Physiology and Ultrastructure of an Oxygen-resistant Chlorella Mutant under Heterotrophic Conditions

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ABSTRACT

The oxygen-resistant strain of Chlorella sorokiniana (Shihira and Krauss), distinguished by its ability to grow autotrophically under high partial pressures of oxygen, was studied and partially characterized in heterotrophic culture. Ultrastructural analysis of glucose-grown oxygen-resistant strain and wild type cells reveals that osmiophilic deposits (possibly polyphosphate) are present only in wild type, while oxygen-resistant strain apparently contains increased amounts of starch and endoplasmic reticulum. Of major physiological significance are the observations that: (a) oxygen-resistant strain requires 6 to 8 days to completely adapt to dark, heterotrophic conditions, whereas wild type acclimates in 1 day; (b) oxygen-resistant strain is resistant to high oxygen tension only when grown on glucose, but not on acetate; and (c) the respiratory rate, but not the photosynthetic rate, of heterotrophic oxygen-resistant strain is abnormal compared to wild type.

Heterotrophic culture of green algae of the genus Chlorella represents a useful experimental technique by which metabolic regulatory mechanisms can be investigated (2, 17, 18, 20). Significant changes in response to such conditions have been described in these organisms, especially for photosynthetic and dark, oxidative enzyme systems (3, 8, 21).

In this paper, we report the use of this technique to characterize more a fully a mutant strain of the high temperature species, C. sorokiniana. The strain, ORS 1 has been observed to grow under 95% O2/5% CO2 in the light or under the same gas phase in the dark on glucose (10, 16), in contrast to the (+) whose growth is rapidly impaired. These observations suggest that oxygen-resistance in ORS is not mediated by a light-dependent mechanism. Therefore, the general physiology and ultrastructure of ORS and (+) were compared under heterotrophic conditions because of the manipulative control which growth on a specific substrate (e.g., glucose) offers over growth via photosynthesis.

MATERIALS AND METHODS

Organisms. The (+) of Chlorella sorokiniana (Shihira and Krauss) was obtained from the University of Maryland Culture Collection through R. W. Krauss. ORS, partially described previously (10, 16, 23), has been maintained in this laboratory. Axenic stock cultures of both strains were kept at room temperature on agar slants containing 1.2% Tryptic Soy Broth (Difco).

Experimental Culture Conditions. Batch cultures were usually grown at 37 C with constant agitation in 2.5-liter flasks inside a darkened New Brunswick psychrometer incubator-shaker. Gases were bubbled continuously into the medium. Fresh medium was added by means of siphon tubing connected to a feed reservoir. For substrate screening experiments, batch cultures were grown in screw-cap culture tubes in a dark incubator at 37 C.

Culture Media. Modified Knops salt medium (formulated by J. Myers, Laboratory of Algal Physiology, University of Texas, Austin) supported good dark growth when supplemented with 0.3% (w/v) glucose. Glucose was routinely sterilized by autoclaving it in the MKM at 121 C for 15 min. Heat-sensitive compounds were filter-sterilized through 0.45 μ Millipore filters.

When cells were grown on 0.25% (w/v) sodium acetate in MKM, the pH rose faster than in glucose-grown cultures. In order to maintain culture pH between 6.5 and 7.0, 1 N HCl was added as necessary.

Gas Mixtures. Except for compressed air, all gases were supplied and analyzed by Big Three Industrial Gas and Equipment Co., Houston, Texas. All concentrations of gases are expressed in units of volume per volume per cent at 1 atm pressure.

Cell Counts and Cell Mass Measurements. Cell numbers were determined with a Spencer Brightline hemacytometer or a Coulter Model B particle counter with a 70 μ orifice. Cell mass was based on dry weight or absorbance measurements. For dry weight, samples were filtered through preweighed 0.45 μ Millipore filters, dried at 102 C for 12 hr, and tared. Absorbance of cell suspensions around 600 nm was read using an Evelyn photoelectric colorimeter.

Growth Rates. Cultures were diluted daily with fresh medium to an initial density of less than 0.01 mg/ml and allowed to grow to a density of 1 to 1.2 mg/ml. For determination of exponential growth rates, growth was followed over the time interval from 0.1 to 1.0 mg/ml. Thus the curves in Figures 2 and 3 represent only a portion of the total exponential growth occurring in a batch run. Growth rates were


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3 Abbreviations: ORS: oxygen-resistant strain; (+): wild type strain; MKM: modified Knops medium; GAPD: glyceraldehyde 3-phosphate dehydrogenase; poly-P: polyphosphate.
accepted only if they remained stable for at least two consecutive batch runs.

