The Heterotrophic Dinoflagellate *Cryptecodinium cohnii* Defines a Model Genetic System To Investigate Cytoplasmic Starch Synthesis\(^\text{V}\)↓

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The nature of the cytoplasmic pathway of starch biosynthesis was investigated in the model heterotrophic dinoflagellate *Cryptecodinium cohnii*. The storage polysaccharide granules were shown to be composed of both amylose and amylopectin fractions with a chain length distribution and crystalline organization very similar to those of green algae and land plant starch. Preliminary characterization of the starch pathway demonstrated that *C. cohnii* contains multiple forms of soluble starch synthases and one major 110-kDa granule-bound starch synthase. All purified enzymes displayed a marked substrate preference for UDP-glucose. At variance with most other microorganisms, the accumulation of starch in the dinoflagellate occurs during early and mid-log phase, with little or no synthesis witnessed when approaching stationary phase. In order to establish a genetic system allowing the study of cytoplasmic starch metabolism in eukaryotes, we describe the isolation of marker mutations and the successful selection of random recombinant populations after homothallic crosses.

Alveolates comprise one of the largest and most diverse groups of protists (over 10^5 species are currently recognized), which includes three major lineages consisting of dinoflagellates, ciliates, and apicomplexan parasites, together with a number of other lesser-known and -studied genera (for a general review, see references 10 and 20).

Ciliates are characterized by their complex feeding apparatus, their nuclear dimorphism (macro- and micronuclei), and their complex cilature. They are all nonphotosynthetic.

Dinoflagellates are distinguished by their specialized flagellar dimorphism and their highly unusual molecular features, such as the presence of permanently condensed chromosomes with a unique form of closed mitosis. They also harbor some of the largest known eukaryotic genomes. Dinoflagellates contain both photosynthetic and heterotrophic species that prey quite aggressively by phagocytosis. The photosynthetic dinoflagellates harbor plastids with a diverse array of evolutionary origins, suggesting that they have, at least in some instances, originated through tertiary endosymbiosis (for a review of dinoflagellate biology, see reference 12).

Apicomplexa comprise nonphotosynthetic obligate intracel-

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the sequenced genomes of the ciliates *Tetrahymena thermophila* and *Paramecium tetraurelia* (1, 9). There is thus a distinct possibility that the secondary-endosymbiosis event occurred after the ciliates diverged from the common ancestor and before the apicomplexan parasites diverged from their dinoflagellate sisters. All three alveolate lineages store glucose in the form of α-1,4-linked glucose chains, which are branched through α-1,6 branches. Interestingly, *T. thermophila*, and *P. tetraurelia* synthesize glycogen (18), the most widespread form of storage polysaccharide found in heterotrophic eukaryotes and in bacteria and archaea (for a review of glycogen structures, see reference 17). While glycogen accumulates as small hydrolysable particles whose diameters cannot exceed 40 nm, dinoflagellates and apicomplexans store a more complex macrogranular insoluble form of storage polysaccharide that has a strong resemblance to plant starch (7; for a review of starch structure, see reference 6). In our previous experiments, we characterized the starch pathway in the model cryptophyte *Guillardia theta* (8) and the model glaucophyte *Cyanophora paradoxa* (22). We showed that these algae synthesized starch by a pathway distinct from those evidenced in green algae and land plants and more similar to that which was recently proposed for the Rhodophyceae (7, 21, 31). The latter is based on the use of UDP-glucose as a substrate, while green algae and land plants use ADP-glucose, as do cyanobacteria, to build storage polysaccharides. *G. theta* and *C. paradoxa* were both shown, in addition, to contain a granule-bound starch synthase that was able to use both types of glycosyl nucleotides for amylose biosynthesis (8, 22).

An important difference in starch metabolism that distinguishes the red algae and glaucophytes from the green algae and land plants can be found in the cellular localization of starch. Indeed, green algae and higher plants store starch within their plastids, while red algae synthesize it within their cytoplasm. This difference entails the existence of different controls exerted at the level of starch synthesis and degradation. Those investigating the starch structure within Rhodophyceae coined the term floridean starch to qualify the polysaccharides that accumulate in the cytoplasm of the Florideophyceae, a group of complex multicellular red algae (reviewed in reference 31). The structure of floridean starch was believed to differ from that of the classical plastidial starch by the absence of amylose and the sole presence of amylopectin, the major semicrystalline fraction of starch. Since these pioneering studies, amylose has been found in the Porphyridiales, a group of unicellular red algae (19), and part of a *Porphyridium purpureum* granule-bound starch synthase I (GBSSI) sequence, the enzyme responsible for amylose synthesis, has been reported (25). Because of its widespread use, we will use the name “floridean starch” when referring to all forms of cytoplasmic or periplastidial starch accumulated in Rhodophyceae, cryptophytes, glaucophytes, and alveolates, notwithstanding the presence or absence of amylose.

