Photosystem II breakdown induced by reactive oxygen species in freshly-isolated *Symbiodinium* from *Montipora* (Scleractinia; Acroporidae)

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ABSTRACT: Freshly-isolated *Symbiodinium* (FIS) have been used to study cnidarian–alga symbiosis based on the assumption that their physiological performance is comparable to that of the algae *in hospite*. This assumption was tested with 15 species of scleractinian corals, using pulse-amplitude modulation (PAM) chlorophyll *a* (chl *a*) fluorescence to compare maximum quantum yields (F_v/F_m) of photosystem II (PSII) in *Symbiodinium in hospite* and after isolation in seawater. FIS from *Montipora* spp. exhibited rapid and dramatic decreases (by up to >95%) in PSII activity within 30 min of isolation. In contrast, PSII activities of FIS from 8 other coral species decreased by only 5 to 21% after >4 h in seawater. To investigate possible reasons for this variation, the variation in *Symbiodinium* genetic type (ITS-2 types), transmission modes and several physiological indices were considered. The rapid loss of PSII activity in FIS from *Montipora* spp. was not correlated with *Symbiodinium* ITS-2 type, the mode of symbiont transmission, or ionic regulation capability, nor could consistent chemical effects in host extracts be demonstrated. PSII inactivation was correlated with increased levels of reactive oxygen species (ROS), degradation of pigments in peridinin-chl *a* protein, and chloroplast disruption, indicating that the FIS was under physiological collapse. The specific mechanism(s) causing *Montipora*-associated *Symbiodinium* to fail during the isolation methods used here remain unknown. However, these data indicate that care should be taken when using FIS to represent *Symbiodinium in hospite*, especially when comparing different species of corals.

KEY WORDS: *Montipora* · *Symbiodinium* · PSII inactivation · ROS · Symbiont transmission mode · Host extract · Cnidarian · Symbiosis

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INTRODUCTION

Symbiodinium spp. are symbiotic dinoflagellates that reside within corals, sea anemones, and many other marine invertebrates (see review in Coffroth & Santos 2005). These unicellular algae perform intensive interactions with their animal hosts, including nutrient ex-

change (Muscatine 1967, 1990, Dubinsky & Jokiel 1994, Wang & Douglas 1997, 1998), enhancement of coral calcification (Pearse & Muscatine 1971, Barnes & Chalker 1990), responses to stress (Jones et al. 1998, 2008), and habitat selection (Venn et al. 2008). Increasing seawater temperature causes the breakdown of these symbiotic associations (especially in reef-building corals),

making them an area of special biological interest (Hoegh-Guldberg 1999, Hoegh-Guldberg et al. 2007, Weis 2008, Weis et al. 2008).

Determining the contribution of host and *Symbiodinium* to the holobiont physiology is problematic without separation of *Symbiodinium* from the host. Since culturing of *Symbiodinium in vitro* may not be representative of conditions *in hospite*, many studies instead use freshly isolated *Symbiodinium* (FIS) suspended in seawater, which is assumed to be more physiologically similar to *Symbiodinium in hospite* (Muscatine 1967, Hinde 1988, Sutton & Hoegh-Guldberg 1990, Wang & Douglas 1997). However, this assumption is questionable. The intracellular environments of marine invertebrates have lower concentrations of Na^+ and Ca^{2+}, higher concentrations of K^+, and lower pH than seawater (Kirschner 1991, Goiran et al. 1997). Consequently, changes in these conditions can elicit stress responses, such as a transient Na^+ shock, as observed in *Symbiodinium* isolated from the coral *Galaxea fasicularis* into seawater (Goiran et al. 1997). The process of making FIS could therefore shock *Symbiodinium*. This effect might be more significant in the corals with vertical transmission of *Symbiodinium*, because these algae may not encounter seawater environments for many generations, and hence may lose their ability to adapt to changing salinities. Furthermore, the specialized environment of *Symbiodinium in hospite*, defined by the symbiosome membrane, is lost when symbioses are disrupted, potentially exposing the algae to a variety of novel host-derived enzymes. Even though these enzymes may have minimal affects on some *Symbiodinium*, which have been documented in many studies by directly using the host tissue extract as 'host factor' to induce photosynthate release from *Symbiodinium* (Sutton & Hoegh-Guldberg 1990, Gates et al. 1995, 1999, Wang & Douglas 1997), they might be significant in other cases. For example, Bhagooli & Hidaka (2003) observed that *Symbiodinium* from the coral *Montipora digitata* lost maximum quantum yield (F_v/F_m) of photosystem II (PSII) quickly after isolation into seawater, whereas *Symbiodinium* from 4 other species retained normal PSII function. The ultrastructure of the *Symbiodinium* from a soft coral *Capnella gaboensis* was observed to be severely damaged after isolation, which was also assumed to be the effects of host-tissue extracts (Sutton & Hoegh-Guldberg 1990).

