Determination of the Thermal Tolerance of *Symbiodinium* Using the Activation Energy for Inhibiting Photosystem II Activity

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Jih-Terng Wang, Pei-Jie Meng, Yi-Yun Chen, and Chaolun Allen Chen (2012) Determination of the thermal tolerance of *Symbiodinium* using the activation energy for inhibiting photosystem II activity. *Zoological Studies* **51**(2): 137-142. Holobionts with different *Symbiodinium* clades or subclades display varying levels of thermal tolerance; however, an index to quantify and standardize this difference has not yet been formulated. In this study, the potential for the activation energy (Ea) to inhibit photosystem (PS)II being used to represent the heat tolerance of *Symbiodinium* was investigated. As the Ea required for PSII heat denaturation increased, the PSII apparatus in the algae remained stable at higher temperatures; thus, PSII activity was maintained at higher temperatures. The Ea was determined by fitting the kinetics data of the decrease in the maximum quantum yield (F_v/F_m) of freshly isolated *Symbiodinium* (FIS) at an elevated temperature to the Arrhenius equation. The results indicated that the PSII activity of FIS linearly decreased with an increase in the incubation time under thermal stress ($r^2 > 0.95$), and the rate of PSII denaturation significantly fit the Arrhenius equation ($r^2 > 0.95$) after a logarithmic transformation. Comparisons between 5 *Symbiodinium* subclades indicated that D1a, known as the most heat-tolerant subclade, showed the highest Ea value (348 ± 16 kJ/mole), which was significantly ($p < 0.05$) higher than those of B1, C1, C3, and C15 (126-262 kJ/mole). The reliability of the Ea calculation was confirmed by the low coefficient of variation (< 10%), suggesting that it can reliably be used to quantify the thermal tolerance of *Symbiodinium*. http://zoolstud.sinica.edu.tw/Journals/51.2/137.pdf

Key words: Coral bleaching, Activation energy, PSII activity, *Symbiodinium*.

S*ymbiodinium* algae, the dinoflagellates mostly found in symbiosis with corals and sea anemones, are widely considered to underpin the ecological success of cnidarian-alga symbioses in shallow, nutrient-poor waters (Muscatine and Porter 1977, Falkowski et al. 1993). However, thermal stress caused by increasing seawater temperatures results in a breakdown of symbiotic associations and seriously threatens coral reefs worldwide (Hoegh-Guldberg et al. 2007, Lesser 2007). Thermal breakdown of coral-*Symbiodinium*

symbioses causing coral bleaching was found to be closely related to the thermal inhibition of photosystem (PS)II activity of symbiotic algae (Hill et al. 2004, Takahashi et al. 2008). With the 9 currently described clades (A-I) and numerous subclades within *Symbiodinium* (see review in Coffroth and Santos 2005), the algae were also shown to exhibit different extents of tolerance to thermal stress in culture or in hospite (Bhagooli and Hidaka 2003, Rowan 2004, Tchernov et al. 2004, Robinson and Warner 2006, Sampayo et al.

2008). Selecting thermally-tolerant *Symbiodinium* clades or subclades; therefore, was proposed as a way to promote the survival of corals in the coming century (Chen et al. 2005a, Berkelmans and van Oppen 2006). This proposal was based either on the biogeographic distribution of thermal-tolerant *Symbiodinium* in historically warming regions (Chen et al. 2005a b, LaJeunesse et al. 2010) or on thermal-tolerant experiments under controlled laboratory conditions (Rowan 2004, Tchernov et al. 2004, Sampayo et al. 2008). However, conflict occurs when thermal tolerance is determined among different *Symbiodinium* clades or subclades. For example, both biogeographic and physiological experiments showed that *Symbiodinium* clade D (specifically subclade D1a) is the most heat-tolerant clade compared to clades A, B, and C (Rowan 2004, Chen et al. 2005b, LaJeunesse et al. 2010). However, analysis of the thylakoid membrane integrity showed that there are also thermal-tolerant subclades within clades A, B, and C, suggesting that a priori ribosomal DNA phylotyping is not diagnostic for thermal sensitivity of *Symbiodinium* associations (Tchernov et al. 2004). To resolve this conflict, it is necessary to develop a quantitative comparison with a single parameter or index to determine the thermal tolerance among *Symbiodinium* clades and subclades.