Genetic approaches to the study of starch metabolism in *Chlamydomonas* were successful in paving the way for a deeper understanding of starch metabolism in plants (reviewed in reference 2). A similar powerful unicellular genetic system allowing mutant selection, sexual crosses, and biochemical investigations is needed to study floridean-starch metabolism. No such system is presently available within unicellular red algae, glaucophytes, or cryptophytes, although many species are available for standard biochemical approaches. Within alveolates, the apicomplexan parasites with their sequenced genomes and their targeted gene disruption techniques offer an attractive potential (26). However amylopectin synthesis is not a universal feature of apicomplexans (7). In addition, amylopectin-producing parasites are not easy to grow in vitro and certainly do not offer a good potential for biochemical studies. Among dinoflagellates, mutant selection and successful crosses of *Cryptothecodinium cohnii*, a heterotrophic species, were reported years ago (4, 5, 14, 27, 29, 33). Since these pioneering studies, while some additional mutants (15) have been reported, such genetic approaches have not been reproduced in this fast-growing organism or any other dinoflagellate species. In addition, all the original mutant strains, including albinoblasts, purified, and adenine-requiring auxotrophs, have been lost, precluding the use of marker genes.

The present work investigates the suitability of *C. cohnii* as a genetic system to investigate the major features of floridean-starch metabolism. We report a detailed characterization of starch structure and metabolism in the model heterotrophic dinoflagellate. We show that the multiple dinoflagellate soluble and granule-bound starch synthases use UDP-glucose to polymerize glucans, as do all presently characterized starch synthases of floridean-starch-accumulating species. In stark contrast with other microorganisms, *C. cohnii* is now reported to accumulate starch in early to mid-log phase. With the aid of canavanine resistance and albinoblast marker mutations, we were able to set up homothallic crosses routinely and report a straightforward procedure for the selection of recombinant populations, allowing a “random-spore” type of genetic analysis in dinoflagellates. These techniques can be used to study the segregation of any mutant trait in dinoflagellates and may be applicable to many different dinoflagellate species, provided they can grow on solid media.

**MATERIALS AND METHODS**

**Materials.** ADP[U-14C]glucose, UDP[U-14C]glucose, and Percoll were purchased from GE Healthcare (formerly Amersham, United Kingdom, and Pharmacia, Sweden). ADP-glucose, UDP-glucose, rabbit liver glycogen, and Sepharose CL-2B were obtained from Sigma-Aldrich (St. Louis, MO). Starch assay kits were obtained from DiDia (Lyon, France). TSK HW50 and Fractogel TSK DEAE-650 (M) were obtained from Merck (Darmstadt, Germany). The protein assay kit was purchased from Bio-Rad (Munich, Germany). Instant Ocean (Aquarium Systems, Sarbebour, France) was used as sea salts and was purchased from a local store.

**Algal strain and growth conditions.** *C. cohnii* (strain ATCC 40750, purchased from LGC Promochem, France) was grown in the dark at 27°C in either rich medium (33.3 g liter−1 sea salts, 12 g liter−1 glucose, and 4 g liter−1 yeast extract) or MLH medium (28). The strains were maintained in the dark at 27°C on solid rich medium (1.2% agarose) and transferred onto new plates every 2 months. The Saccharomycetes cerevisiae strain JC482 (MATa leu2 ura3-52 his4) was grown in YPAD medium (1% yeast extract, 2% peptone, 2% glucose, 0.003% adenine sulfate) at 30°C with constant shaking. The cell density was measured using a Coulter particle counter. For the growth curve-monitoring experiments, late-stationary-phase precultures of known cell density were used to inoculate large independent cultures. *Cryptothecodinium* cultures were inoculated at 25,000 cells ml−1 and *Saccharomycetes* cultures at 500,000 cells ml−1. Extractions of polysaccharide from cells in the latency phase showed that both species start to grow with an undetectable amount of polysaccharide.