Since FIS is frequently used in exploring the physiological interactions between cnidarians (e.g. coral) and their symbiotic alga, it is important to clarify these phenomena and determine whether they are general properties of FIS. In the present study, we examined the variation in PSII activity (F_v/F_m) in *Symbiodinium* isolated into seawater from 15 species of scleractinian

corals for correlations with coral species, *Symbiodinium* ITS-2 type, the mode of symbiont transmission between host generations (vertical versus horizontal), and the differences in host response to the stress derived from physical disruption. The effects of increased Na^+ influx, loss of photosynthetic pigment concentration, loss of plastid membrane integrity, and the production of reactive oxygen species (ROS) in the algae were also examined in detail to reveal the possible cause(s) of PSII inhibition in the FIS from some coral hosts.

MATERIALS AND METHODS

Collection and maintenance of corals. Multiple colonies of 15 species of scleractinian corals (see Table 1) were collected from the shallow water (<5 m) reefs located at the outlet of a nuclear power plant in the Kenting National Park, Taiwan (21° 55' 54'' N, 120° 44' 45'' E) between October 2008 and June 2009. The seawater temperature at the sampling site rises to over 30°C during mid-summer, which is 1.5 to 2.0°C higher than the ambient water in Kenting reefs (Meng et al. 2008). Coral colonies were transferred to the laboratory within 3 h in an aerated plastic box, and maintained in an aquarium tank (60 × 45 × 45 cm) equipped with illumination (12 h light:12 h dark regime and ca. 50 μmol photons m^{-2} s^{-1} photosynthetically active radiation), temperature control (25°C), filtration (EHEIM), and a protein skimmer. Corals were utilized for experiments within 2 wk, even though others remained healthy for at least 1 mo in this aquarium system. Replicate number (n) in the following description indicates the number of coral colonies used.

Isolation of *Symbiodinium* and fluorescence methodology. Immediately prior to isolating *Symbiodinium*, maximum quantum yields of PSII ($F_v/F_m = [F_m − F_0]/F_m$) of the same *Symbiodinium in hospite* were determined as follows: coral fragments were transferred to a separate tank covered with a black sheet for dark-adaptation for 30 min at 25°C (Ralph 2005), after which minimum (F_0) and maximum (F_m) fluorescence was measured using a DIVING-PAM fluorometer (Walz) at the DIVING-PAM setting of 8 for measuring-light and a saturating flash of actinic light.

Coral fragments with 5 to 10 cm^2 of live tissue were stripped of tissue using a modified air blast technique (Rowan 1991). After mixing with 2 to 3 volumes of 0.45 μm filtered seawater (FSW) in a 25 ml syringe, the resultant slurry was passed through 15 μm nylon mesh to remove debris. *Symbiodinium* were then isolated by repeat centrifugation at 860 × *g* for 3 min and washed 3 times with FSW (Wang & Douglas 1997). Since the time required for FIS preparation varied

between 10 and 20 min, initial measurements of F_v/F_m were made at 30 min following air blasting of coral tissue. One ml of FIS (ca. 10^5 cells ml^{-1}) was suctioned onto 0.45 μm membrane filters (Advantec) at the set time. F_v/F_m on 0.45 μm membrane filters were measured in a transparent plastic bag immediately after collection, according to the method modified from Bhagooli & Hidaka (2003). The stability of PSII activity in the FIS was then examined by measuring F_v/F_m repeatedly at 30 or 60 min intervals, for a total of 4 h, during which time the FIS preparations were kept in the dark at 25°C. In order to avoid anaerobic conditions, the algal suspension was mixed by gently inverting every 10 to 20 min. Measurements of F_v/F_m of FIS were expressed as indices of PSII inactivation (%):

$$\text{PSII inactivation} = [(F_v/F_m)_c - (F_v/F_m)_{FIS}]/(F_v/F_m)_c \times 100\,\%$$

where $(F_v/F_m)_c$ and $(F_v/F_m)_{FIS}$ are the values measured in intact coral and FIS, respectively.

PCR-DGGE analysis of *Symbiodinium* ITS-2 types. The FIS were preserved in 70% ethanol at −20°C before DNA extraction with the Viogene Plant Genomic DNA Extraction Miniprep System (Viogene). The *Symbiodinium* DNA was extracted from ca. 10^6 cells of FIS and suspended in 150 μl distilled water, and then used to identify *Symbiodinium* ITS-2 types as described by LaJeunesse (2002). Briefly, the internal transcribed spacer region 2 of ribosomal DNA (ITS2) was amplified by PCR using the designed primer set ITSintfor2 (5′-AAT TGC AGA ACT CCG TG-3′) and ITS2 clamp (5′-CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GGG ATC CAT ATG CTT AAG TTC AGC GGG T-3′), and products were analyzed by denaturing-gradient gel electrophoresis (DGGE). Diagnostic PCR products were eluted from each gel, reamplified, and sequenced. The final identification of ITS-2 type with sequence data was conducted by E. Sampayo in Dr. LaJeunesse's laboratory at the Mueller Laboratory, Department of Biology, Pennsylvania State University, USA.

