol | 1.21 | 486 | 129703316 | |
| Stigmastanol    | 1.22 | 484 | 5280794 | 331824 |
| Ergosta-5,7,24(28)-trienol | 1.23 | 468 | 42608412 | 23726 |
| Ergosta-5,7-dienol | 1.27 | 470 | 5326970 | |
| Ergost-7-enol   | 1.32 | 472 | 5283646 | 17938 |
| 7-Dehydroproriferasterol | 1.35 | 482 | 20843908 | 17146 |
| 4x,14x-Dimethylergosta-8,22-dienol | 1.35 | 498 | 10161432 | |
| Oubusilfoliol   | 1.37 | 498 | 65292 | 16573 |
| β-sitosterol    | 1.37 | 486 | 222284 | 331677 |
| 24(28)-Dihydrobousilfoliol | 1.39 | 500 | 23258269 | |
| Cycloartenol    | 1.40 | 500 | 313075 | |
| 24(28)-Dihydroxycoleucaenol | 1.56 | 500 | 6427296 | |
| 24-Methylenecoleartenol | 1.57 | 512 | 94204 | |
| 24-Methylcyloartenol | 1.62 | 514 | 13784482 | |
| 9,19-Cyclostanol-25-en-3β-ol, 24-methyl | 1.66 | 512 | |
| Cycloaludanol    | 1.75 | 512 | 101729 | |

*a Relative to cholestenol standard.
*b Doyle et al. (1971).
*c Retention time from Zhou et al. (2006).
*d Isomer.
*e Spectrum of free alcohol.
*f Bowden and Palmer (1975).
*g Refers to acetate derivative.
OSC structure prediction. The tertiary structure of the Coccomyxa subelliptoidea CAS protein was predicted using the template-based structure prediction from the RaptorX webserver (Källberg et al. 2012). The predictions were based primarily on the human OSC in complex with lanosterol (PDB 1W6K). The key structures were confirmed using the template-free contact prediction from the RaptorX webserver (Wang et al. 2017). The predicted structure was visualized and annotated using version 2.3.2 of PyMOL (Schrödinger 2019).

RESULTS

We took a 4-prong approach to study sterol biosynthesis in four unicellular algae. We determined [A] which sterols were synthesized under standard growth conditions, [B] which of six known antifungal antibiotics were also inhibitory toward the microalgal, [C] how these inhibitors influenced the sterol compositions of the respective algae, and [D] which homologs of known sterol biosynthetic genes from Arabidopsis thaliana, Saccharomyces cerevisiae, and Chlamydomonas reinhardtii were present in C. subelliptoidea, C. variabilis and C. sorokiniana.

Sterol composition of four microalgae. The major sterols were identified by GC/MS for four unicellular, freshwater green algae Chlorella variabilis, C. sorokiniana, Chlamydomonas reinhardtii, and Coccomyxa subelliptoidea. Four different patterns were observed (Table 2). Chlamydomonas reinhardtii and the two Chorella sp. contained ergosterol as the dominant sterol. Chlamydomonas reinhardtii contained ergosterol (65%), ergost-7-enol (10%), and 7-dehydroepisterol (27%; Table 2). Note that ergosterol (C24β-methyl) and 7-dehydroepisterol (C24β-ethyl) differ by only a single carbon. Our results for C. reinhardtii serve as an internal control in that they confirm prior reports of ergosterol and 7-dehydroepisterol as the major sterols (Miller et al. 2012, Brumfield et al. 2017). The distinction between C. variabilis having both ergosterol and ergostatetraenol and C. sorokiniana having ergosterol only (Table 2) reflects in part the absence of the final enzyme C24 (28) sterol reductase in C. variabilis (Table 3) with the concomitant necessity of recruiting a less efficient reductase to make ergosterol. Ergostatetraenol is the precursor for ergosterol (Fabris et al. 2014, Birkow et al. 2015, Brumfield et al. 2017; Fig 1) and the two molecules have very similar retention times by GC/MS (Table 1). Thus, a small difference in the % completion of the last step could determine whether ergostatetraenol appeared as a separate peak (C. variabilis) or as a shoulder on the ergosterol peak (C. sorokiniana). In contrast, C. subelliptoidea resembled the vascular plants because it only contained the phytosterols campesterol (48%), ß-sitosterol (43%), and stigmasterol (9%; Table 2).

