Desiccation tolerance of Botryococcus braunii (Trebouxiophyceae, Chlorophyta) and extreme temperature tolerance of dehydrated cells

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Desiccation tolerance of *Botryococcus braunii* (Trebouxiophyceae, Chlorophyta) and extreme temperature tolerance of dehydrated cells

Mikihide Demura · Motohide Ioki · Masanobu Kawachi · Nobuyoshi Nakajima · Makoto M. Watanabe

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Abstract *Botryococcus braunii* Kützing, a green colonial microalga, occurs worldwide in both freshwater and brackish water environments. Despite considerable attention to *B. braunii* as a potential source of renewable fuel, many ecophysiological properties of this alga remain unknown. Here, we examined the desiccation and temperature tolerances of *B. braunii* using two newly isolated strains BOD-NG17 and BOD-GJ2. Both strains survived through 6- and 8-month desiccation treatments but not through a 12-month treatment. Interestingly, the desiccation-treated cells of *B. braunii* gained tolerance to extreme temperature shifts, i.e., high temperature (40 °C) and freezing (−20 °C). Both strains survived for at least 4 and 10 days at 40 and −20 °C, respectively, while the untreated cells barely survived at these temperatures. These traits would enable long-distance dispersal of *B. braunii* cells and may account for the worldwide distribution of this algal species. Extracellular substances such as polysaccharides and hydrocarbons seem to confer the desiccation tolerance.

Keywords Algal dispersal · *Botryococcus braunii* · Desiccation tolerance · Polysaccharide · Temperature tolerance

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M. Demura · M. Ioki · M. Kawachi · N. Nakajima · M. M. Watanabe
Graduate School of Life and Environmental Sciences, University of Tsukuba, Tennoudai 1-1-1, Tsukuba, Ibaraki 305-8577, Japan

M. Demura (✉) · M. Ioki · M. Kawachi · N. Nakajima
National Institute for Environmental Studies, Onogawa 16-2, Tsukuba, Ibaraki 305-8506, Japan
e-mail: demura.mikihide@nies.go.jp

Introduction

Botryococcus braunii Kützing (Trebouxiophyceae, Chlorophyta), a green colonial microalga, is a cosmopolitan species that occurs worldwide in both freshwater and brackish water environments and robustly produces hydrocarbons (Banerjee et al. 2002). The hydrocarbon content of *B. braunii* colonies is much higher than that of any other oil-producing microalga and can reach up to 75 % of the dry weight (Chisti 2007). Therefore, *B. braunii* has received considerable attention as a potential source of renewable fuels. Recent studies have drastically advanced our understanding of the biochemical, molecular biological, and applied aspects of this organism (Baba et al. 2012; Demura et al. 2012; Ioki et al. 2012a, b, c, d; Magota et al. 2012; Matsushima et al. 2012; Molnar et al. 2012; Niehaus et al. 2011; Ranga Rao et al. 2012; Shiho et al. 2012; Xu et al. 2011, 2012; Yonezawa et al. 2012). However, several basic ecophysiological properties of this alga related to its worldwide occurrence still remain unknown.

The desiccation tolerance of microalgae has been previously reported in terrestrial cyanobacteria (Cameron 1962; Dodds et al. 1995; Sakamoto et al. 2009), desert green algae (Gray et al. 2007), phototrophic biofilms algae (Häubner et al. 2006; Rindi 2007; Gustavs et al. 2010), and alpine soil algae (Karsten et al. 2010; Holzinger et al. 2011). Desert biological soil crusts contain many unicellular green algae belonging to three major classes, i.e., Chlorophyceae, Trebouxiophyceae, and Charophyceae (Lewis and Flechtner 2002). Gustavs et al. (2010) found five trebouxiophytes in aeroterrestrial biofilms. Trebouxiophycean algae have also been detected in the air as "aeroalgae" (Handa et al. 2007). Because most of these algae are distributed worldwide, desiccation tolerance appears to be a key ecophysiological trait pertaining to the dispersal of microalgal species on a global scale.

B. braunii is widely distributed in the freshwater environments of Europe, Africa, Asia, Australia, North America, and South America (Aaronson et al. 1983). In Japan, *B. braunii* is found over a wide geographical range across the Japanese islands (unpublished data). Phylogenetic analysis of 31 *B. braunii* strains collected from various localities in Japan failed to detect any reliable correlation between phylogeny and localities, suggesting that *B. braunii* is dispersed between ponds, presumably by wind or birds (Kawachi et al. 2012). However, desiccation tolerance of *B. braunii* is still unknown. In this study, we demonstrate that *B. braunii* cells could survive dehydrating conditions for over 6 months and that desiccation-treated cells gained tolerance to extreme temperatures.