Determination of physiological status of isolated *Symbiodinium*. To reveal cell integrity and physiological condition of the FIS, Na$^+$ channel stability (an indicator to show if cells could maintain lower Na$^+$ level than external environment), the concentration of photosynthetic related pigment (an indicator representing the photosynthetic integrity within *Symbiodinium* cells), and of ROS (an indicator representing stress occurring in the cells) in the FIS were examined. According to a method modified from Goiran et al. (1997), FIS used for Na$^+$ channel stability were isolated from corals by blasting with 60 mM Na$^+$ artificial seawater (ASW, modified from Wang & Douglas 1997), and washed 3 times by repeat suspension and centrifu-

gation ($860 \times g$, 3 min) with the same ASW medium to remove host debris. Determination of Na$^+$ influx began with suspending these FIS into FSW, followed by collecting 1 ml aliquots (corresponding to about 10^6 cells) at 15 or 30 min intervals for determination of intracellular Na$^+$ ion concentration. Then, the FIS suspensions were washed 3 times with 1.2 ml of ice-cold isotonic glycine buffer (glycine 1.1 M, Tris 5 mM, pH 8.2) using quick spins ($6120 \times g$ for 7 s) and re-suspension. Total washing procedure was controlled within 3 min. The resulting cell pellet was re-suspended in 1 ml distilled water and disrupted by ultrasonication (Sonics & Materials) for 5 min on ice. The concentration of Na$^+$ was then determined by atomic absorption spectrophotometry (Hitachi, model Z-5000), and expressed as μequiv mg^{-1} protein.

Contents of the total chlorophyll *a* (chl *a*), of peridinin and chl *a* in peridinin-chl *a* protein (PCP), and of total soluble protein in the FIS were then determined. Prior to pigment and protein determination, FIS were incubated at 25°C under dim light. At 30 min intervals, cells were collected by centrifuging 1 ml aliquots (about 10^6 cells). The samples for PCP and protein determination were extracted with 1 ml 1 mM Tris-HCl (pH 8.0), and those for total chl *a* determination were extracted with 1 ml 90% acetone. Exact cell numbers in each sample were measured using a Neubauer improved haemocytometer (Marienfeld). Pigments of PCP in the water-soluble fraction were quantified by spectrum analysis using a spectrophotometer (Hitachi U-1900) and expressed as absorbance per 10^6 cells at specific wavelength (459 nm for peridinin; 438 nm and 673 nm for chl *a*) following the spectrum data obtained by Haxo et al. (1976). Total chl *a* content in the acetone extract was determined spectrophotometrically using the equation developed by Jeffrey & Haxo (1968). Protein content was quantified using a protein assay kit (Bio-Rad) according to the manufacturer's instruction for microassay, using bovine serum albumin as the standard.

ROS in FIS were detected by incubating 1 ml of algal suspension with 5 μl 2′,7′-dichlorofluorescin diacetate (H$_2$DCFDA, 10 mM in dimethylsulfoxide) for 30 min in the dark, followed by fluorescence microscopy (Mydlarz & Jacobs 2004). For quantifying ROS, H$_2$DCFDA-treated FIS were washed twice with FSW, frozen as cell pellets in liquid nitrogen to stop the reaction, and then thawed in 200 μl methanol for 10 min. After vortexing and centrifugation at $21\,500 \times g$ for 5 min (4°C) to pellet the cells, 20 μl of the methanol extract was injected into a fluorescence detector (Hitachi L-2485) for measuring the fluorescence intensity (excitation at 488 nm and emission at 525 nm) (Mydlarz & Jacobs 2004). The data were expressed in arbitrary fluorescence units per 10^6 cells.

Transmission electron microscopy (TEM). FIS from *Galaxea fasicularis* and *Montipora aequituberculata* were harvested by centrifugation (3 min at $860 \times g$) immediately after isolation and fixed in cacodylate buffer containing 4% paraformaldehyde and 8.6% sucrose for 2 h. Pellets of the algal cells were washed in a series of 0.1 M phosphate buffers with descending sucrose concentration and postfixed in 1% OsO_4 (in 0.1 M phosphate buffer) for 4 h. After dehydration in an ascending acetone series (30 to 100%), samples were embedded in Spurr's resin, sectioned (50 nm thickness) with a Leica ultramicrotome (EM UC6), stained with 5% uranyl acetate and 0.5% lead citrate, and examined with a Phillips CM-100 transmission electron microscope.

***Montipora* extract and PSII inactivation.** To test whether the host tissue slurry from *Montipora* spp. contained detrimental factors which inactivate the PSII function of *Symbiodinium*, FIS from *Galaxea fasicularis*, the most stable FIS found in this study, was incubated with the host extract of *M. aequituberculata*. Various concentrations of host tissue extract (measured with protein concentration) were prepared from air-blasted *M. aequituberculata* tissue and the first run of centrifugation (3 min at $860 \times g$). The incubation of FIS from *G. fasicularis* mimicked the preparation procedure of FIS from *M. aequituberculata*: the FIS was resuspended in *Montipora* extract for 10 min, and then collected by centrifugation and transferred to fresh FSW for a further 20 min incubation. F_v/F_m of the treated FIS was then measured (as described in 'Isolation of *Symbiodinium* and fluorescence methodology').