Growth rates were calculated as doublings per day (d), using the formula

\[ d = \frac{\log N_2 - \log N_1}{3.3(24, t)} \]

where \( N_1 \) and \( N_2 \) are values for cell mass or cell numbers at times 1 and 2, over the time interval, \( t \), in hours.

**Growth Yield.** Yield (= mg dry weight cells produced per mg glucose consumed) was computed by measuring glucose utilization and increase in cell mass simultaneously during log growth. Glucose in growth medium was assayed using the Glucostat reagent (Worthington Biochemical Corp.).

**Morphological Studies.** Fully dark-adapted, log-phase cells, growing on 0.3% glucose at 38.5 °C, were harvested in very dim light (< 10 ft-c), centrifuged, and washed in MKM prior to fixation.

Cells were fixed at room temperature for 2 hr in 6% (v/v) glutaraldehyde in 10 mM cacodylate buffer at pH 6.8, washed four times over a 1-hr period with 10 mM cacodylate buffer, and postfixed for 2 hr in 2% (w/v) OsO₄ in 10 mM cacodylate buffer (9). After washing in tap water, cells were dehydrated in a graded ethanol series, transferred to propylene oxide, and finally embedded in Epon 812. Thin sections, cut with a diamond knife, were stained with 2% (w/v) uranyl acetate for 15 min, poststained with lead citrate for 7 min, and photographed in an RCA EMU-3F electron microscope.

**Adaptation to Dark Growth.** The period of adaptation to dark, heterotrophic conditions was studied simply by transferring exponentially-growing autotrophic cells to glucose medium in the dark. Standard conditions for dark growth (37.5 °C and 1.7% CO₂ in air) were subsequently maintained. Light-grown cells were cultured as previously described (10). Adapting cultures were diluted with fresh glucose medium every 24 hr.

At various times during the dark exposure, samples were taken for analysis. Several parameters were monitored: growth rate, respiratory rate, and the enzymes, NAD- and NADP-linked glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12 and EC 1.2.1.13).

Growth rates were determined as doublings per day over 12 hr periods based on dry weight increase. GAPD was assayed in cell-free homogenates, prepared by grinding washed cells with 0.1 mm glass beads in a Bronwill MSK homogenizer at temperatures of 5 °C or lower. The homogenizing medium consisted of 0.05 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA and 0.1% (w/v) Triton X-100. The supernatant from a 30-min centrifugation at 32,000g was used for determination of both NAD- and NADP-linked GAPD by the spectrophotometric method of Schulman and Gibbs (19). The oxidative GAPD reaction was followed at 26 °C. A unit of GAPD corresponds to the amount of enzyme which oxidizes 1 μ mole of reduced pyridine nucleotide per hr.

**Respiration and Photosynthesis.** Oxygen consumption or evolution was followed at 37.5 °C with a Model 53 oxygen monitor (Yellow Springs Instrument Co.) calibrated against air level oxygen.

Glucose respiration was measured on samples of cell suspension straight out of the growth chamber. Cells were protected from light during all stages of manipulation by a black cloth.

Photosynthesis studies were done on heterotrophic cells harvested in log growth, centrifuged in the cold at 2000g for 10 min, and washed twice and suspended in cold MKM. During this time cells were kept in darkness or very dim light (< 10 ft-c). Oxygen evolution was measured upon illumination of a cell suspension of 1 × 10⁵ cells/ml in the oxygen electrode chamber with a GE tungsten photoflood lamp (500 w) providing saturating light intensity.

**RESULTS**

Heterotrophic culture conditions were optimized by testing various parameters such as temperature, gas conditions, and substrates for the proper combination promoting maximum logarithmic growth in the dark.

The optimal temperature was around 38 °C for both (+) and ORS (Fig. 1). Moreover, the temperature requirements did not differ between air and 100% O₂ as parallel curves for both atmospheres were constructed.