Sensitivity to known antifungal compounds. Chlorella sorokiniana cultures (1x10^6 cells·mL^-1) were tested for their sensitivity to six antifungal antibiotics, five of which (terbinafine, clotrimazole, ketoconazole, fluconazole, and atorvastatin) block sterol biosynthesis in fungi (Macreadie et al. 2006), whereas thiolutin inhibits RNA polymerases. Two of the antibiotics, fluconazole and atorvastatin, were not inhibitory at any concentrations tested up to 50 and 100 µM, respectively. This finding is not surprising for atorvastatin, a well-known inhibitor of HMG CoA reductase used to lower cholesterol levels in humans, because neither Chlorella sp. has the gene for HMG CoA reductase (Table 3). For another inhibitor, terbinafine, the cultures did not have differing sterol profiles, but, compared with the controls, their total ergosterol levels decreased by ca. 40%, from 4.3 nmol·mg^-1 to 2.5 nmol·mg^-1 in C. variabilis and 3.2 nmol·mg^-1 to 2.0 nmol·mg^-1 dry weight for C. sorokiniana (Table 4). These results are close to those of Lu et al. (2014) who reported a 20% decrease in total sterol content for terbinafine-treated Nannochloropsis oceanica. Together these observations are consistent with a mode of action for terbinafine in algae identical with that established in fungi, that is, inhibition of squalene epoxidase. This mode of action would reduce the entry of carbon into stage III of the sterol pathway but not alter the remaining sterol profiles.

Terbinafine, clotrimazole, thiolutin, and ketoconazole, blocked growth of Chlorella sorokiniana at minimal inhibitory concentrations of 2, 2, 10, and 20 µM respectively. Each of these four active drugs was algistatic rather than algicidal, in agreement with the fungistatic nature of their activity toward fungi (Odds et al. 1985). Thus, if their modes of action in algae parallel those in fungi, terbinafine, clotrimazole, and ketoconazole should be effective inhibitors of algal sterol synthesis while thiolutin, as a transcription inhibitor, would allow the determination of algal mRNA half-lives (Kebaara et al. 2006). The two inhibitory azoles, clotrimazole and ketoconazole, are both imidazoles whereas fluconazole is a triazole, and this structural difference may contribute to their sterol inhibition, or lack thereof.

Sterol biosynthesis inhibition. Theazole antifungals inhibit key enzymes in sterol biosynthesis, leading to the accumulation of diagnostic intermediates that allow us to study the intermediates, and in doing so better understand their sterol biosynthetic pathways. Thus, Chlorella sorokiniana, C. variabilis, Chlamydomonas reinhardtii, and Coccomyxa subelliptoidea were treated with clotrimazole (5 µM) and ketoconazole (30 µM). These concentrations were chosen to be slightly higher than those needed for 50% inhibition of cell growth for C. sorokiniana in liquid culture. Methanol-only controls were used in each case. Although the clotrimazole and ketoconazole concentrations were chosen based on their activity toward C. sorokiniana, their general suitability for the other green algae is shown by their dramatically altered sterol profiles in all cases (Tables 4 and 5, Table S1 in the Supporting Information).
Table 2. Percentage of major sterols in four microalgae.

| Name                        | Chlorella variabilis | Chlorella sorokiniana | Coccomyxa subellipsoidea | Chlamydomonas reinhardtii |
|-----------------------------|----------------------|-----------------------|--------------------------|---------------------------|
| Ergostatetraenol            | 42%                  | 99%                   | 48%                      | 63%                       |
| Ergosterol                  | 53%                  |                       | 99%                      | 63%                       |
| Campesterol                 |                      |                       | 48%                      | 9%                        |
| Stigmasterol                |                      |                       | 2%                       | 9%                        |
| Ergosta-5,7,24(28)-triienol | 2%                   |                       | 1%                       | 10%                       |
| Ergosta-5,7-dienol          | 3%                   |                       | 1%                       | 27%                       |
| Ergost-7-enol               |                      |                       | 1%                       | 10%                       |
| 7-dehydroporiferasterol     |                      |                       | 43%                      |                           |
| beta-sitosterol             |                      |                       |                          |                           |
| Total Sterols (nmol · mg dw⁻¹) | 2.5                 | 3.2                   | 3.6                      | 3.4                       |