The thermal tolerance between different *Symbiodinium* clades or subclades has been compared by estimating the temperature-dependent performance of the photosynthesis-irradiation response (Iglesias-Prieto et al. 1992, Rowan 2004), the degree of decrease in PSII activity during heat treatment (Bhagooli and Hidaka 2003, Rowan 2004, Robinson and Warner 2006, Sampayo et al. 2008), or thermal sensitivity to the induction of stress proteins (or enzymes) and their related genes (Downs et al. 2000, Brown et al. 2002, Souter et al. 2011). However, comparing results between studies has been difficult due to the experimental designs and conditions used. In this study, we attempted to develop a universal index, as indicated by the activation energy (Ea) for thermally inhibiting PSII activity, to represent the thermal tolerance of members of *Symbiodinium*, since the PSII activity of *Symbiodinium* is closely associated with photosynthesis of the alga and its symbiotic stability with corals (Robinson and Warner 2006, and references therein). Moreover, the function of the PSII apparatus is determined by the natural state of several proteins, such as the D1 protein, peridinin-chlorophyll-*a*-binding proteins,

the chlorophyll-*a*/chlorophyll-*c₂*/peridinin protein complex, etc. (Takahashi et al. 2008). Thus, the loss of PSII activity is expected to follow the process of protein denaturation. If the denaturation of PSII proteins follows a first-order reaction, then the Ea for thermally inhibiting PSII activity could be calculated from the Arrhenius equation (a kinetic equation for measuring the Ea by linearly regressing the rate constant on the reaction temperature in °K). This Ea value could potentially represent the thermal tolerance of *Symbiodinium*. Based on this idea, this study was conducted by subjecting freshly isolated *Symbiodinium* (FIS) to elevated temperatures, measuring the rate of decline in PSII activity (as indicated by F_v/F_m over time), and then fitting the rate constants to the Arrhenius equation to calculate the Ea.

MATERIALS AND METHODS

Symbiodinium isolation and subclade typing

Freshly isolated *Symbiodinium* (FIS) samples used in this study were designated *Symbiodinium* C1, C3, C15, D1a, and B1 from 4 hard corals (*Stylophora pistillata*, *Acropora humilis*, *Porites lutea*, and *Galaxea fasicularis*) and a sea anemone (*Aiptasia pulchella*), respectively, based on a recent study (Wang et al. 2011). The corals were collected by scuba diving in Kenting National Park, Taiwan (21°55'54"N, 120°44'45"E), and the sea anemone was obtained from a laboratory culture as described in Wang et al. (2011). Isolation of *Symbiodinium* from each replicate of the animal host was conducted as previously described (Wang and Douglas 1997, Wang et al. 2011). Briefly, coral fragments having about 100 cm² of live tissue were stripped of tissue using an air blast, and tentacles of 10 *Aiptasia pulchella* were homogenized with a tissue grinder. After mixing with 2-3 volumes of artificial seawater (Instant Ocean, Sarrebourg Cedex, France), the resultant slurry was passed through a 15-μm nylon mesh to remove debris. *Symbiodinium* was then isolated by centrifugation at 860 xg for 3 min and washed with artificial seawater 3 times. *Symbiodinium* was preserved in 80% ethanol before conducting the phylotype analysis by resolving the polymerase chain reaction (PCR) product of the ribosomal internal transcribed spacer (ITS) 2 in denatured gel gradient electrophoresis (DDGE) developed by LaJeunesse et al. (2003) and modified as described in Wang et al. (2011). Since the co-

existence of multiple clades or subclades in a single coral host is well documented (Chen et al. 2005a b, Berkelmans and van Oppen 2006), FIS phylotyping of the ITS2 gene by PCR-DGGE represented the dominant *Symbiodinium* population from which the host was isolated. To obtain Ea data from a single subclade, the kinetics data were abandoned if the PCR-DGGE suggested the possibility of a mixture of clades or subclades of FIS in the preparation (data not shown).