**Polysaccharide extraction.** (i) Starch. *Cryptothecodinium* cultures were centrifuged (3,000 × g; 10 min). The cells were resuspended in extraction buffer (20 mM Tris/HCl, pH 7.5, 5 mM EDTA, 1 mM dithiothreitol [DTT]) and disrupted by sonication. The extract was centrifuged (10,000 × g; 15 min), and the super-
natant was kept and used for further enzymological analysis, while the pellet (starch and cell fragments) was resuspended in 90% Percoll and centrifuged (10,000 × g; 30 min) to separate high-density starch granules from cell debris of lower density. The last step was repeated once to ensure complete removal of cell debris. The starch was then washed twice in sterile water. Clean, dry starch pellets were stored at 4°C for up to 1 month.

(6) Glycogen. Saccharomyces cells were collected by centrifugation (10 min at 5,000 × g and 4°C) and washed once with sterile water. The pellets were resuspended in water, and the cells were disrupted by ultrasonic treatment and incubated at 95°C for 20 min. The supernatant was then centrifuged at 10,000 × g, and the supernatants were kept to assay the glycogen content. Starch, glycogen, and glucose amounts were assayed using the Dinitellammonium Enzymat Chk kit.

Determination of starch structure: X-ray diffraction, differential-scanning calorimetry, and SEM. The Sepharose CL-2B gel chromatography procedure was previously described for G. theta (8). Eluted glycans were de
tected by measuring the optical density at 340 nm, (the maximal-absorbency wave
length) after interaction with 0.2% KI, 0.02% I2. Analysis of debranched amylo-
pectin/amylase by gel permeation chromatography was performed as follows. A total of 2 mg of diazylated and modified polysaccharide purified by gel perme-
ation chromatography was suspended in 55 mM sodium acetate, pH 3.5, and hydrol
yzed by 10 units of Pseudomonas amylopodim isomylase (Hayashibara Biochemical Laboratory, Okayama, Japan) at 42°C for 12 h. The reaction was stopped by boiling the mixture, and the mixture was raised to 10% dimethyl sulfoxide to avoid retrogradation of long glucans and immediately subjected to TSK HW50 g gel permeation chromatography (8). X-ray diffraction, differential-scanning calorimetry, and scanning electron microscopy (SEM) were performed as described previously.

Zymogram analysis of starch synthases, starch-metabolizing enzymes, and starch phosphorylases. For starch synthesize zymograms, 200 µg of crude extract proteins was loaded on 35% (acylamide-bisacylamide) 7.5% polyacrylamide gels containing 0.3% rabbit liver glycogen. Electrophoresis was carried out at 4°C under non-denaturating conditions at 15 V cm⁻¹ for 180 min, using the Mini-
Protean II cell (Bio-Rad), in 25 mM Tris-glycine, pH 8.3. The gel was then
incubated overnight in 50 mM Tris/Cl, pH 7.5, 100 mM (NH₄)₂SO₄, 20 mM β-
mercaptoethanol, 5 mM MgCl₂, 0.5 mg/ml bovine serum albumin, and 1.2 mM 
UDP-Glc at 25°C. After incubation, the gel was stained by immersion in an iodine solution (0.25% KI and 0.025% I2). For detection of starch-metabolizing enzymes and starch phosphorylases, the same procedure was followed. “In-gel” glycogen was replaced by 0.3% hydrolyzed potato starch (Sigma), and the gels were incubated in 25 mM Tri-Gly, pH 8.3, for starch-hydrolyzing activities or in 50 mM Tris/Cl, pH 7.5, 100 mM (NH₄)₂SO₄, 20 mM β-mercaptoethanol, 5 mM MgCl₂, 0.5 mM bovine serum albumin, 20 mM glucose-1-phosphate for starch phosphorylase zymograms.

Extraction and separation by sodium dodecyl sulfate-polyacrylamide gel elec
trophoresis (SDS-PAGE) of granule-bound starch proteins. Granule-bound pro-
teins were extracted by denaturing 5 mg of starch granules in 200 µl of dena-
turation buffer (62.5 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 20 mM DTT, 0.01% bromophenol blue) for 20 min at 90°C. The sample was centrifuged at 20 min at 10,000 × g, and the supernatant was loaded onto a 7.5% SDS-
polyacrylamide gel. The proteins were stained with Roti-blue (Carl Roth, Ger-
many).

Separation and partial purification of starch synthase activities from crude extract: solubil starch synthase assays. Every buffer used for experiments was derived from the main extraction buffer: 20 mM Tris/Cl, pH 7.5, 5 mM EDTA, 1 M DTT, 0.5 mM benzamide, and 1 mM phenylmethylsulfonyl fluoride. The complete procedure was performed at 4°C. Two liters of algal culture was loaded on a Fractogel TSK DEAE-650 (M) anion-exchange column (1 ml; 0.5-cm diameter). The column was washed with 10 ml extraction buffer to eliminate Glucidex and the proteins that did not interact by anion exchange. The elution was performed using 5 ml of elution buffer plus 1 M NaCl.