In *Chlorella sorokiniana*, the decreased ergosterol in the ketoconazole treated cells was accompanied by the appearance of 10% obtusifoliol, 18% 14α-methylergosta-8,24(28)-dienol (also known as 4-desmethyl obtusifoliol), and 24% 14α-methylergosta-8-enol (Table 4). This observation is consistent with ketoconazole inhibiting the P450 CYP51 C-14 demethylase whose normal substrate is obtusifoliol (step 4 in Fig. 1). CYP51 is an ortholog of ERG 11, the primary target of theazole drugs in fungi. Thus, inhibition of CYP51 leads to the accumulation of obtusifoliol and subsequent conversion to 14α-methylergosta-8-enol by the combined action of Erg 4/24 and the SM02/BSD1/Erg28 complex, acting in either order. Next, the sterols detected in clotrimazole treated cells (Table 4) suggest that clotrimazole inhibits CYP710, the P450 C-22 sterol desaturase, as well as CYP51 because clotrimazole treated cells also accumulated ergost-7-enol and ergost-5,7-dienol (Table 4). Inhibition of CYP710 (step 8 in Fig. 1) would lead to the accumulation of episterol and ergosta-5,7,24(28) trienol which can both act as substrates for Erg 4/24, being converted to ergost-7-enol and ergosta-5, 7-dienol, respectively (Fig. 1).

The sterol profiles for *Chlorella variabilis* following azole treatment are very similar to those for *C. sorokiniana* (Table 4) but in this case ketoconazole also appears to inhibit CYP710 as well as CYP51. Previously, Doyle et al. (1971) noted that triparanol-treated *Chlorella emersonii* accumulated both 14α-methylergosta-8-enol and 14α-methylergosta-8, 24 (28)-dienol while Chan et al. (1974) observed that triparanol-treated *C. sorokiniana* accumulated large amounts of ergost-7-enol and ergost-8-enol in addition to their normal ergosterol and ergosta-5, 7-dienol.

The sterol inhibition patterns for the other two algae were more complicated. For *Chlamydomonas reinhardtii*, ketoconazole and clotrimazole dramatically reduced the levels of both ergosterol and ergost-7-enol with the concomitant appearance of several intermediates from the cycloartenol side of the oxidosqualene cyclase pathway (Table S1). However, the levels of 7-dehydroporiferasterol remained relatively unchanged. At present we have no explanation for the different responses of the two major sterols in *C. reinhardtii*. Finally, in the presence of the azoles, *Coccomyxa subellipsoidea*, which normally synthesizes the phytosterols campesterol, stigmasterol, and β-sitosterol (Table 5), produced seven new intermediates from the cycloartenol pathway including obtusifoliol and cyclolaudenol. We will revisit why *C. subellipsoidea* synthesizes a different suite of sterols a little later when we analyze the amino acid sequence of the *Coccomyxa* CAS1.

Sequence retrieval and curation. Protein sequences from known *Arabidopsis thaliana* sterol biosynthetic genes were used in BLASTp searches against the *Chlorella sorokiniana* genome to identify their orthologs. The *C. sorokiniana* protein sequences identified were then used in BLASTp searches against the remaining algal sequences to identify their orthologs in each species. All of the identified orthologs were used in reciprocal BLASTp searches of the NCBI database to confirm the identities of these genes. Genes for sterol biosynthesis in the three model systems, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, and *Chlamydomonas reinhardtii*, are shown in Tables S2 and S3 in the Supporting Information while the biosynthetic genes for the four unicellular algae are in Table 3. Complete BLASTp statistics along with values for gene length in amino acids, % GC content, % identity, and numbers of exons and transmembrane domains are shown in Tables S4-S7 in the Supporting Information. All of the identified orthologs were used in reciprocal BLASTp searches of the NCBI database to identify their orthologs in each species. All of the identified orthologs were used in reciprocal BLASTp searches of the NCBI database to confirm the identities of these genes. Genes for sterol biosynthesis in the three model systems, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, and *Chlamydomonas reinhardtii*, are shown in Tables S2 and S3 in the Supporting Information while the biosynthetic genes for the four unicellular algae are in Table 3. Complete BLASTp statistics along with values for gene length in amino acids, % GC content, % identity, and numbers of exons and transmembrane domains are shown in Tables S4-S7 in the Supporting Information. All of the identified orthologs were used in reciprocal BLASTp searches of the NCBI database to confirm the identities of these genes. Genes for sterol biosynthesis in the three model systems, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, and *Chlamydomonas reinhardtii*, are shown in Tables S2 and S3 in the Supporting Information while the biosynthetic genes for the four unicellular algae are in Table 3. Complete BLASTp statistics along with values for gene length in amino acids, % GC content, % identity, and numbers of exons and transmembrane domains are shown in Tables S4-S7 in the Supporting Information.
has only the cytosolic MVA pathway (Table S2). In contrast, all of the algae lack the last four enzymes (HMG, MK, PMK, and MVD) of the MVA pathway including HMG CoA reductase; instead, they only contain the plastidal MEP pathway for IPP synthesis (Table 3). The absence of HMG CoA reductase was supported by the negligible effect of atorvastatin on growth and sterol production (as previously discussed), suggesting that Chlorella spp. only use the plastidic MEP pathway to produce IPP (Table 3). This feature was also demonstrated for Chlamydomonas reinhardtii by Schwender et al. (1997) and for the diatom Haslea ostrearia by Massé et al. (2004); however, Fabris et al. (2014) showed that the diatom Phaeodactylum tricornutum retained and used the MVA pathway to make IPP.