It appears that only hexoses and acetate are adequate carbon sources for heterotrophic growth of (+) and ORS (Table 1). Compounds which failed to support growth when tested at a concentration of 0.1% were: Bacto-Casitone, D-xylose, D-arabinose, sucrose, glyceral, D-glucosamine, L-glutamine, L-

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![Fig. 1. Effect of temperature on growth of C. sorokiniana, (+) and ORS strains, on 0.3% glucose in darkness. Air (= 1.7% CO₂ in air) or O₂ (= 100% O₂) was bubbled continuously into the cultures. Growth was measured as described in "Materials and Methods."](image-url)
proline, and pyruvic, citric, succinic, and α-keto-glutaric acids. There were no apparent substrate differences between the two strains, although ORS grew slower on glucose.

A check of CO₂ requirements showed no difference in growth rate for ORS on air alone or air fortified with 1.7% CO₂, whereas (+) was dependent on CO₂ in excess of air level for maximum growth. At high oxygen tensions, added CO₂ (e.g., 98% O₂/2% CO₂) slightly retarded growth of ORS, when compared to growth on 100% O₂.

The growth curves in Figure 2 depict the basic physiological differences between dark-, glucose-grown (+) and ORS, and also the quantitative, rather than qualitative, nature of oxygen resistance under these conditions. (+), growing at 7 doublings/day on air, is severely inhibited to less than 0.8 doublings/day by exposure to 100% O₂. Apparently, some (+) cells remain viable and adapt somewhat to the high oxygen environment after numerous transfers; but their growth rate under 100% O₂ remains lower than that for ORS. ORS, when transferred to 100% O₂, grows immediately at a rate of 2 to 3 doublings/day, a decrease of only about 50% from its air rate of 5 doublings/day. After 1 day under oxygen, ORS attains the stable exponential growth rate of 3 doublings/day.

In contrast to growth rates, qualitative metabolic differences between heterotrophic (+) and ORS are apparent. Only a transient drop in pH occurs in ORS cultures exposed to 100% O₂, while a drastic decrease is evident in (+) cultures (see inset, Fig. 2).

Growth yield data indicate that ORS converts glucose to cell mass more effectively under 100% O₂ than does the (+) strain. Yield under air is quite similar for both strains (average 0.500), whereas only ORS maintains this level of glucose incorporation under 100% O₂. Yield of (+) under high oxygen drops to about 0.240.

ORS does not manifest resistance to 100% O₂ if grown on acetate and thus responds identically to (+) (Fig. 3). Growth on acetate ceases completely for both strains within 16 hr after initial exposure to high oxygen. This characteristic of ORS contrasts with the response observed by Begin-Heick and Blum (1) for the chloric euglenoid, Astasia longa.

COMPARATIVE ULTRASTRUCTURE OF HETEROTROPHIC (+) AND ORS

Wild Type on Air. The electron micrographs of (+) on air represent typical dark-grown C. sorokiniana (Fig. 4). Organelles and fine structure common to chlorellas (2, 7, 14, 15, 17) and other eukaryotic cells are discernible.

Several well developed mitochondria are present in each cell, often in close association with the single chloroplast of the cell (Fig. 4C). They often appear elongated and "sausage-like," and possess prominent laminar cristae. The cup-shaped chloroplast is found in a partially dedifferentiated condition, characterized by very few internal membranes or thylakoids.

Electron opaque deposits, often granular in texture, occurring in the cytoplasm, are characteristic of (+) (Fig. 4, A and B). This dense material sometimes is in close proximity to mitochondria or the chloroplast. Other investigators have described similar looking material in Chlorella (14), Trebouxia (4), and Myxococcus (22) and identified it as insoluble polyphosphate deposits.

A rather nondescript inclusion, evident in Figure 4, A and C, bears a strong resemblance to peroxisomes in higher plants (5), and to the microbodies recently identified in several green algae (7).

ORS on Air. ORS on air presents several outstanding differences (Fig. 5). ORS cells generally are more variable in size and shape than (+). The osmiophilic (possibly poly-P) deposits, so abundant in the cytoplasm of (+), are conspicuously lacking in ORS. In many cells, the chloroplast shows a more compact, spindle or disc shape (Fig. 5, A, E, and F), and thylakoids retain a more characteristic stacked appearance. Starch grains often fill much of the chloroplast (Fig. 5B).

Mitochondria present a less expanded configuration than in (+), with the intracristal space appearing less extensive (Fig. 5, C, D, and E).