Fluorescence methodology

FIS samples with about $(0.5-0.8) \times 10^6$ cells/ml, counted with an improved Neubauer hemocytometer (Marienfeld, Germany), were maintained at 25°C under dim light (< 5 μE/m^2/s, PAR) for 1 h before proceeding with heat treatment. Measurement of changes in the maximum quantum yield [$F_v/F_m = (F_m - F_o)/F_m$] of FIS at the elevated temperatures began by suspending an algal pellet, collected from centrifugation of 10 ml of an algal suspension at 860 xg for 2 min, in the original volume of artificial seawater which had been prewarmed to the experimental temperature (of 31, 33, 35, 37, 39, or 41°C). The value of F_v/F_m of the FIS suspension was directly measured at 2-min intervals with a DIVING-PAM fluorometer (Walz, Germany) at the DIVING-PAM setting of 8 for measuring the light and saturating flash of the actinic light. The F_v/F_m of treated FIS was measured under indoor illumination (< 10 μE/m^2/s, PAR), and heat treatment was completed within 12 or 14 min depending on the temperature used. The F_v/F_m value of a control FIS that remained at 25°C under dim light for 4 h was examined to evaluate the quality of FIS used in the experiment.

Kinetics and statistical analyses

The rate constant, k (1/min), of PSII protein denaturation at each temperature was obtained from the slope of the linear regression of F_v/F_m values against incubation times. Then, each k value was natural-logarithmically (ln) transformed to produce an Arrhenius plot with 1/T in °K. The fitness of the kinetics data to the Arrhenius equation, [$\ln(k) = \ln(A) - (Ea/R)(1/T)$], was examined by a linear regression of $\ln(k)$ against 1/T. Therefore, the Ea of each sample was calculated from the Arrhenius equation obtained above with the gas constant, R (= 8.314 J/mol/°K). The coefficient of variation (CV) was used to examine the reproducibility between experiments.

Comparisons of Ea values between *Symbiodinium* subclades were made using a one-way analysis of variance (ANOVA) following by Fisher's least significance difference (LSD) test, with a significance level of $p < 0.05$.

RESULTS

F_v/F_m values of FIS from each preparation, which ranged 0.618-0.675, were comparable between *Symbiodinium* subclades with a 4-h incubation at 25°C under dim light. When data from mixed populations of *Symbiodinium* subclades were excluded, F_v/F_m values of all subclades tested (C1, B1, C3, C15, and D1a) linearly decreased with incubation time at an elevated temperature, as shown by data for *Symbiodinium* subclade D1a in figure 1A. Coefficients of the linear regression (r^2) of the decrease in F_v/F_m values with incubation time at each treated temperature were all significant ($p < 0.05$), and were 0.982 ± 0.008 (mean ± S.D., $n = 40$) for D1a, 0.979 ± 0.010 ($n = 35$) for C15, 0.981 ± 0.015 ($n = 30$) for C3, 0.980 ± 0.019 ($n = 30$) for B1, and 0.988 ± 0.007 ($n = 40$) for C1. When the logarithmically transformed denaturation rate (k) of PSII was plotted against 1/T, coefficients of the linear regression were also significant ($p < 0.05$), and showed a good fit to the Arrhenius equation ($r^2 = 0.942$-0.985), as shown by D1a data in figure 1B. The regression coefficients obtained were 0.961 ± 0.019 ($n = 8$) for D1a, 0.966 ± 0.012 ($n = 7$) for C15, 0.965 ± 0.020 ($n = 6$) for C3, 0.965 ± 0.017 ($n = 6$) for B1, and 0.966 ± 0.013 ($n = 8$) for C1. The Ea for PSII denaturation was then calculated from each Arrhenius equation (Table 1). The Ea values significantly differed among the 5 different *Symbiodinium* subclades ($F_{4,30} = 288.3$, $p < 0.001$). The post-hoc analysis with Fisher's LSD test also indicated that *Symbiodinium* D1a displayed the highest Ea, followed in order by C15, C3, B1, and C1 (Table 1). Ea values for D1a and C15 were almost 2-fold higher than those of B1 and C1.