Stage 2 of sterol biosynthesis – IPP to 2, 3-oxidosqualene – has three enzymatic steps and homologous genes were present in the three model systems (Table S2) and all four of the algae for each of these steps (Table 3). Thus, our data agree with the conclusion from Benveniste (2004) that the biosynthetic pathway from IPP to 2, 3-oxidosqualene is the same in all eukaryotes.

Stage 3, from 2, 3-oxidosqualene to completion, there are at least six points of interest: 1/ Arabidopsis thaliana has two oxidosqualene cyclase (OSC) genes, designated CAS1 for cycloartenol synthase and LAS1 for lanosterol synthase (Tables S2 and S3), while the other organisms have only one. The yeast Saccharomyces cerevisiae has ERG7, a lanosterol synthase (Tables S2 and S8), while the four algae have only a single cycloartenol synthase (Table 3). The assignment of these algal genes as CAS rather than LAS is based primarily on their functionality and the chemical identity of their downstream products and is supported by the bioinformatic analyses shown later. 2/ The presence of

| Enzyme | Chlamydomonas reinhardtii | Chlorella sorokiniana | Chlorella variabilis | Coccomyxa subellipsoidea |
|--------|--------------------------|----------------------|---------------------|--------------------------|
| Stage I plastid - MEP Pathway | | | | |
| 1-deoxy-D-xylulose 5-phosphate synthase | Cre07.g356530 (736) | sca185.g101550 (712) | 59788 (721) | 16525 (733) |
| 1-deoxy-D-xylulose 5-phosphate reductoisomerase | Cre12.g346050 (456) | sca079.g101000 (469) | 29723 (456) | 47418 (476) |
| 2-C-methyl-D-erythritol 4-phosphate cytidytransferase | Cre16.g679660 (320) | s0a01.g102250 (304) | 14190 (168) | 35670 (260) |
| 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase | Cre02.g145050 (348) | sca085.g104500 (366) | 33454 (354) | 35952 (301) |
| 2-C-methyl-D-erythritol 2,4-cyclo-diphosphate synthase | Cre12.g503550 (208) | s0a53.g102250 (140) | 12213 (167) | 18339 (208) |
| 1-hydroxy-2-methyl-2E-butenyl-4-diphosphate synthase | Cre12.g490550 (682) | s127.g100200 (1157) | 144676 (699) | 27377 (749) |
| 4-hydroxy-3-methyl-2E-butenyl-4-diphosphate reductase | Cre08.g372950 (466) | s0a12.g106300 (402) | 59658 (506) | 53820 (439) |
| Isopentenyl pyrophosphate isomerase | Cre11.g467544 (254) | sca136.g102550 (306) | 53578 (244) | 36851 (230) |
| Stage I cytosol - MVA pathway | | | | |
| Acetoacetyl-CoA thiolase | Cre02.g146050 (491) | sca243.g102350 (533) | 27161 (401) | 37141 (404) |
| 3-Hydroxy-3-methylglutaryl-CoA synthase | Cre16.g678850 (718) | sca110.g104250 (476) | 138158 (513) | 27385 (520) |
| 3-Hydroxy-3-methylglutaryl-CoA reductase | N.F. | N.F. | N.F. | N.F. |
| Mevalonate kinase | N.F. | N.F. | N.F. | N.F. |
| Phosphomevalonate kinase | N.F. | N.F. | N.F. | N.F. |
| Mevalonate diphosphate decarboxylase | N.F. | N.F. | N.F. | N.F. |
| Stage II ER | | | | |
| Farnesyl diphosphate synthetase | Cre03.g207700 (361) | sca140.g100250 (358) | 33543 (347) | 22862 (340) |
| Farnesyl diphosphate farnesyl transferase | Cre03.g175250 (462) | sca085.g101650 (437) | 11287 (187) | 35284 (348) |
| Monoxygenase/hydrolase | Cre17.g734644 (524) | sca094.g100900 (545) | 137251 (525) | 53930 (509) |
| (squalene epoxidase) | | | | |
| Stage III ER | | | | |
| Cycloartenol synthase | Cre01.g011100 (763) | s0a81.g101700 (695) | 2290 (762) | 54267 (754) |
| Lanosterol synthase | N.F. | N.F. | N.F. | N.F. |
| Sterol C-24 methyltransferase | Cre12.g500500 (388) | sca033.g100500 (412) | 26131 (333) | 27964 (389) |
| Cyclohexanol cycloisomerase | Cre16.g657300 (276) | s0a47.g103200 (298) | 11379 (282) | 65481 (298) |
| Sterol C-14 demethylase | Cre02.g99250 (496) | s139.g104650 (498) | 142513 (613) | 54016 (492) |