A major difference between (+) and ORS pertains to cell division. ORS routinely demonstrated a type of division figure almost never seen in (+); binary division in which one cell (Fig. 5F) gives rise to two daughter cells which remain joined by a common portion of the cell wall. The resulting "dumb-
FIG. 4. Electron micrographs of (+) type C. sorokiniana grown on 0.3% glucose in darkness under air + 1.7% CO₂. A: Structure visible: nucleus (N), nucleolus (nu), chloroplast (C), polyphosphate deposit (PP), vacuole (V), microbody (mb), starch (S) in chloroplast, and cell wall (cw). × 36,700. B: Note variety of mitochondria (m) and their proximity to chloroplast and polyphosphate. × 30,000. C: A polyphosphate granule and mitochondria appear within a “pocket” of cytoplasm in the chloroplast. × 27,500. D: Note relationship of microbody, mitochondria, and chloroplast. A dictyosome (d) is visible, and chloroplast appears in two parts. × 27,500.
FIG. 5. Electron micrographs of ORS strain *C. sorokiniana* grown on 0.3% glucose in darkness under air + 1.7% CO₂. A: Note irregular shape of cell. × 27,500. B: Abundant starch is frequently visible in the chloroplast. × 33,000. C, D, and E: Note variability in mitochondrial morphology, and close apposition of mitochondria to chloroplast. × 27,500. F and G: Two stages in ORS “binary” division cycle. F: Nucleus and chloroplast appear to have divided and separated to opposite poles of the cell. Slight indentation of the cell wall (arrow) occurs early in division. × 24,700. G: “Dumb-bell” cell, characteristic of very late stage in ORS division. Common portion of cell wall is shared by two separate cells (arrows). × 27,500.
FIG. 6. A and B: Electron micrographs of heterotrophic (+) type exposed to 100% oxygen for 72 hr. A: Note apparent increase in dictyosomes (d). X 30,000. B: Note mitochondria, starch and polyphosphate deposits under high O₂. X 24,700. C and D: Electron micrographs of ORS grown on 0.3% glucose in darkness under 100% oxygen. C: General structural dedifferentiation is evident. Chloroplast appears diffuse, cytoplasmic vesicles (CV) are abundant, and mitochondria (m) are poorly developed. X 30,400. D: Note quantity of starch in the chloroplast and prominent endoplasmic reticulum (er) around the cell periphery. X 27,500.

HETEROTROPHIC GROWTH OF CHLORELLA

343

bell” cells (Fig. 5G) comprised roughly 5 to 10% of ORS cultures at all times. It seems likely that this phenomenon represents incomplete autospore separation.

Wild Type on 100% Oxygen. Figure 6B represents a typical intact cell of (+) exposed to 100% O₂ for about 72 hr. Mitochondria, dictyosomes, and amorphous osmiophilic deposits apparently increase in numbers.

ORS on 100% Oxygen. These cells (Fig. 6, C, and D) differ vastly from their air-grown counterparts. Striking changes in morphology reflect an apparent shift in metabolism. Deposits of reserve or storage material (e.g., starch, cytoplasmic vesicles of medium density) are greatly increased.

Around the edges of the cell, a large quantity of endoplasmic reticulum, both smooth and rough, is prominent (Fig. 6D). In cross section, the latter, studded with ribosomes, forms a network of cisternae, which may contain visible material.

Mitochondria show a pronounced change. Small rudimentary mitochondria (consisting primarily of matrix and very few cristae) are discernible, especially around the cell periphery (Fig. 6C). They resemble the repressed, inactive mitochondria of yeasts grown on high concentrations of fermentable sugars (11).

Kinetics of Adaptation to Dark Growth. It became apparent early in the course of studies that ORS required a much longer period to completely adapt to dark conditions. In view of the findings of previous workers (2, 6, 8, 13), documenting fundamental changes in photosynthetic organisms introduced into the dark, the adaptation process appeared significant for characterization of ORS.

Figure 7 presents the adaptation of (+) and ORS to dark conditions based on growth rate. By the 2nd day, (+) reaches maximum log growth. In contrast, ORS grows at a constant rate of less than 3 doublings/day until day 6, when its growth rate starts to increase. By day 8, ORS has attained its maximum dark-growth rate. Such a long lag period in response to glucose in darkness could be characteristic of an induction or derepression phenomenon.