DISCUSSION

This study proposes that the activation energy for inhibiting PSII activity under thermal stress could be used to represent the thermal tolerance of *Symbiodinium*. With such an index, thermal tolerances among *Symbiodinium* subclades could be compared on a universal scale. In order to

test the hypothesis, 5 *Symbiodinium* subclades, for which the tolerance or sensitivity to heat was compared in the literature (LaJeunesse et al. 2003, Fabricius et al. 2004, Rowan 2004, Berkelmans and van Oppen 2006), were selected for testing in this study.

When a *Symbiodinium* alga is stressed due to an elevated temperature, many physiological responses are evoked, including upregulation of stress protein synthesis, downregulation of normal protein synthesis, and an increase in protein denaturation (as reviewed by Brown et al. 2002). It would be easy to obtain the correlation between heat-stress indicators of the tested organism and temperature, but none of them can be summarized to a constant value to reflect the heat-stress response or tolerance of a *Symbiodinium* alga without a kinetics analysis. Kinetics studies on the

increase and subsequent collapse in the rate of respiration or heart beat were used to represent the thermal tolerance of a snail (Stenseng et al. 2005), crab (Stillman 2002), and shellfish (Dahlhoff and Somero 1993). Increases in the rates of respiration and heart beat usually follow Q_{10} over a wide range of temperatures. With photosynthetic algae, a decline in PSII activity during heat treatment was found, in this study, to be very suitable for a kinetics analysis of the thermal deterioration of *Symbiodinium* algae for 4 reasons. First, the PSII activity of *Symbiodinium* can be instantly determined *in situ*; therefore, the time interval for the kinetic analysis can be precisely controlled. Second, the PSII activity of *Symbiodinium* was proven to be closely related to the photosynthetic capability of the alga (Robinson and Warner 2006, and references therein). Third,
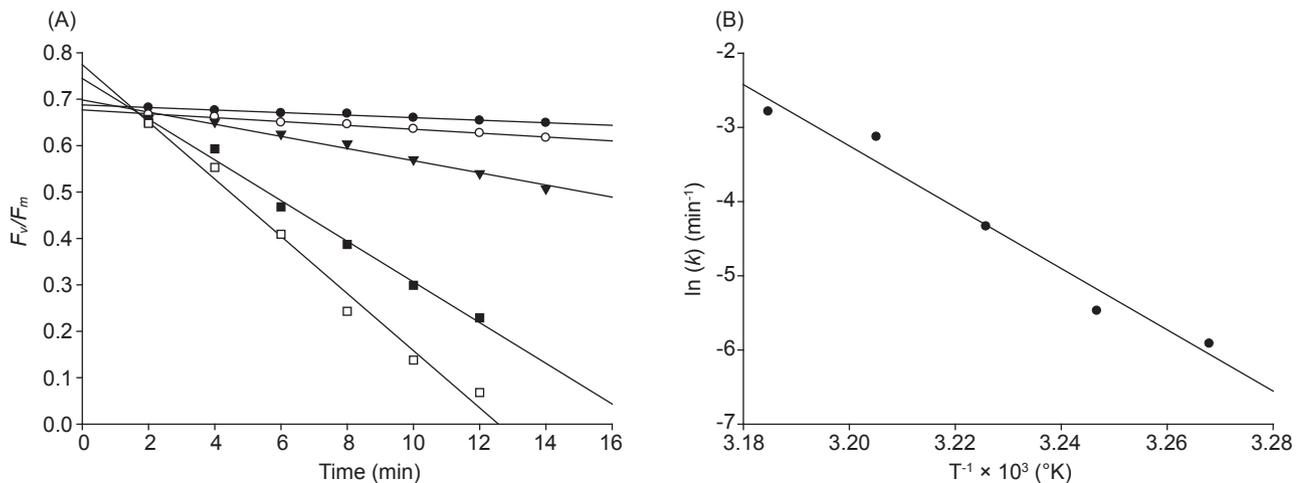