| C(8,7) sterol isomerase | Cre12.g537900 (219) | sca034.g101250 (297) | 34946 (224) | 13584 (235) |
| C-4 sterol methyl oxidase | Cre06.g261200 (308) | s0a222.g101000 (518) | 57760 (290) | 11014 (308) |
| C-3 sterol dehydrogenase | Cre12.g518650 (402) | s0a89.g108000 (277) | 49861 (361) | 54657 (357) |
| 3-keto sterol reductase | N.F. | N.F. | N.F. | N.F. |
| Endoplastic reticulum protein | Cre13.g567901 (74) | s0a39.g105150 (149) | 59539 (149) | 14819 (124) |
| C-5 sterol desaturase | Cre16.g663950 (346) | s134.g102400 (337) | 37407 (286) | 15746 (275) |
| C-22 sterol desaturase | Cre11.g467927 (516) | s146.g107050 (528) | 30281 (422) | 29437 (539) |
| C-24 (28) sterol reductase | Cre02.g076800 (426) | s232.g100350 (444) | N.F. | 26214 (436) |
FIG. 1. Putative sterol pathway in Chlorella variabilis and Chlorella sorokiniana showing structures of the biosynthetic intermediates and the enzymes which catalyze each step, starting for convenience with cycloartenol, the product of Cas1p. 1: SMT1-sterol C-24 methyltransferase; 2: SMO2-C-4 sterol methyloxidase; 3: CPI1-cycloeucanol cycloisomerase; 4: CYP51-sterol C-14 demethylase; 5: ERG4/24-C-24(28) sterol reductase; 6: HYD1-C (8, 7) sterol isomerase; 7: STE1-C-5 sterol desaturase; 8: CYP710-C-22 sterol desaturase. The biosynthetic pathway follows that suggested by Brumfield et al (2017) for C. reinhardtii, overlaid with our data showing the sterol intermediates which accumulate following treatment with ketoconazole or clotrimazole, drugs thought to inhibit the two cytochrome P_{450} enzymes, CYP51 and CYP710, here labeled 4 and 8, respectively.

the three genes CAS1, CPI1, and HYD1 in the genomes for Chlorella sorokiniana, C. variabilis, Coccomyxa subellipsoidea, and Chlamydomonas reinhardtii strongly suggests that these algae use the higher plant cycloartenol branch of sterol biosynthesis, which is likely a general feature of the green algae. 3/ In yeasts, the C-4 demethylation step is catalyzed by a complex encoded by ERG 25, 26, 27, and 28; however, C. reinhardtii (Brumfield et al. 2017) and the other algae have orthologs to ERG 25, 26, and 28, but not to ERG 27 (Table 3). 4/ The ergosterol biosynthetic pathway is composed of membrane-associated enzymes assembled as a multi-enzyme complex. In yeasts, the noncatalytic protein ERG28 functions as a scaffold protein, anchoring enzymes and creating a “hub” for enzymatic interactions with substrates (Mo and Bard 2005, Winkel 2009). We have identified an ERG28 homolog in C. variabilis (59539), C. sorokiniana (039.g105150.t1), and C. subellipsoidea (141819) as well as in C. reinhardtii and other algae (Brumfield et al. 2017). 5/ For C. sorokiniana, the 11 genes for stages 1 and 2 are all
high % GC; they range from 63.6 to 70.8% GC, averaging 67.1% GC. Similarly, the 11 predicted genes for Stage 3 are also high % GC, ranging from 61.8 to 67.1% GC, averaging 65.1% GC (Table S4). There are no apparent differences in % GC for the genes encoding plastid-localized components versus ER-localized components (Table S4). The only notable difference based on localization is the number of transmembrane domains (Table S4). No further conclusions can be drawn from these high % GC values because the genomic GC value for C. sorokiniana is 63% GC. RNA-Seq experiments with C. sorokiniana showed that all 22 of the predicted sterol biosynthetic genes were expressed as full-length transcripts during growth (W. Riekhof, unpub. data). Thus, the direct gene comparison reinforces the premise that the green algae as a group produce sterols using the cycloartenol pathway of higher plants and algae rather than the lanosterol pathway of fungi and animals.