During dark adaptation, respiratory rates changed as shown in Figure 7. The (+) demonstrated a rapid 200% increase in O₂ uptake over the light level as would be expected for an increase in mitochondrial metabolism. Although O₂ uptake by ORS increased only moderately (50%) over a 5-day period, ORS was previously respiring vigorously in the light, somewhat more than (+). Evidently, heterotrophic conditions exert very stringent metabolic control on ORS respiratory pathways, a point of major difference between (+) and ORS.

Separate functions are ascribed to NADP- and NAD-linked GAPD (6, 19). The NADP enzyme, localized in the chloroplast, participates in the photosynthetic carbon cycle. The NAD specific enzyme is a component of the glycolytic pathway found in the cytoplasm. Several workers (6, 13) have documented relative changes in the levels of these two enzymes in Chlamydomonas, finding that in general they show opposite responses to light and heterotrophic conditions.

NADP-GAPD behaved as would be predicted, decreasing in darkness to a constant level (Fig. 8). While (+) enzyme content fell to about 50% of the light level, ORS activity dropped dramatically by 80%. This dark-grown level of chloroplastic GAPD is, however, sufficient to sustain photosynthesis, as both heterotrophic ORS and (+) photosynthesized immediately out of the dark, at approximately 10% of the rate of light-grown cells (dark-grown rates, 6.6 and 3.2 μl O₂/hr·10⁶ cells for ORS and (+), respectively). These data suggest that (+) and ORS chloroplastic metabolism is similarly effected by dark heterotrophic conditions.

ORS contains a high level of the cytoplasmic enzyme. NAD-GAPD, even in the light (Fig. 8). Conversely, (+) has a very low level in the light (only 33% of the ORS level), but rapidly forms it upon adaptation to darkness. Both strains contain about the same amount of the enzyme in the dark. This suggests that ORS actively glycolyzes in the light, indeed close to its maximum capability. It is significant, too, that NAD-GAPD activity in heterotrophic (+) under high O₂ is severely depressed (7 μmol/hr·10⁶ cells), while the level in corresponding ORS (16 μmol/hr·10⁶ cells) remains comparable to the air-grown level. The constant level of this glycolytic enzyme in ORS (light or dark, air or high oxygen) could indicate an
apparent dependence on cytoplasmic metabolism for ORS, and perhaps reflects a lack of normal feedback control between cytoplasmic and chloroplasmic metabolism.

**DISCUSSION**

The selective dark-growth requirements of both (+) and ORS confirm that *C. sorokiniana*, like many chlorellas, possesses a minimal repertoire of heterotrophic pathways, either biosynthetic and/or energy-producing (20, 24). Failure of amino acids (Bacto-Casitone) and organic acids to support growth may not reflect a permeability problem, but rather metabolic disturbance (12), since growth on glucose plus amino acids was higher than on glucose alone.

Comparison of ORS and (+) reveals that the two strains differ appreciably in metabolic control over oxidative portions of carbohydrate pathways, specifically those portions from glucose to acetate. This is based on (a) the fact that high oxygen has a selectively different action on (+) and ORS growth on glucose only when glucose catabolism is involved and (b) the adaptation kinetics of NADP-GAPD for ORS. Evidently, cytoplasmic metabolism in ORS is a major buffer against inhibition by high oxygen.

The ultrastructure and NADP-GAPD adaptation experiments for (+) and ORS are notable exceptions to the reports for *C. pyrenoidosa* (van Niel's strain) by Budd et al. (2), who showed that the chloroplast regressed to the proplastid stage under heterotrophic conditions, and Devlin and Galloway (3), who concluded that there was more NADP-GAPD in heterotrophic than autotrophic cells. These variations between species of *Chlorella* merely reflect the species specificity involved in the heterotrophic behavior of photosynthetic organisms transferred into the dark.

ORS is easily recognized on both the structural and physiological levels. Certainly, under 100% O₂ ORS shows independence from the same control of cell metabolism by O₂ seen in (+). At the same time, even on air level O₂, mutant properties are manifested. Based on the lag in dark growth and the low respiratory rate under heterotrophic conditions, it appears that a fundamental difference between ORS and (+) is directly related to control of dark oxidative and energy metabolism. This alteration in control would not seem to affect ORS photosynthetic light reactions in the chloroplast, in particular oxygen evolution, which continues at a rate similar to that of (+). Therefore, aspects of ORS energy metabolism should be examined in detail. Moreover, the absence of osmophilic deposits in ORS makes imperative investigation of cell composition, especially phosphorus and lipid content.

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**LITERATURE CITED**