Statistical analysis. Comparisons between treatments on PSII-inactivation were made using a 1-way analysis of variance (ANOVA) followed by Fisher's least significance difference test, with a significance level of 0.05. Pair comparison of Na^+ concentration between samples at each time interval was conducted with Student's *t*-test. The correlation between protein concentration and PSII inhibition was evaluated using the Pearson pair-wise test.

RESULTS

PSII inactivation in isolated *Symbiodinium*

FIS obtained from 15 species of scleractinian corals across 6 genera and 4 families were compared. These coral species represented horizontal and vertical symbiont transmission, and 5 different ITS-2 types of *Symbiodinium*

(Table 1). Due to the detection limit of DGGE, a *Symbiodinium* ITS-2 type population less than 10% is difficult to detect. In this study, therefore, the detectable ITS-2 type of *Symbiodinium* only represented the dominant population. Overall, PSII function, measured with maximum quantum yield, in *Symbiodinium* from all coral species examined was as high as previous findings by Bhagooli & Hidaka (2003) immediately before they were isolated into seawater, in which the values of F_v/F_m ranged from 0.640 to 0.720 *in hospite* (n = 123).

However, when isolated into seawater, the FIS from most *Montipora* spp., including *M. aequituberculata*, *M. efflorescens*, *M. foliosa*, *M. digitata*, and *M. monasteriata*, displayed 81 to 100% inactivation in PSII activity (Table 1). The FIS from *M. gaimardi* and *M. stellata* displayed 62 and 67% PSII inactivation, respectively. In contrast to *Symbiodinium* from *Montipora* spp., inactivation of PSII in FIS from *Stylophora pistillata*, *Pocillopora damicornis*, *Porites lutea*, *Galaxea fasicularis*, and 4 species of *Acropora* (*A. humilis*, *A. yongei*, *A. nana*, and *A. intermedia*) was no more than 21% (Table 1). Longer-term inactivation of PSII followed the same trends (Fig. 1). F_v/F_m in FIS from *M. gaimardi* and *M. stellata* decreased to near 0 at 1 h and 4 h, respectively, whereas F_v/F_m in FIS from *G. fasicularis*, *P. damicornis*, *A. yongei*, and *P. lutea* remained relatively high (>0.5).

Table 1. Coral species used in the present study and their mode of *Symbiodinium* transmission between generations (V: vertical; H: horizontal), dominant *Symbiodinium* identities (ITS-2 type), number of colonies from which a particular ITS-2 type was identified, and the extent of PSII inactivation in *Symbiodinium* 30 min after isolation into seawater. In the coral species with 2 genetic types of *Symbiodinium* between colonies, ITS-2 type and its corresponding colony number are separately recorded (before and after '/')

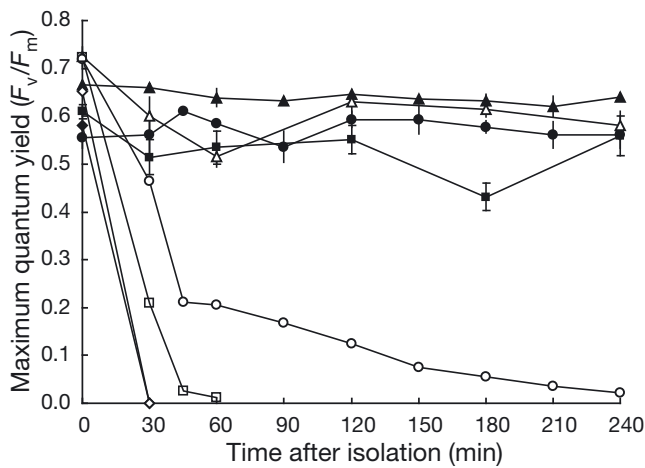| Species	Transmission mode	Symbiont ITS-2 type	n	PSII inactivation (%)
Acroporidae				
Montipora efflorescens	V	C15/D1a	8/3	99.9 ± 0.1
M. aequituberculata	V	C15/D1a	12/12	98.7 ± 3.4
M. monasteriata	V	C15	3	96.8 ± 5.6
M. foliosa	V	C15	7	95.3 ± 9.0
M. digitata	V	C15	10	81.0 ± 20.0
M. gaimardi	V	C15	3	67.3 ± 28.8
M. stellata	V	D1a	12	61.9 ± 18.7
Acropora yongei	H	C3	3	21.0 ± 11.2
A. intermedia	H	C3h	3	14.2 ± 8.0
A. nana	H	C3	5	10.8 ± 8.0
A. humilis	H	C3	9	10.8 ± 7.3
Pocilloporidae				
Stylophora pistillata	V	C1	11	17.6 ± 3.6
Pocillopora damicornis	V	C1	4	12.0 ± 5.1
Poritidae				
Porites lutea	V	C15	10	16.5 ± 4.4
Oculinidae				
Galaxea fasicularis	H	D1a	8	4.8 ± 2.5