**Comparison of Chlorella sp.**, Coccomyxa subellipsoidea, and Chlamydomonas reinhardtii. Sterol biosynthesis in the two Chlorella spp. and Coccomyxa subellipsoidea (Table 3) are very similar to Chlamydomonas reinhardtii both biochemically and genetically (Table S2; Miller et al. 2012, Brumfield et al. 2017). All four algae make IPP via the plastid MEP pathway and are missing the cytosolic MVA pathway (Table 3). Likewise, all four contain the same suite of 14 stage II and III genes (Table 3). These 14 genes (Tables S4-S7) are roughly equivalent in their percent identity, length of coding sequence, and number of introns present. One notable exception is SMO2 (C-4 sterol methyl oxidase), which stands out by being only 29% identical between C. sorokiniana (Table S4) and C. reinhardtii (Table S6). Additionally, C. sorokiniana is notable for having several more exons (up to twice as many) than the other species for nearly all sterol-related genes, consistent with the increased number of exons across the rest of its genome (Table S4).

**Phylogenetic analysis of OSC.** Saccharomyces cerevisiae has a single oxidosqualene cyclase OSC (ERG 7) which cyclizes 2, 3-oxidosqualene to make lanosterol, while Arabidopsis thaliana has 13 OSCs, one of which (LAS1, At3g45130) makes lanosterol and another (CAS1, At2g07050) makes cycloartenol (Tables S2 and S3; Xue et al. 2012). To determine the number of OSC genes in the four algae, we performed BLASTp searches against all 13 A. thaliana OSCs (Table S9 in the Supporting Information). For all four species, the only significant hits to any of the A. thaliana OSCs is the single OSC reported. Additionally, in every case, the alignment for the algal OSC protein is 5-6% higher with CAS1 than LAS1.

To investigate the algal OSCs further, we constructed a phylogenetic tree for forty OSCs (Fig. 2). The sequences separated into four clades or clusters. The first included OSCs that preferentially produce the sterol precursor cycloartenol, forming the branch for higher plants and algae. The second clade included Metazoa using lanosterol, while the third clade included the fungi using lanosterol. A fourth clade included two OSCs from the diatoms Thalassiosira pseudonana and Fistulifera solaris (Fig. 2). All nine of the microalgal OSCs were part of the cycloartenol-forming plant OSCs (Fig. 2). The Chlorella spp. have ergosterol as their major sterol (Table 2) and it is likely synthesized via CAS

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### Table 4. Antifungal inhibition of Chlorella spp. sterol biosynthetic pathway and resulting sterol compositions.

| Sterol                          | Control | Clotrimazole | Ketoconazole |
|--------------------------------|---------|--------------|--------------|
| Ergostatetraenol                | 41.7%   | 24.1%        | 28.2%        |
| Ergosterol                      | 53%     | 38.8%        | 44.5%        |
| 14α-Methylergosta-8,24(28)-dienol| 2.3%    | 3.6%         |              |
| 14α-Methylergost-8-enol         | 2.9%    | 6.9%         | 5.9%         |
| Ergosta-5,7,24(28)-triolenol    | 13%     | 6%           | 0.4%         |
| Ergosta-5,7-dienol              | 1%      | 6%           | 0.4%         |
| Obtusifoliol                    | 1%      | 6%           | 0.4%         |
| Cyclolaudenol                   | 1%      | 6%           | 0.4%         |
| Total Sterols (nmol · mg dw⁻¹)  | 3.2     | 2.6          | 2.6          |

### Table 5. Antifungal inhibition of Coccomyxa subellipsoidea sterol biosynthetic pathway and resulting sterol compositions.