The purification procedure for the low-mobility, high-affinity isoform was as
follows. The amylose reagent containing the low-mobility, high-affinity isoform was washed with 15 ml of extraction buffer, and the proteins were eluted using 10 ml extraction buffer plus 10 mg · ml⁻¹ Glucidex 19 (Roquet frères, France). This fraction was then loaded on a Fractogel TSK DEAE-650 (M) anion-exchange column (1 ml; 0.5-cm diameter). The supernatant was kept to assay the glycogen content. Starch, glycogen, and glucose amounts were assayed using the Dinitellammonium Enzymat Chk kit.

UV mutagenesis and mutant screening. UV mutagenesis was performed using a Transluminator UV LightBox (UV Products Inc.) that delivered 302-nm wavelength light. In order to build a cell survival curve, a known number of wild-type cells from liquid rich-medium cultures were spread on rich-medium petri dishes. These dishes were then exposed to UV at 5 cm from the source for various exposure times and immediately incubated in darkness. Survival was assayed by counting the colonies on treated and untreated petri dishes. A time of exposition corresponding to approximately 10% survival was used for high-rate mutagenesis. After mutagenesis, colonies were picked onto new agar plates for sensitivity against drugs and on rich medium for the starch deficiency screen and albin mutant screen. The drugs added to MLH medium were fluoroorotic acid (50 µg · ml⁻¹ plus 200 µg · ml⁻¹ uracil), methylanthranilic acid (50 µg · ml⁻¹ plus 200 µg · ml⁻¹ tryptophan), diaminopurine acid (45 µg · ml⁻¹ plus 200 µg · ml⁻¹ adenine), canavanine (200 µg · ml⁻¹), and cycloheximide (1 µg · ml⁻¹).

For screening of starch-deficient strains, petri dishes were exposed to iodine vapors after 7 days of growth. A pinch of iodine crystals was laid down at the bottom of a glass jar with a sealed top approximately the size of a petri dish. Iodine vapors appeared after a few minutes at room temperature. The process could be speeded up by warming the bottom of the glass jar on an electrical plate. When the jar was saturated with iodine vapors, the lid was lifted under a chemical hood and replaced by an open petri dish with the agar turned face down for one to a few seconds. The intensity of the staining could be monitored by watching the stain expanding progressively to the center of the colonies as the dishes were being stained. If some viability was to be maintained on the screened plates, care was taken to stain only once and as lightly as possible. Photographs of the phenotype were taken immediately, and the petri dishes were quickly destained by drying them under a sterile hood. Otherwise, the plates could be retained at will and kept sterile. For screening of carotene-deficient strains, 5-day-old colonies were exposed to continuous light (10 µE/m² s⁻¹) for 5 days to induce pigmentation.

Cell crossing and genetic analysis. To find the best conditions for cell crossing, two types of crosses were investigated. In the first type of cross, starch-deficient mutants were mixed together to look for the presence of wild-type recombiantins. In the second type of experiment, the canavanine-resistant mutant was mixed with the albin mutant to look for double-mutant recombiantins. Cells were mixed (with identical cell densities for each partner) under four conditions: (i) MLH medium at 25°C, (ii) minimal low-glucose medium (33.3 g · liter⁻¹ sea salt, 0.1 g · liter⁻¹ yeast extract, 0.1 g · liter⁻¹ glucose) at 25°C to induce nutrient stress, (iii) MLH at 15°C, and (iv) minimal low-glucose medium at 15°C to induce cold stress. After 5 days, the cells were counted and spread on rich-medium petri dishes. The colonies were stained with iodine vapors or were picked on both rich-medium plates under light and canavanine MLH plates to look for double mutants. Genetic analysis of the mutation in the PP314 strain was carried out as follows. PP314 was crossed with the canavanine-resistant mutant CAN1. The RB1 strain carrying both phenotypes was selected. RB1 was then crossed with the albin ABL1 strain. The progeny were first selected on canavanine plates. Resistant strains were picked on rich-medium plates and grown under light to look for pigmentation. Strains carrying both mutations were then stained with iodine to look for the segregation of the starch deficiency phenotype.

