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Reduced expression of the rate-limiting carbon fixation enzyme RuBisCO in the benthic foraminifer *Baculogypsina sphaerulata* holobiont in response to heat shock

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ABSTRACT

Baculogypsina sphaerulata (Parker and Jones, 1860) is a common large benthic foraminifer (LBF) and is an important calcifier in coral reef ecosystems. As there are concerns that global increasing temperatures may compromise the survival of this species, which forms a symbiotic relationship with the diatom *Nitzschia* sp., we investigated the response of the *B. sphaerulata* holobiont from the intertidal algal flats of Xiao Liu Chiu Island, Taiwan to heat shock. *B. sphaerulata* specimens were incubated at 26 (ambient), 28, 30, 32, or 34 °C for 5 h designed to simulate short pulses of elevated temperature that occur in situ from subaerial exposure at low tide. To assess the molecular-level response, we measured the expression of the ribulose 1-5-bisphosphate carboxylase/oxygenase (RuBisCO) protein in the diatom symbiont. There was a significant decrease in expression of this rate-limiting carbon fixation enzyme in *B. sphaerulata* holobionts incubated at 34 °C/8 °C above ambient (~50% decline relative to controls), but expression was resilient to levels of warming up to 6 °C above ambient. This suggests that exposure to high temperatures occasionally experienced in nature may diminish the capacity for carbon fixation of the diatom symbiont. Given the importance of photosynthesis and carbon fixation in these marine calcifiers, these data suggest that climate-driven ocean warming may exert deleterious effects on the *B. sphaerulata* holobiont.

1. Introduction

Symbiotic associations between marine animals and algae are common in the phyla Cnidaria, Mollusca, and Foraminifera, many of which reside in shallow, tropical waters (Trench, 1993). Recently, there have been major concerns as to how calcifying organisms with symbiotic algal associations, in particular corals and Foraminifera, will fare in a warming and acidifying ocean (Hoegh-Guldberg, 1999; Sinutok et al., 2011; Uthicke et al., 2012). In corals, increased temperature can compromise dinoflagellate (genus *Symbiodinium*) photosynthesis (e.g. Warner et al., 1999), leading to increases in reactive oxygen species production (Lesser, 1997), and, in severe cases, symbiotic disintegration (i.e., bleaching) and host mortality (Gates, 1990; Hoegh-Guldberg, 1999). Acute (<48 h) thermal stress decreases photosynthetic efficiency and expression of the photosystem II protein D1 (Jones et al., 2000; Warner et al., 1999). Although photoinhibition due to thermal stress is well documented in corals (reviewed in Smith et al., 2005), little is known about how other marine symbioses, such as the foraminiferan-algae association, will respond to increased temperature in the context of climate change.

Large benthic Foraminifera (LBF) are major contributors to calcium carbonate production in coral reef ecosystems and perform a crucial role in buffering against changes in ocean chemistry (Hohenegger, 2006; Langer et al., 1997; Scoffin and Tudhope, 1985). They host a variety of algal symbionts that are deleteriously affected by prolonged exposure to increased temperature, as evidenced by decreased photosynthetic efficiency and calcification (Hallock, 1981; Lee, 2006; Schmidt et al., 2011; Sinutok et al., 2011). For the diatom-hosting species, *Amphistegina radiata*, *Heterostegina depressa* and *Calcarina hispida*, incubation at 8 °C above ambient for 30 days led to decreased photosynthetic efficiency and growth, resulting in bleaching (Schmidt et al., 2011). Similar experiments with *Marginopora vertebralis*, a *Symbiodinium*-bearing species found that incubation at +5 °C above ambient for 7 days resulted in significant morality (Uthicke et al., 2012), as well as decreases in calcification. These changes were suggested to be due to a decrease in physiological performance of the dinoflagellate symbionts (Sinutok et al., 2011).

While previous long-term elevated temperature studies have enhanced our understanding of the foraminiferal response to environmental changes, no studies have been conducted on their response

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to the acute increases in temperature that they may encounter in the field at low tide from subaerial or near subaerial exposure. Furthermore, only one other study to date has used molecular tools to gauge the sub-cellular response of these critically important, and potentially threatened, calcifiers (Heinz et al., 2012). To address these knowledge gaps, we investigated the heat shock response of the *Baculogypsina sphaerulata* holobiont from the intertidal algal flats of Xiao Liu Chiu Island, Taiwan. Samples were exposed for 5 h to temperatures aimed to simulate pulses of warming experienced during summer day-time low tides in their intertidal environment; 26 (ambient), 28, 30, 32, and 34 °C. The upper level of warming (34 °C) potentially represents a "tipping point" that results in photosystem degradation, as observed in other foraminiferan species exposed to long-term (weeks) increases in temperature (Schmidt et al., 2011).

The physiological response of the *B. sphaerulata* holobiont was documented by measuring protein expression of ribulose 1-5-bisphosphate carboxylase/oxygenase (RuBisCO), a highly conserved, rate-limiting Calvin cycle (carbon fixation) enzyme in the diatom symbionts *Nitzschia* sp. (Lee and Correia, 2005) that are commonly associated with this foraminifer host. Given the well-documented photoinhibition and decreased carbon fixation in both corals and Foraminifera exposed to elevated temperature (Schmidt et al., 2011; Warner et al., 1999), we hypothesized that heat shock treatments would lead to a decrease in expression of RuBisCO. Previous studies of heat shock response in marine species along a thermal gradient have found higher thermotolerance of intertidal species compared to subtidal, although many intertidal species may be living near their maximum thermal tolerance (Somero, 2010). Therefore, by investigating the thermal biology of an intertidal foraminifer holobiont already adapted to life with significant temperature fluctuations, we provide insights into how these organisms may respond to future climate-driven ocean warming.