Materials and methods

Botryococcus braunii colonies were isolated from freshwater bodies in the Okinawa Prefecture of Japan using a micropipette. Two strains, BOD-NG17 and BOD-GJ2, were established in a unialgal culture and a clonal state. The 18S rDNA sequences of these strains, which we used for species confirmation, were deposited in the GenBank/EMBL/DDBJ database with following accession numbers: AB758446 and AB758447 for BOD-NG17 and BOD-GJ2, respectively. These strains were maintained in test tubes containing AF-6 medium (Kasai et al. 2004) at 22 °C under a 12-h light/12-h dark cycle with white fluorescent illumination (approximately 100 μmol photons m^{-2} s^{-1}).

Desiccation treatment *B. braunii* cultures (500 μL) were transferred to a 1.5-mL plastic tube. Each tube contained ca. 2,500 colonies for both strains. The tube was placed with its lid open in the growth chamber at 22 °C. The culture medium evaporated within 2 weeks. The cell viability after 6-, 8-, and 12-month desiccation was examined using a growth test.

Histochemical staining of polysaccharides To stain polysaccharides, 3 μL of a crystal violet solution (10 mg mL^{-1} in methanol) was added to a 100-μL stationary-phase culture (Tanoi et al. 2013). The stained cells were photographed using a microscope equipped with a digital camera.

High temperature and freezing treatment After 2 weeks of desiccation, the tubes containing the desiccation-treated or untreated (control) cultures (500 μL containing ca. 2,500 colonies) were incubated at 40 or −20 °C in the dark for 1, 4, 10, and 20 days. Cell viability was then examined using the growth test.

The growth test To examine cell survival after desiccation treatment with or without subsequent exposure to an extreme temperature, growth tests were performed using three independent tubes for each measurement. Cells in each tube were suspended in 1 mL AF-6 medium, and 30-μL aliquots containing ca. 75 colonies each were then dispensed into 32 wells of a 96-well plastic plate containing 100 μL each of AF-6 medium. After incubation for 1 month at 22 °C under a 12-h light/12-h dark cycle with white fluorescent illumination (approximately 100 μmol photons m^{-2} s^{-1}), each well was observed for *B. braunii* growth under an inverted light microscope. Wells showing more than a fivefold increase in the colony number were counted as "wells with viable *B. braunii*" and their percentage was calculated for each tube. This method allowed us to compare culture viability between the treatments because it was otherwise difficult to estimate the cell viability due to the tightly packed colonies of *B. braunii*.

Results

Desiccation tolerance Both BOD-NG17 and BOD-GJ2 remained viable under dehydrating conditions for at least 8 months. After 6 months of desiccation, the percentages of wells with growing *B. braunii* cells reached 100 % in the growth tests for both strains. After 8 months of desiccation, the percentages decreased to 13.5±11.8 % (n=3) and 3.1±2.1 % (n=3) for BOD-NG17 and BOD-GJ2, respectively. For both strains, no growth of the rehydrated cells was detected after 12 months of desiccation (Fig. 1).

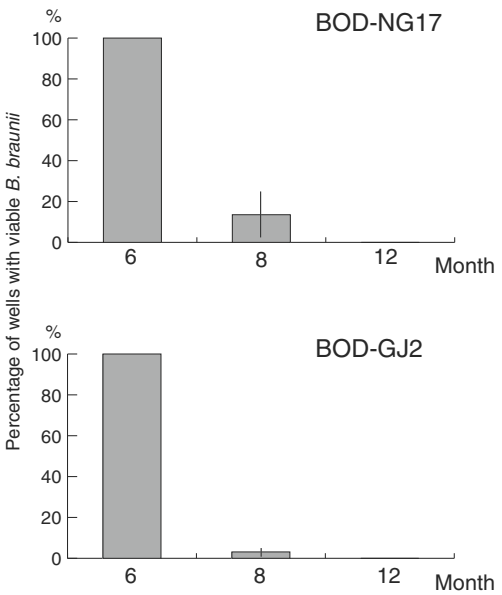

Fig. 1 Tolerance of *B. braunii* to long-term desiccation. *Bar* = S.D. (n=3)