Fig. 1. Changes in maximum quantum yield of PSII (F_v/F_m) with time in *Symbiodinium* from different corals after isolation into seawater. The data from hosts *Galaxea fasicularis* (▲) (n = 4), *Pocillopora damicornis* (△) (n = 4), *Acropora yongei* (●) (n = 4), *Porites lutea* (■) (n = 4), *Montipora efflorescens* (◆) (n = 4) and *M. aequituberculata* (◇) (n = 4) are means ± SD, and those from *M. stellata* (○) (n = 2) and *M. gaimardi* (□) (n = 2) are means only

Fig. 3. Intracellular Na$^+$ concentrations in *Symbiodinium* freshly isolated into 60 mM Na$^+$ artificial seawater and transferred into filtered seawater at Time 0. *Symbiodinium* were isolated from *Galaxea fasicularis* (○) (n = 6) and *Montipora aequituberculata* (●) (n = 6). Data are mean ± SD

Table 1 also indicates that symbiont transmission mode has little bearing on PSII inactivation in FIS. Although *Montipora* spp. are all vertical transmitters, not all vertical transmitters produced high PSII inactivation in FIS. For example FIS from *Stylophora pistillata*, *Pocillopora damicornis* and *Porites lutea* displayed low PSII inactivation. In addition, the analysis of FIS using PCR-DGGE ITS-2 fingerprints (Fig. 2) and partial 5.8S DNA sequencing (data not shown) showed

that *Symbiodinium* ITS-2 types found in *Montipora* spp. (D1a, n = 27; and C15, n = 43) could also be found among FIS that did not display high PSII inactivation (D1a, n = 8; and C15, n = 10) (Table 1).

Cellular events in isolated *Symbiodinium*

The fates of FIS cells from *Montipora aequituberculata* during PSII inactivation were examined using parallel analyses of FIS from *Galaxea fasicularis* as
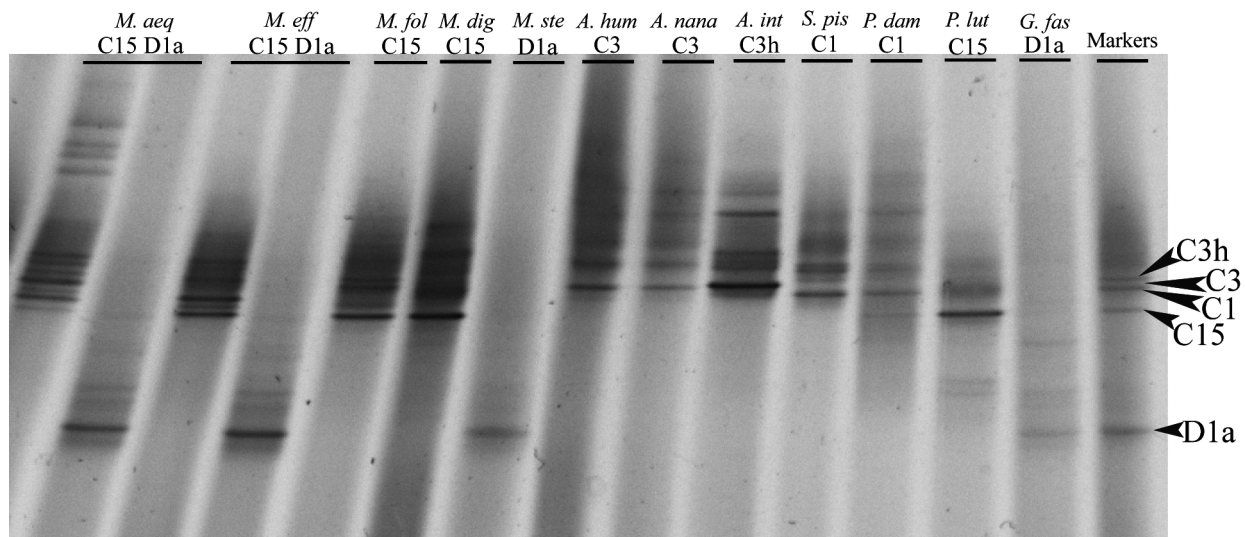

Fig. 2. Representative PCR-DGGE ITS2 fingerprints of *Symbiodinium* freshly isolated from the corals listed in Table 1

negative controls. The intracellular Na⁺ pools in these FIS were first compared (n = 6) (Fig. 3). After transfer from 60 mM Na⁺ ASW to FSW, Na⁺ influx was detected at 45 to 60 min in FIS from *G. fasicularis* and at 30 to 45 min in FIS from *M. aequituberculata*, with significant 2- to 3-fold increases in intracellular Na⁺ concentration ($t_{1,5}$ = 3.920 and 3.424 for *M. aequituberculata* and *G. fasicularis*, respectively; p < 0.05). Influx continued subsequently in both types of FIS, but only the FIS

from *G. fasicularis* maintained intracellular [Na⁺] lower than seawater (0.67 to 0.76 µequiv mg⁻¹ algal protein) after 4 h.