RESULTS

Morphology and structure of C. cohnii starch granules. Two previous studies on the nature of the storage polysaccharide accumulated by dinoflagellates (7, 32) established that the or-
Organisms accumulated α-1,4-linked and α-1,6-branched glucans with fractions consisting of both high-mass amyllopectin-like and small amylose-like components that could be separated by gel permeation chromatography. Like plant amyllopectins, the debranched high-mass amyllopectin-like component displayed a typical multimodal distribution when examined by fluorescence-assisted capillary electrophoresis (7). In order to get a more detailed picture of the storage polysaccharides, we resolved to separate the low-molecular-weight amylose from amyllopectin through gel permeation chromatography (Fig. 1) and to subject both amylose and amyllopectin to a more complete chain length distribution analysis through separation of the chains on a TSK-HWS0-type gel filtration column (Fig. 2). This technique enables one to access the average chain length distribution of all glucans, including the extra-long chains that escape both the high-performance anion exchange chromatography with pulsed amperometric detection and the fluorescence-assisted capillary electrophoresis techniques. The chromatograms displayed in Fig. 2 show that both C. cohnii and Chlamydomonas reinhardtii amyllopectin fractions contained a substantial amount of glucose within chains with average degrees of polymerization (DP) above 50. These chains were likely due to the action of GBSS on amyllopectin outer chains. The chromatograms shown for the dinoflagellate and green-alga model species are nearly identical, proving that the simplified monomodal chain length distribution evidenced recently for the apicomplexan cousins of C. cohnii (7) does not represent a general case for alveolate starch.

Starch differs from glycogen in its insoluble nature. The starch polymers (amylopectin and amylose) are packed into large (from 0.1- to 10-μm diameter in most instances) insoluble granules whose numbers, shapes, and sizes are genetically controlled. We investigated the morphology of such granules by electron microscopy. As seen in Fig. 3, when observed by SEM, all granules displayed a regular round or ovoid smooth shape. We were unable to find granules harboring the highly unusual ball-shaped cavity found in cryptophytes (8) or the other unusual “glove” shapes found in some Chlamydomonas starch granules. These unusual features were in both instances correlated with the presence of pyrenoidal starch.

Amylopectin is responsible for building the backbones of starch granules. Neighboring chains within clusters interwine into parallel double helices that assemble into highly specific crystalline lattices (reviewed in reference 6). These diffract and give two types of X-ray diffraction patterns corresponding to two different assembly geometries that have been named A type and B type (6). Starch is typically semicrystalline, as only sections of amyllopectin assemble into crystals, while amylose is usually thought of as a mostly amorphous fraction. The C. cohnii starch granules were further analyzed using X-ray diffraction. The results, displayed in Fig. S1 in the supplemental material, establish that C. cohnii synthesizes semicrystalline starch polymers of the A type, with a crystallinity around 24%. Together with the results displayed in Fig. 1, Fig. 2, and Fig. 3, these data demonstrate that dinoflagellates accumulate starch with a structure very similar to that of the vascular plants and green algae and very different from that of their apicomplexan sisters (7). We also probed crude extracts for the presence of water-soluble polysaccharides or oligosaccharides and were unable to find levels amounting to more than 0.1% of the starch accumulated by these organisms.

C. cohnii synthesizes starch in early and mid-log phase. Heterotrophic microorganisms synthesize storage polysaccharides at specific times in their growth curves. The synthesis of glycogen in late log phase and entry into stationary phase defines the responses of most bacteria and yeasts examined (reviewed in reference 23). This coincides with the consumption of the bulk of the reduced carbon available in the medium. Nutrient limitation or starvation-induced accumulation of carbohydrate stores is indeed a very common response among microbes (23). Figure 4B shows the pattern of glycogen accumulation exhibited by the wild-type S. cerevisiae JC482 yeast strain grown onYPAD medium (20 g · liter⁻¹ of glucose). Figure 4A shows similar curves for the wild-type C. cohnii strain ATCC MK8805 (12 g · liter⁻¹ of glucose). The curve shows an immediate response to the elevated glucose supply, followed after 3 days by a slow and steady decline. Thus, starch synthesis appears to be maximal in early log phase and declines from mid- to late log phase.