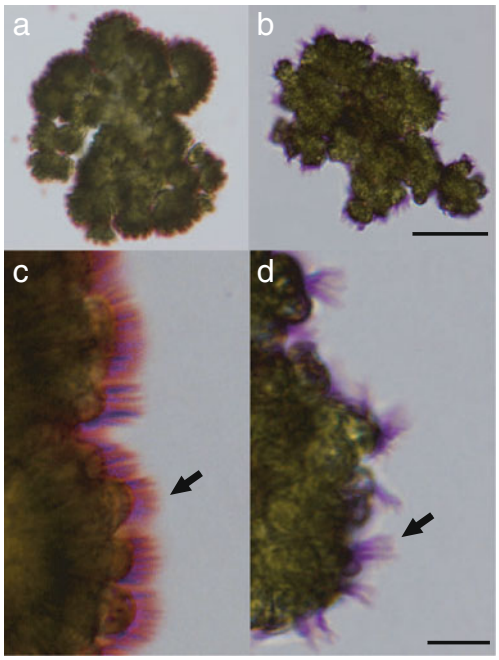

Fig. 2 Visualization of extracellular polysaccharide arrays by crystal violet staining. Polysaccharide arrays surrounding the colonies appear purple. Whole colonies (**a**, **b**) and enlarged views (**c**, **d**) are shown for BOD-NG17 (**a**, **c**) and BOD-GJ2 (**b**, **d**). *Bars* indicate 50 and 10 μm in the upper and lower micrographs, respectively

Extracellular polysaccharide arrays Histochemical staining of polysaccharides in BOD-NG17 and BOD-GJ2 revealed that the cells of both strains are surrounded by radially oriented arrays of polysaccharides. BOD-GJ2 exhibited sparser arrays than BOD-NG17 (Fig. 2).

Acquired tolerance of desiccation-treated cells to high temperature and freezing Extreme temperatures damage *B. braunii* cells. Incubation of nondesiccation-treated BOD-NG17 or BOD-GJ2 cells at 40 °C for only 1 day completely inhibited growth. Incubation of BOD-NG17 and BOD-GJ2 at −20 °C for 10 and 4 days, respectively, resulted in no growth recovery after thawing. However, *B. braunii* desiccation-treated cells survived under these extreme temperatures. Desiccation-treated cultures of both strains remained viable at 40 °C for at least 4 days. After incubation at 40 °C for 1 day, the percentages of wells with growing *B. braunii* cells reached 100 and 47.9±21.5 % (n=3) in the growth tests of desiccation-treated BOD-NG17 and BOD-GJ2, respectively. The percentages decreased to 21.9±8.3 % (n=3) and 8.3±2.8 % (n=3) after incubation of BOD-NG17 and BOD-GJ2, respectively, at 40 °C for 4 days. For both strains, no growth was detected after incubation at 40 °C for 10 and 20 days. At −20 °C, desiccation-treated cultures of both strains survived for at least 10 days but no longer than 20 days. For BOD-NG17, the percentage of wells with growing *B. braunii* cells in the growth tests were 100 %, 100 %, 28.3±6.3 % (n=3), and 0 % following 1-, 4-, 10-, and 20-day incubation, respectively. For BOD-GJ2, the percentages were 58.3±38.9 % (n=3), 6.3±2.1 % (n=3), 7.3±3.5 % (n=3), and 0 % following 1-, 4-, 10-, and 20-day incubation (Fig. 3).

Discussion

In this study, we determined the culture viability, rather than the cell viability, after desiccation and extreme temperature treatments because the tendency of *B. braunii* to form tightly packed colonies made estimation of the cell viability