FIS from *Montipora aequituberculata* (but not from *Galaxea fasicularis*) also appeared to become less pigmented during incubation in FSW. When examining the pigments, mean (± SD) total chl *a* contents were similar in FIS from both species (6.83 ± 0.35 and 7.28 ± 0.34 µg per 10⁶ cells for *G. fasicularis* and *M. aequitu-*
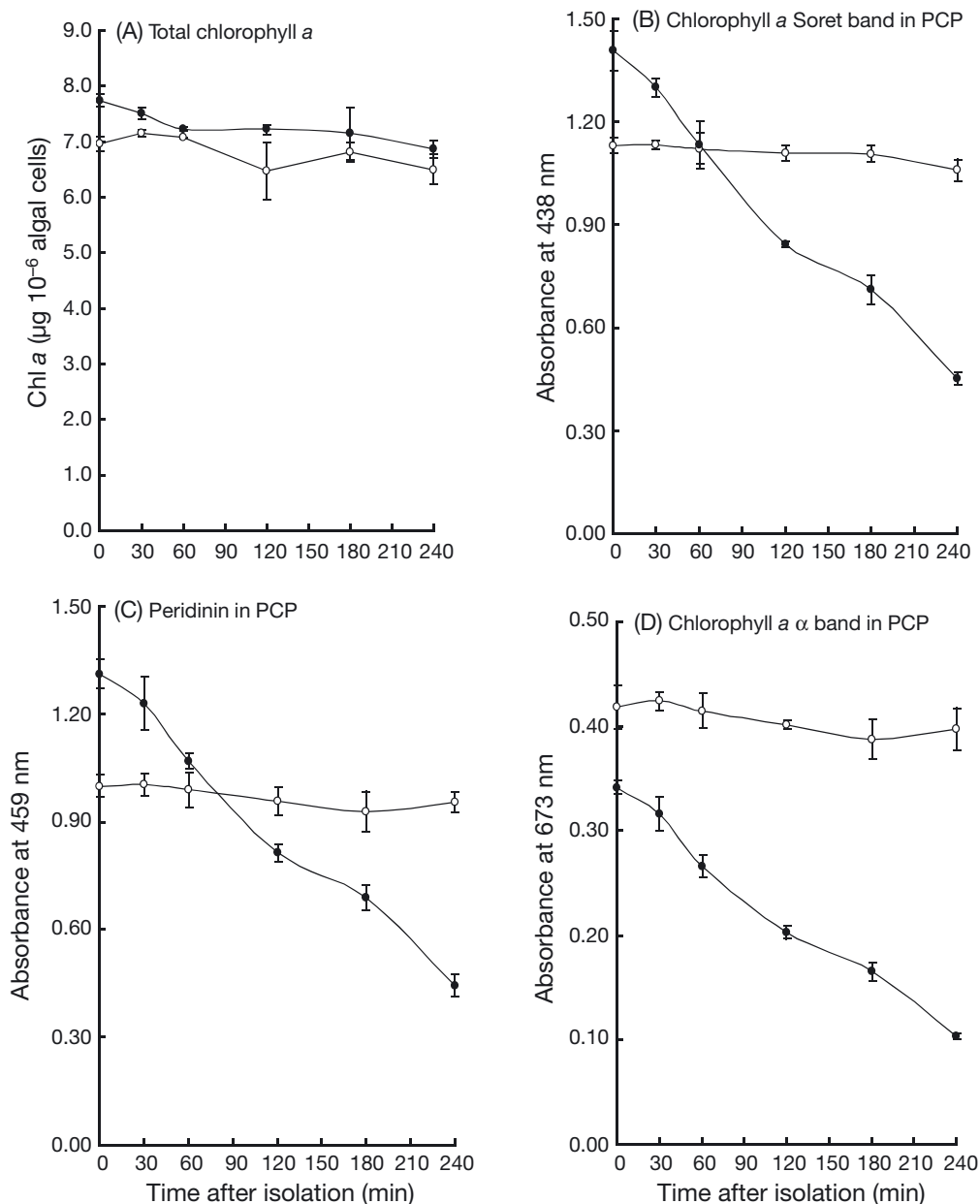