C. cohnii contains multiple UDP-glucose-utilizing soluble starch synthases and a 110-kDa GBSSI-like protein. The biochemical properties of dinoflagellate crude extracts were investigated by a complete set of zymogram procedures. The results displayed in Fig. 5A demonstrate that C. cohnii contains a minimum of three UDP-glucose-utilizing soluble starch synthase activity bands. In addition, the dinoflagellate exhibits two phosphorylase activity bands (Fig. 5B) and a set of hydrolases with diverse specificities, as suggested by the distinctive staining yielded on zymogram gels (Fig. 5C). Indeed, the iodine-
staining properties of the starch hydrolase and transferase bands suggest the presence of distinct branching enzymes, amylases, and direct debranching enzyme. However, these iodine color interactions are at best suggestive and will require further characterization to define them. To further investigate the properties of the UDP-glucose-utilizing soluble starch synthases, we partly purified these enzymes by a two-step affinity and anion-exchange chromatography procedure. The results, displayed in Fig. 5E, show that we were successful in purifying two sets of activities. The first set (purified 106-fold) corresponded to the low-mobility activity band, while the second (purified 30-fold) corresponded to the multiple high-mobility migrating forms. The extracts were essentially devoid of contaminating starch hydrolases or phosphorylases and thus could be further characterized with respect to their substrate preferences (Table 1). We did, however, detect traces of some unidentified faint hydrolase or, more likely, transferase band binding tightly to glycogen on our zymogram gels (Fig. 5E, top). However, these activities could not be scored through the standard quantitative reducing end assays and therefore were assumed not to interfere with the starch synthase assays. In addition to the soluble starch synthases, we were able to identify the major 110-kDa starch-associated protein as a GBSS through peptide sequencing (Fig. 5D; see Fig. S2 in the supplemental material). This correlated with the presence of a granule-associated starch synthase activity that selectively used UDP-glucose. At variance with the results obtained with cryptophytes and glaucophytes, the dinophyte GBSS was unable to

FIG. 2. Separation of isoamylase-debranched glucans from purified amylpectin and amylose. Amylopectin and amylose from *C. cohnii* (A and C, respectively) and *C. reinhardtii* (B and D) were debranched by isoamylase, and linear glucans were separated by TSK HW50 size exclusion chromatography. The fractions were allowed to interact with iodine, the optical density (OD) at 626 nm was measured, and the total glucose content of each fraction was assayed (solid lines). An additional upper curve is individually scaled to show the average DP of the corresponding glucans. This average DP was calculated from the λ_{max} values of the debranched glucans using the scale published by Banks et al. (3).

FIG. 3. SEM of native purified starch granules from *C. cohnii*. 
indicates the residual glucose concentration in the medium. The amount of starch synthesized by *C. cohnii* during culture development (A) was compared to glycogen accumulation in a growing culture of *S. cerevisiae* (B). Means ± standard deviations of three separate experiments are displayed. Cell density (●) is indicated on the left y axis, and the relative polysaccharide amount (∆) is displayed on the right y axis. An additional upper curve is individually scaled and indicates the residual glucose concentration in the medium.

FIG. 4. Kinetics of polysaccharide accumulation during growth. The amount of starch synthesized by *C. cohnii* during culture development (A) was compared to glycogen accumulation in a growing culture of *S. cerevisiae* (B). Means ± standard deviations of three separate experiments are displayed. Cell density (●) is indicated on the left y axis, and the relative polysaccharide amount (∆) is displayed on the right y axis. An additional upper curve is individually scaled and indicates the residual glucose concentration in the medium.

use ADP-glucose at significant rates. Indeed, in the presence of 3 mM nucleotide sugar and 50 mM maltotriose, the ratio of incorporation with ADP-glucose versus that of UDP-glucose was less than 2%. Taken together, these results demonstrate that *C. cohnii* synthesizes starch through a UDP-glucose-based pathway close to those recently proposed for Rhodophyceae, Glaucophyta, and Cryptophyceae.