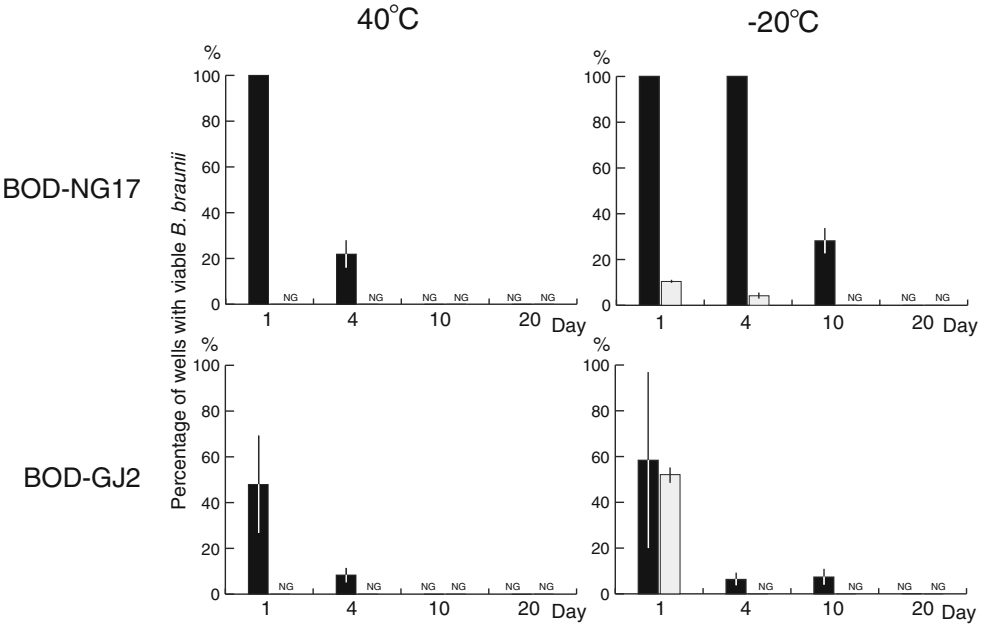

Fig. 3 Increased tolerance of desiccation-treated cells of *B. braunii* to high temperature and freezing. *Black and gray columns* indicate desiccation-treated and untreated cells, respectively. *NG* indicates no detectable growth. *Bar* = S.D. (n=3)

difficult. Effects of different treatments were successfully evaluated using a growth test that determined the culture viability based on the percentage of wells containing growing *B. braunii*. Both BOD-NG17 and BOD-GJ2 exhibited tolerance to desiccation for over 6 months. This is the first report on desiccation tolerance of *B. braunii*.

The extensive extracellular matrices of *B. braunii* colonies seemed to be associated with the desiccation tolerance. In the present study, histochemical staining revealed that *B. braunii* colonies are surrounded by radial arrays of polysaccharides. Extracellular polysaccharides protect algal cells against desiccation by preventing cellular water loss (Cameron 1962; Clegg 2001; Oren 2007; Sakamoto et al. 2009; Tamaru et al. 2005). The radial arrays of polysaccharides were more abundant in BOD-NG17 than in BOD-GJ2. Robustness of the polysaccharide arrays may be associated with relatively high desiccation tolerance of BOD-NG17. In addition, the extracellular matrix also retains a large quantity of liquid hydrocarbons and a network of cross-linked hydrocarbons, and each *B. braunii* cell is surrounded by a cup-shaped "retaining wall" outside its cell wall (Weiss et al. 2012; Wolf 1983). The retaining walls and hydrocarbons may guard the *B. braunii* cell from various stresses.

Cellular events associated with the desiccation tolerance of *B. braunii* are presently ambiguous. Speculations include (a) involvement of sugar alcohols (polyols) like sorbitol and ribitol as in the case of aeroterrestrial trebouxiophytes such as *Stichococcus* sp., *Coccomyxa* sp., *Chlorella* spp., and *Apatococcus lobatus* (Chodat) J.B. Petersen (Gustavs et al. 2010). (b) Various saccharides, such as arabinose, galactose, fucose, glucose, mannose, and deoxyhexoses, have been detected from *B. braunii* (Banerjee et al. 2002; Metzger et al. 1990; Weiss et al. 2012) and they also might be involved in anhydrobiosis. In *Nostoc commune Vaucher*, physiological activities including trehalose accumulation change under desiccation (Fukuda et al. 2008; Tamaru et al. 2005; Sakamoto et al. 2009; Reina-Bueno et al. 2012). (c) Flexibility of the cell walls is critical for the desiccation tolerance of an aeroterrestrial green alga *Klebsormidium crenulatum* (Kütz.) Lokhorst (Holzinger et al. 2011) and similar mechanisms may be in action in *B. braunii*. Physical characteristics of *B. braunii* cell walls need to be clarified.

The dehydrated cells of both BOD-NG17 and BOD-GJ2 gained tolerance to high (40 °C) and freezing (−20 °C) temperatures. However, the underlying mechanisms remain unclear. It is possible that polyols are involved because they act not only as osmolytes, but also as antioxidants, heat protectants, and rapidly available respiratory substrates (Gustavs et al. 2010).

In conclusion, *B. braunii* is tolerant to desiccation and the dehydrated cells gain tolerance to extreme temperatures. The tolerance of *B. braunii* to desiccation and extreme temperatures, which algal cells often encounter when carried by birds or wind, enables its dispersal on a global scale.

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