Fig. 4. Contents of photosynthetic pigments in *Symbiodinium* isolated from *Galaxea fasicularis* (○) or *Montipora aequituberculata* (●) during incubation in filtered seawater. Pigments are (A) total chlorophyll *a* (chl *a*) and that in peridinin-chl *a* protein (PCP) (B) chl *a* Soret band (438 nm), (C) peridinin (459 nm), and (D) chl *a* α band per 10⁶ *Symbiodinium* cells. Data are mean ± SD (n = 3)
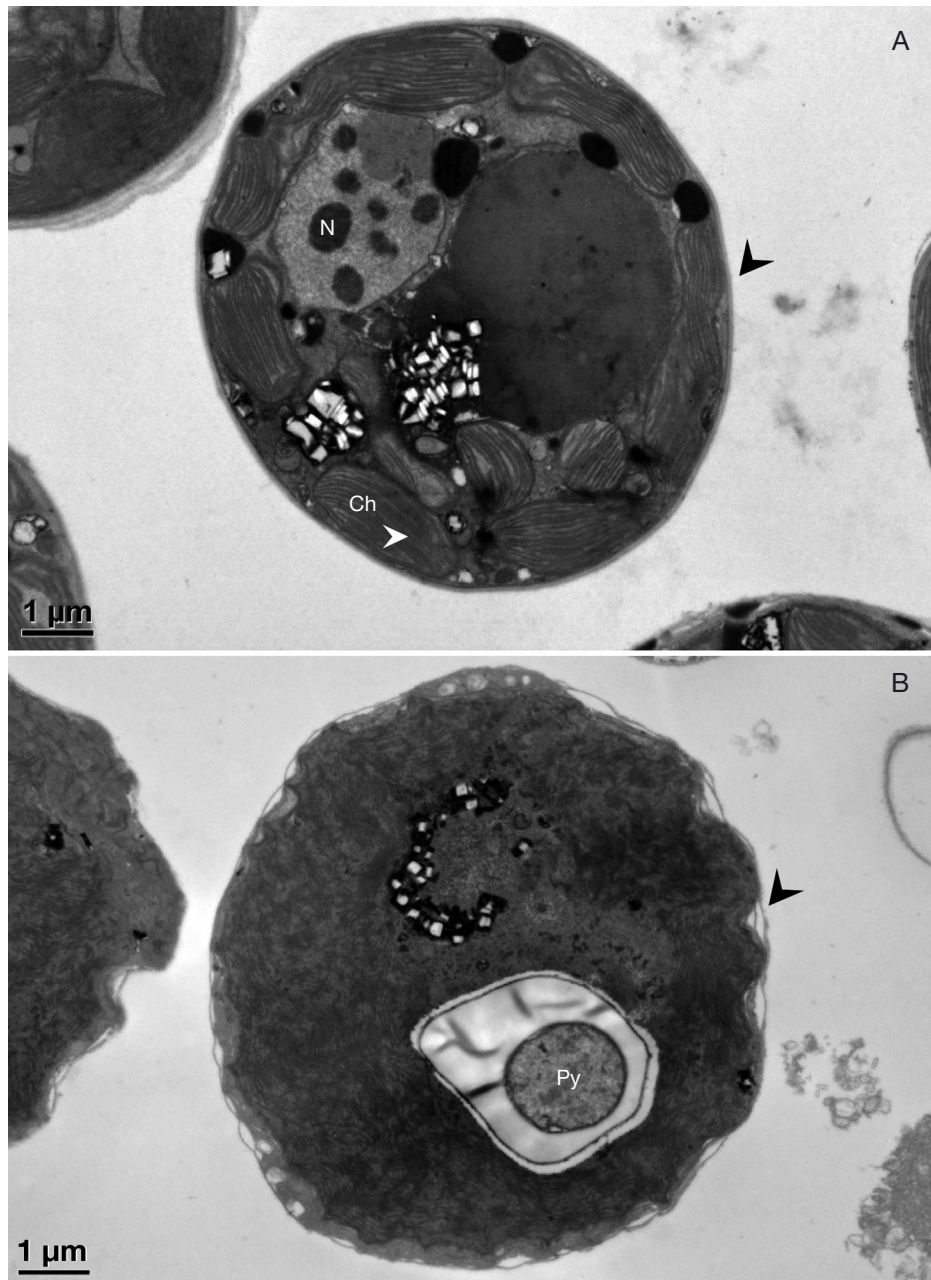
Fig. 5. Transmission electron micrographs of sections of *Symbiodinium* after isolation in filtered seawater. Algae isolated from (A) *Galaxea fasicularis* and (B) *Montipora aequituberculata*. Arrowheads indicate cell wall (black arrow) and plastid membrane (white arrow). Py: pyrenoid; N: nucleus; Ch: chloroplast

berculata, respectively; both n = 3) during incubation (Fig. 4A). However, only FIS from *M. aequituberculata* lost PCP pigments (Fig. 4B−D), which displayed 68 ± 2, 66 ± 2 and 70 ± 1% (mean ± SD, n = 3) pigment loss for the chl *a* Soret band (438 nm), peridinin (459 nm) and chl *a* α band (673 nm). Moreover, soluble protein in FIS from *M. aequituberculata* decreased by 60 ± 3% (mean ± SD, n = 3) after 4 h of incubation, compared to