| Sterol                          | Control | Clotrimazole | Ketoconazole |
|--------------------------------|---------|--------------|--------------|
| Campesterol                     | 48.1%   | 40.1%        | 31.7%        |
| 14α-Methylergost-8-enol         | 42.9%   | 42.7%        | 31.1%        |
| Stigmasterol                    | 9.0%    | 6.1%         | 5.2%         |
| 4α,14α-Dimethylergosta-8,22-dienol| 24.25-dihydrolanosterol | 2.5% | 9.2%         |
| β-Sitosterol                    | 42.9%   | 42.7%        | 31.1%        |
| Obtusifoliol                    | 4.3%    | 2.6%         | 0.9%         |
| 24-Methylencycloartenol         | 0.9%    |              |              |
| 9,19-Cycloartenost-22-en-β,ol,  | 0.9%    |              |              |
| 24-methyl                      | 14.3%   |              |              |
| Cyclolaudenol                   | 3.6     | 4.6          | 3.7          |

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**Table 4. Antifungal inhibition of Chlorella spp. sterol biosynthetic pathway and resulting sterol compositions.**

**Table 5. Antifungal inhibition of Coccomyxa subellipsoidea sterol biosynthetic pathway and resulting sterol compositions.**
and cycloartenol because only cycloartenol/obtusifoliol intermediates were formed after inhibition by ketoconazole and clotrimazole (Tables 4 and 5). It is well known that single-site mutations in the Arabidopsis thaliana CAS gene can produce lanosterol instead of cycloartenol (Segura et al. 2002, Benveniste 2004). However, as pointed out by Brumfield et al. (2017), multiple sequence alignment shows that the four algal OSCs all have the highly conserved amino acids expected for cycloartenol synthase (CAS) rather than for lanosterol synthase (LAS; Fig. 3).

The Coccomyxa question. Coccomyxa subellipsoidea appears to be intermediary between the other algae and higher plants, both in its OSC phylogeny (Fig. 2) and in its dramatically different sterol composition (Tables 2 and 6). Additionally, its % GC composition ranges from 51 to 62% for the sterol biosynthetic genes in the MEP pathway and from 49 to 63% for the genes for stages 2 and 3 (Table S7) whereas the genes for the two Chlorella sp. (Tables S4 and S5) were more tightly clustered around their average values of 66% GC. To investigate possible causes for this transition, we conducted a multiple sequence alignment of the OSC proteins from Arabidopsis thaliana (both CAS1 and LAS1), Saccharomyces cerevisiae, and the four algae we have studied (Fig. 3). The three residues used by Brumfield et al. (2017) as characteristic of cycloartenol directed synthesis (marked in orange) and the catalytic aspartic acid (marked in blue) are identical for the four algae and the A. thaliana CAS (Fig. 3), and thus they cannot explain the synthesis of the three phytosterols by C. subellipsoidea. Instead, we have identified 22 amino acid positions in the CAS protein that are differentially conserved. That is, the C. subellipsoidea CAS and A. thaliana CAS1 have one amino acid, but the three ergosterol-containing algae have a different amino acid. These 22 amino acids are shown in yellow on the multiple sequence

Fig. 2. Phylogenetic tree for 40 oxidosqualene cyclase (OSC) proteins showing the position of genes from 9 algal species relative to their plant, animal, fungal, and diatom equivalents. Acetobacter tropicallis (not shown) was used as an out-group to derive the root.
alignment (Fig. 3). Most of these 22 amino acids are located either around the catalytic site or on the outer surface of the protein (Fig. 4).

**DISCUSSION**

Unicellular algae as an evolutionary transition point for sterols. The biosynthesis of sterols in animals, fungi, and plants differs in terms of precursors, enzymatic steps, and final sterol composition. It is widely accepted that the major sterols present in animals and fungi are cholesterol and ergosterol, respectively, and that both employ the cytosol localized MVA pathway leading to the 5-carbon precursor IPP. Subsequent steps are shared in animals and fungi up to lanosterol, after which a bifurcated pathway produces their respective distinct sterols.

Plants differ in that they use the plastid-localized MEP pathway to make IPP and then use the precursor cycloartenol (instead of lanosterol) to produce a wide array of phytosterols. Currently, algae have been grouped with plants as producing cycloartenol and utilizing the phytosterol biosynthetic pathway. We studied ergosterol biosynthesis in *Chlorella variabilis* and *C. sorokiniana* because of their recently sequenced and annotated genomes (Blanc et al. 2010; H.D. Cerutti, unpub. data). Coccomyxa subellipsoidea was included because its sterol lipid profile appears to be transitional between algae and plants, which is supported by the OSC phylogenetic analysis (Table 2). We combined these bioinformatic data (Table 3) with the antibiotic inhibition of key steps in sterol biosynthesis (Table 4) to create a putative ergosterol biosynthetic pathway for *C. variabilis* and *C. sorokiniana* (Fig. 1).