2. Methods

2.1. Field collection and heat shock experiment

Specimens of *B. sphaerulata* were collected from the intertidal algal flat during low tide in an exposed cove on the coral reef island Xiao Liu Chiu (22° 21′ 3″N, 120° 23′ 17″E), off the southwest coast of Taiwan (Fig. 1A–B). In the field, *B. sphaerulata*, which typically attach firmly to algal substrates, were located on macroalgae on rocky reef flats that were regularly or nearly exposed during spring tides. To characterize the thermal regime of the field site, a HOBO® Pendant (Onset, Pocasset, MA, USA) temperature logger was deployed nearby (22° 20′ 53.4″N, 120° 23′ 26.6″E) at 8 m depth for two months (June–July 2011) around our sampling date with measurements every 6 min. In addition, because this species typically has minimal water cover during summer low tides, air temperature data were also collected from the Central Weather Bureau (22° 19′ 55.4″N, 120° 21′ 44.0″E) (Fig. 1C–E).

Specimens were collected in July 2011 and transported to the National Museum of Marine Biology and Aquarium (NMMBA) in Pingtung County, Taiwan. Samples were placed in aquaria set to ambient (26 °C) temperature for two days prior to use in the experiments. Thirty *B. sphaerulata* were placed haphazardly into 20 ml glass scintillation vials with 8 replicates per treatment. The vials were sealed to prevent evaporation and immersed in ~20 l temperature-controlled water baths with recirculating water set at either 26 (control), 28, 30, 32, or 34 °C for 5 h. Temperature was measured with a digital thermometer (model 15-077-8, Fisher Scientific, Pittsburg, PA, USA), and photosynthetically active radiation (PAR) was measured with a Li-Cor meter (LI-1400, Li-Cor Biosciences, Lincoln, NE, USA). After the treatment exposure for 5 h, the specimens were quickly transferred into 1.5 ml microcentrifuge tubes, frozen in liquid nitrogen and stored at –80 °C until protein extraction.

2.2. Protein extraction, SDS-PAGE, and western blotting

Soluble protein was extracted from all 30 individuals in each of the 40 samples with 60 μl RIPA buffer (50 mM Tris–HCl pH [7.4], 1% Nonidet-P40, 0.25% Na-deoxycholate, 150 mM NaCl, and 1× complete protease inhibitor cocktail, [Roche, Basel, Switzerland]). All 30 *B. sphaerulata* in each sample were mechanically ground with metal tweezers and frozen at −20 °C for 12 h. They were then centrifuged for 5 min at 12,000×*g*, and the supernatant with the total solubilized protein was transferred to a new microcentrifuge tube. Protein content was quantified using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Soluble protein (5 μg) was dissolved in 1× Laemmli sample buffer (Laemmli, 1970), boiled for 5 min at 95 °C, and spun at 12,000×*g* for 10 min at 4 °C to pellet insoluble material. Forty microliters of the supernatant were loaded into SDS-PAGE gels, which were electrophoresed on ice at 70 V for 30 min followed by 120 V for 1 h through the 5% stacking and 12% separating gels, respectively. Each gel was also loaded with protein extracted from a stock homogenate of *B. sphaerulata* incubated at 26 °C as a positive control to normalize samples across gels.

Following electrophoresis, proteins were transferred to PVDF membranes at 4 °C at 100 V for 90 min in transfer buffer (25 mM Tris–HCl, pH [6.8], 192 mM glycine and 20% methanol). To determine efficacy of transfer, SDS-PAGE gels were stained with SYPRO® Ruby (Invitrogen) after transfer according to the manufacturer's recommendations and visualized on a Typhoon Trio™ Variable Mode Imager (Amersham Biosciences, Little Chalfont, United Kingdom) at 532 nm. In certain cases, the PVDF membranes were stained with Ponceau S (Sigma, St. Louis, MO, USA) according to the manufacturer's recommendations to further visualize degree of protein transfer.

Membranes were blocked in 5% skim milk (w/v) in Tris-buffered saline with Tween-20 (TBST, 100 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20) for 1 h at room temperature (RT). The blocking buffer was decanted, and 10 ml of a 1:2000 dilution of a RuBisCO large subunit (RBCL) primary antibody (forms I and II, Agrisera, Vännas, Sweden) in 5% skim milk (w/v) in TBST was added to the membranes, which were then incubated for 2 h with gentle agitation at RT. Samples were washed 3 times (10 min each) with TBST and then incubated with a 1:5000 dilution of goat anti-rabbit secondary antibody (Millipore, Billerica, MA, USA) for 7 min and washed with TBST as above. Samples were then stained with SuperSignal® West Pico Chemiluminescent Substrate Kit chemiluminescent reagent (Pierce, 34082 Amersham Biosciences), and the chemiluminescent signal immediately visualized on a Fusion FX7 (Vilber Lourmat, Marne-la-Vallée, France).

2.3. Image and statistical analysis

Densitometry measurements were made with ImageJ (NIH), and the data were normalized to the intensity of the positive control RuBisCO band from the same gel to compare expression across blots, as five gels/blots were required to process all 40 samples. Data were analyzed by 1-way analysis of variance (ANOVA) with temperature as the fixed factor after a square root-transformation to meet assumptions of ANOVA (normality and homoscedasticity). Post-hoc analysis was performed using Tukey's Honestly Significant Difference (HSD) tests. A visual inspection of residuals indicated five outliers, which were removed from further analysis. All statistical analyses were performed with JMP® (version 9, Cary, NC, USA).

3. Results

3.1. Field and experimental conditions

Mean seawater temperature values between June and August 2011 were 26.8 °C, with a 2.0 °C mean daily variation around the collection time (range: 24.5–29.8 °C) (Fig. 1C). Mean air temperature was 28.0 °C,