Fig. 1. Representative data obtained from freshly isolated *Symbiodinium* subclade D1a. (A) Decrease in the maximum quantum yield (F_v/F_m) of *Symbiodinium* when incubated at 33 (●), 35 (○), 37 (▼), 39 (■), and 41°C (□). (B) An Arrhenius plot obtained from data in (A). The equations and coefficients of the linear regression of F_v/F_m against time are: y = -0.0027x + 0.6876, r^2 = 0.984 (33°C); y = -0.0042x + 0.677, r^2 = 0.991 (35°C); y = -0.0131x + 0.6984, r^2 = 0.979 (37°C); y = -0.0438x + 0.7445, r^2 = 0.993 (39°C); and y = -0.0551x + 0.7394, r^2 = 0.967 (41°C). That for ln(k) on 1/T is y = -41315x + 128.96, r^2 = 0.968.

Table 1. Activation energy (Ea) for inhibiting photosystem II activity of freshly isolated *Symbiodinium* under thermal stress. *n*, number of replicates from different colonies; Ea, activation energy, the data of which followed by the same superscript letter do not significantly differ at *p* = 0.05 according to Fisher's LSD test; CV, coefficient of variation

Cnidarian host	*Symbiodinium* subclade	*n*	Ea (kJ/mole)	CV (%)
Stylophora pistillata	C1	8	126 ± 10 a	7.6
Aiptasia pulchella	B1	6	144 ± 7 b	4.9
Acropora humilis	C3	6	214 ± 7 c	3.2
Porites lutea	C15	7	262 ± 25 d	9.4
Galaxea fasicularis	D1a	8	348 ± 16 e	4.5

the stability of the PSII apparatus is determined by the natural state of a set of proteins, especially the D1 protein (Waner et al. 1999, Takahashi et al. 2008). Fourth, the kinetics of protein denaturation under heat treatment were reported to follow a 1st-order reaction and comply with the Arrhenius equation (Weijers et al. 2003), and the Ea for the thermal inhibition (or denaturation) of PSII proteins can be easily obtained from the Arrhenius equation. Consequently, the data obtained in this study indicated that Ea values for inhibiting PSII activity of each *Symbiodinium* subclade were consistent with previous studies (LaJeunesse et al. 2003, Fabricius et al. 2004, Rowan 2004, Tchernov et al. 2004, Berkelmans and van Oppen 2006, Díaz-Almeyda et al. 2011), i.e., D1a and C15 were the 2 most thermally tolerant *Symbiodinium* among the tested subclades. Reproducibility of the Ea data for each *Symbiodinium* subclade was determined to be acceptable by examining values of the CV which ranged 3.2%-9.4%.

In summary, a high regression coefficient ($r^2 > 0.95$) obtained from the kinetics data and the low CV between replicates (< 10%) indicated that the calculated Ea values for PSII denaturation were stable and reliable. This evidence suggests that the Ea for inhibiting PSII activity during heat stress can be used to quantify the thermal tolerance of *Symbiodinium*; thus, facilitating ecological, physiological, and evolutionary studies of coral symbiosis and bleaching biology. Based on this idea, it is also possible to develop an index to represent bleaching susceptibility of corals by selecting proper physiological indicator(s), changes in which with an increase in heating temperature or light intensity follow a 1st-order reaction.

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