Establishing random-spore analysis in the dinoflagellate *C. cohnii*. Setting up a genetic system requires the ability to select mutants and perform sexual crosses. Dinoflagellates display a homothallic haplontic life cycle. In theory, simple mixing of any combination of dinoflagellate haploid cultures without any mating-type concern should yield sexual fusions, followed by encystment of the planozygotes and meiosis. In order to generate markers for genetic analysis and selection of the minority recombinant population among the mixed cultures, we selected for mutants resistant to 5-fluorotate, canavanine, and cycloheximide through direct selection on drug-containing plates (supplied with uracil in the case of 5-fluorotate) after UV mutagenesis. This enabled us to select a single canavanine-resistant mutant that was stable upon subcloning and showed no signs of high-frequency reversion. In addition, we screened a mutant population of 10⁶ cells after mutagenesis at 1 to 10% survival for mutants altered in carotenoid biosynthesis (Fig. 6) and starch metabolism. Among the survivors we found one albino mutant and five low-starch mutants (accumulating between 1 and 20% of the wild-type amounts). Again, the traits were stable during subcloning and showed no signs of reversion. We then proceeded to reproduce the mating procedures described years ago (4, 27, 33). We used the canavanine resistance and the albino trait to track the presence of the double-mutant combination after culture mixing; we also used two starch-deficient strains to look for wild-type recombinants after mixing (Table 2). Because we had no success with the isolation of recombinants without any stress, as described in the old procedures (4, 27, 33), we analyzed the impacts of nutrient starvation and cold stress (15°C) on the induction of the sexual cycle. The results listed in Table 2 clearly indicate that the optimal conditions are defined by prolonged (5-day) mixing under both glucose and nitrogen starvation at 25°C. These conditions reproducibly yielded 2 to 3% double-mutant albino and canavanine-resistant recombinants or wild-type recombinants when starch-defective mutants were crossed, suggesting that 8 to 12% of the mixed population resulted from the meiotic offspring of sexual crosses. All of these experiments were performed with similar inputs of both parental strains during the mixing.

Recombinants can be safely isolated through a two-step procedure. In a first step, either the canavanine resistance or the albino marker is crossed into the strain carrying a novel trait to be analyzed. This strain is then crossed with a partner containing either the albino marker (if the canavanine resistance has been coupled with the trait to be analyzed) or the canavanine resistance marker (if the albino mutation has been coupled with the trait to be analyzed). The albino offspring of the cross plated on canavanine-containing solid medium is then analyzed for segregation of the novel trait. We applied this technique to one of the five starch mutants detected in our first mutagenesis campaign. The results yielded 42 wild-type recombinants and 38 low-starch segregants in a total sample of 80 canavanine-resistant albino offspring, demonstrating Mendelian segregation of the starch defect. Ten of these strains are presented in Fig. 6. The starch-defective mutants will be characterized elsewhere.

DISCUSSION

Years ago, crosses were successfully made with dinoflagellates, and genetic recombination was evidenced between marker genes by two distinct approaches. In the first series of experiments, mutants defective for carotenoid biosynthesis were mixed, and wild-type “yellow” recombinants were found among descendants (27, 33). However, these experiments did not afford precise quantifications of phenotypic classes, since no counterselection of parents could be applied. In a second approach, paralyzed mutants were selected and mixed (4). A few hours after mixing the paralyzed parents, the observation of swimming planozygotes with four flagella was taken as evi-
dence of sexual fusion and transcomplementation. Because the diploid cells could not be stably maintained, the swimming planozygotes were isolated by micromanipulation and left to undergo meiosis and germinate on agar plates. The results suggested the presence of an unusual one-division segregation, but nevertheless, Mendelian recombination ratios in two-factor crosses were evidenced (4, 5, 12). Since these pioneering studies, no successful genetic analysis has ever been reported with dinoflagellates, and the initial results obtained with C. cohnii have never been reproduced.

In this work, we chose to reinvestigate gametogenesis and thus the conditions that trigger the sexual cycle. While the conditions previously reported by Beam and Himes (4) did not yield any evidence of genetic recombination in our mixing experiments, we were able to trigger the latter by simply applying both nitrogen and carbon starvation at 25°C in a fashion similar to that reported by Tuttle and Loeblich (27). For this purpose, we chose an alternative strategy to the simple analysis of Tuttle and Loeblich (27) and the more technically demanding methods of Beam and Himes (4). This method is based on the use of simple visual markers, such as colony color, and growth markers, such as drug resistance. In crosses involving two such markers, a double-recombinant population was easily selected, upon which segregation of a third marker could be examined. This required a two-step procedure that included first, an introgression of either the canavanine resistance or the albino marker into the strain containing a mutation under analysis, and second, another cross involving this strain with the reciprocal color or resistance marker combination. In theory, this would allow a direct and fast analysis of any mutant character isolated in C. cohnii. Analogous methods could also be easily applied in most homothallic dinoflagellate species, provided they could be easily plated on solid media.