In terms of their sterol contents, unicellular algae exhibit greater diversity than either multicellular algae or plants. Our results emphasize the unique position of the unicellular algae as an evolutionary transition point for sterols and suggest that the unicellular algae provide the evolutionary precursor for...
the cycloartenol based pathways in higher plants (Fig. 2; Haubrich et al. 2014, Vriet et al. 2015).

**Sterol diversity.** In addition to the four patterns we observed in Table 2, previous studies on multicellular algae reported the presence of cholesterol in the marine red algae and fucosterol in the marine brown algae (Fieser and Fieser 1959) while Lu et al. (2014) reported that the oleaginous microalga *Nanochloropsis oceanica* had both cholesterol and fucosterol/isofucosterol as the major and minor sterols, respectively. These general classifications were extended by Duperon et al. (1983) to identify and quantify the free sterols, sterol esters, steryl glycosides, and acetylated steryl glycosides present in marine algae. More recently, this evolutionary diversity was illustrated by the studies of Rampen et al. (2010) who examined 106 diatom cultures looking for an unambiguous diatom biomarker. They detected 44 sterols, of which 11 were major sterols in that they comprised ≥ 10% of the total sterols in one or more diatom. However, none of the major sterols qualified as a diatom-specific biomarker because all of them were common sterols in other algae (Rampen et al. 2010).

In algae, the intricate roles and functions of sterols are not completely understood. Our putative pathway (Fig. 1) helps explain the diversity in sterols in unicellular algae, which may contribute to our understanding their specific functions. Factors influencing sterol differences among algal species could be morphological, environmental, or evolutionary. From a morphological viewpoint, having an alternating life cycle or multiple distinct cell types may require distinct sterols, possibly using both cycloartenol and lanosterol precursors. Different membrane structures might also be related to whether the algae had evolved as the photosynthetic partner in a symbiosis, with the added complication that both primary and secondary plastid endosymbiotic events may have occurred. From an
environmental perspective, water composition, temperature, salinity, nutrient, and ion composition are highly variable between freshwater and marine ecosystems (Porsbringer et al. 2009). For the algae we have studied, *Chlorella sorokiniana* can grow at warmer temperatures than *C. variabilis* while *Coccomyxa subellipsoidea* is unusual in that it can still grow at extreme, near-polar temperatures. These temperature adaptations likely require different sterol compositions (Starr and Parks 1962).

**Why ergosterol?** Changing environmental conditions, such as light intensity and day length, have been shown to change the ratio of sterols present in higher plants (Rahier and Taton 1997). Algae within the class Chlorophyceae have many species that contain ergosterol and/or other $\Delta^5$-7- sterols not found in higher orders of algae and land plants (Patterson et al. 1991). One idea is based on the absorption spectrum of sterols containing two conjugated double bonds. The evolution away from cell membranes containing $\Delta^5$, 7- sterols to those with either $\Delta^5$- or $\Delta^7$-sterols took place as the earth’s ozone layer developed, because $\Delta^5$, 7- sterols are more efficient in absorbing UV radiation (Patterson et al. 1991). Indeed, ergosterol is commonly assayed by its absorption at 280 nm. Hence, once the ozone layer was established, the selective advantage of the $\Delta^5$, 7- sterols may have disappeared. Higher plants contain a cocktail of three $\Delta^5$ phyto-sterols – campesterol (24-methyl), stigmasterol (24-ethyl), and $\beta$-sitosterol (24-ethyl) (Mercer 1993, Holmberg et al. 2002) and this phytosterol composition also occurs in the unicellular green alga *Coccomyxa* (Table 2). The size and direction of the 24-alkyl group inserted by sterol methyltransferase are indicative of either a primitive (24 $\beta$-methyl) or advanced (24 $\alpha$-ethyl) organism (Zhou et al. 2007).

Another more recent idea is that the sterol composition may be constrained by the other aspects of an interlocking membrane composition (i.e., the need for sterols to partner with particular sphingolipids) providing lipid rafts and other distinctive capabilities for the algal membranes (Guan et al. 2009, Gulati et al. 2010, Hannich et al. 2011). An attractive feature of sterol-sphingolipid pairing is that it provides a rationale why organisms as distinct, ecologically, evolutionarily, and metabolically as algae and Saccharomyces utilize the same sterol, ergosterol.

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**AUTHORS CONTRIBUTIONS**

SLR, JLTE, AV, and KWN designed the research; SLR, NTMC, MK, and AV performed the experiments; all authors analyzed data; and AV and KWN wrote the paper.

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