C. cohnii is an easy-to-grow and fast-growing organism. Colonies can be grown in a week at room temperature, and dense liquid cultures can be produced in a few days. Marker and starch metabolism mutations were easily selected for. This, together with the ease with which sexual offspring and recombinants were produced, should attract those that are interested in studying dinoflagellate biology in general. What are needed to further develop this system are both genomic resources and transgenesis techniques. The large dinoflagellate genomes presently preclude the acquisition of the full nuclear genome sequence. Nevertheless, the speed and cost of genome sequencing are constantly being improved, and this situation may change. Some expressed sequence tags have been produced and analyzed (24), but much more extensive expressed sequence tag libraries would be a very wise investment to propel the study of this very promising model. Another “must” would be

\[
\begin{array}{cccc}
\text{Isoform} & \text{Optimal pH/ temp (°C)} & K_m a/V_{max} b \text{ with UDP-glucose} & K_m a/V_{max} b \text{ with ADP-glucose} & K_m for \\
\text{glycogen} c & K_m for \\
\text{amylopectin} d \\
\hline
\text{Low mobility} & 9.5/25 & 21.3 \pm 3.2/7,100 & 46.9 \pm 7/2,300 & 2.4 \\
\text{High mobility} & 10/25 & 10.7 \pm 1.4/2,600 \pm 180 & 11.5 \pm 1/156 \pm 1 & 4.6 \\
\end{array}
\]

\(a\) Millimoles per liter.
\(b\) Micromoles of glucose incorporated per minute and per milligram of protein.
\(c\) Milligrams per liter.
be to devise vectors and transformation techniques to achieve transgenesis in *C. cohnii*.

Our major goal is to genetically dissect starch metabolism in dinoflagellates as a model to understand the pathway of floridean-starch synthesis that has now been shown to be shared with rhodophyceae and glaucophytes. The relatively primitive status of the genomic resources of *C. cohnii* will not preclude us from finding the biochemical defects in most mutants that we create. Indeed, the identities of the enzymes at work are often suspected and are easy to monitor with the zymogram techniques that we report in this work. Nevertheless, molecular cloning of the genes encoding these activities will be complex because of the poor status of the genomic resources. One might argue that alveolates, and especially dinoflagellates, have a far more complex evolutionary history involving secondary and in some cases tertiary endosymbiotic events to define good models for floridean-starch metabolism. This, in theory, could lead to the existence of a much more complex and diverse pattern of genes used for storage polysaccharide metabolism. Nevertheless, our previous phylogenetic analyses demonstrated that, with the noticeable exception of branching enzymes, which display an alveolate-specific phylogeny, the apicomplexan parasite *Toxoplasma gondii* contains a set of amylopectin metabolism genes that are related to those evidenced in Rhodophyceae and distinct from those found in fungi and animals.

The biochemical characterization of enzymes of starch metabolism reported in this work make a strong case for the presence of a UDP-glucose-based pathway similar to that we have recently reported for cryptophytes and glaucophytes (8, 22). Interestingly, unlike the latter, the dinoflagellate GBSSI did not show any ambivalence toward the use of the ADP-glucose substrate and could use only UDP-glucose at significant rates.

A surprising and interesting finding reported in this paper is the pattern of starch synthesis observed in growing cultures of *C. cohnii*. Instead of inducing storage polysaccharide synthesis while approaching stationary phase, as do most bacteria and yeasts reported to date (23), *Cryptothecodinium* synthesizes starch immediately after its inoculation into fresh medium. Starch synthesis proceeds in early log phase and then progressively declines after mid-log phase. This atypical behavior can be explained when one compares the natural growth conditions of *Cryptothecodinium* to those artificially used by biologists to study the organism. *C. cohnii* encompasses a number of different isolates with different feeding behaviors. While most strains can be grown osmotrophically, some isolates have proven to be strictly phagotrophic and cannot be grown on glucose medium (30). These isolates prey quite aggressively upon a diversity of planktonic species. While it was inferred that most osmotrophically grown *C. cohnii* strains may feed on decaying algae, phagotrophy may, in fact, define the predominant way of life of all strains (for a discussion of this issue, see reference 11). The medium used to grow our *C. cohnii* strain contained 12 g·liter⁻¹ of glucose, among others (see Materials and Methods). The organic substances found in these media display concentrations 2 to 7 orders of magnitude higher than those in seawater! We believe that under natural conditions *Cryptothecodinium* is faced with sudden nutrient bursts, due to the capture and digestion of prey or the availability of decaying algae within an otherwise-unsuitable nutrient-starved environment (the ocean). Under such conditions, it is expected that regulatory mechanisms must exist to cope with these bursts in order to store the energy and carbon required to accomplish the dinoflagellate cell division cycle. We believe that the transfer of dinoflagellites into fresh rich medium mimics those nutrient bursts and therefore immediately triggers starch biosynthesis.

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