19 ± 2% (mean ± SD, n = 3) in FIS from *G. fasicularis*. Further examination of the *Symbiodinium* ultrastructures using TEM also indicated greater loss of internal cellular integrity in FIS from *M. aequituberculata*, compared to that from *G. fasicularis* (Fig. 5); the plastid membranes in FIS from *M. aequituberculata* appeared disrupted, whereas plastid structure in FIS from *G. fasicularis* appeared intact.
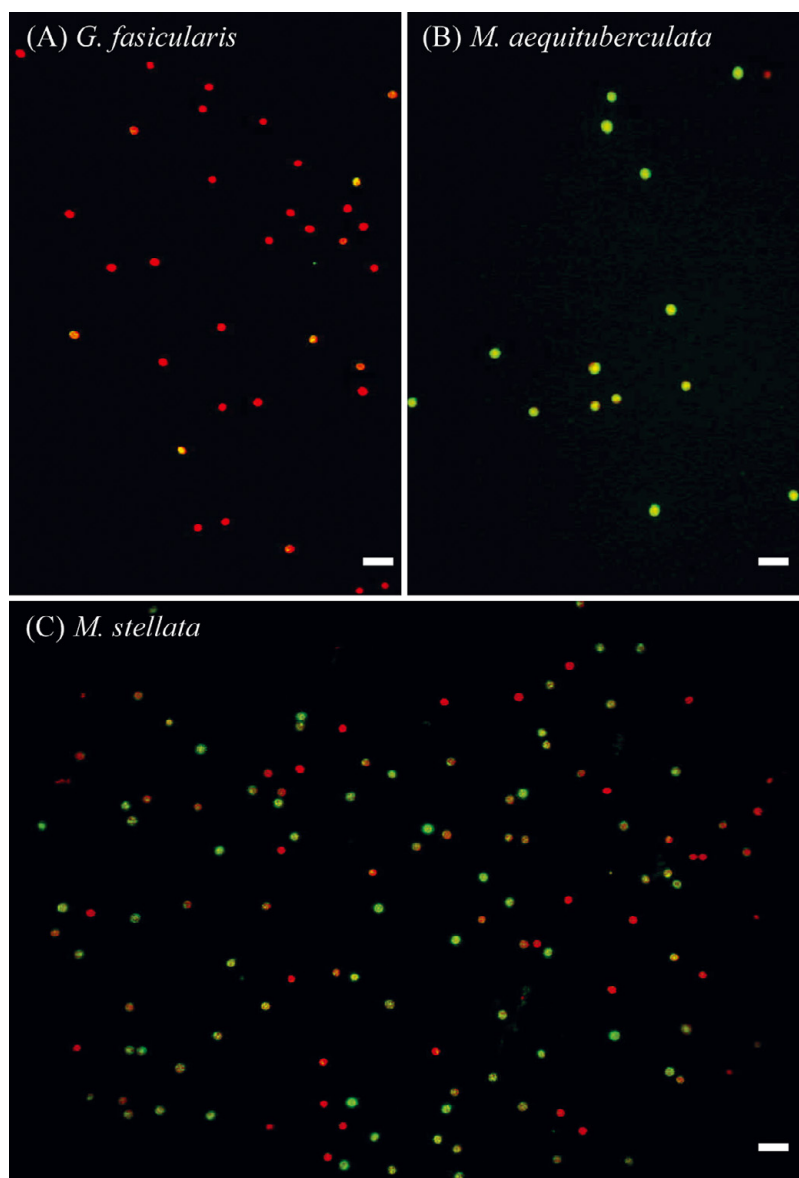
Fig. 6. Reactive oxygen species (ROS) in isolated *Symbiodinium* detected with the specific indicator 2′,7′-dichlorofluorescein. FIS were obtained from (A) *Galaxea fasicularis*, (B) *Montipora aequituberculata*, and (C) *Montipora stellata*. Green fluorescence is diagnostic of ROS; red is chlorophyll autofluorescence. Scale bars = 50 μm

Since cellular damage in *Symbiodinium* associated with coral bleaching is attributed to ROS, the levels of ROS in FIS from *Montipora aequituberculata* versus *Galaxea fasicularis* were further compared. In qualitative analyses of FIS incubated for 30 min by fluorescence microscopy, using the probe H$_2$DCFDA (Fig. 6), a strong ROS signal was observed in highly inactivated FIS from *M. aequituberculata* (Fig. 6A; F_v/F_m < 0.100; green fluorescence); no ROS were observed in FIS

from *G. fasicularis* (Fig. 6B; F_v/F_m = 0.624; red autofluorescence of chl *a* only); and a wide range of ROS signal was seen in moderately inactivated FIS from *Montipora stellata* (Fig. 6C; F_v/F_m = 0.460; a mix of green, yellow, and red fluorescent cells). To quantify ROS in FIS, oxidized (fluorescent) DCF was extracted with methanol and measured. The quantitative results indicated that the ROS level displayed significantly positive correlation with PSII inactivation (r = 0.915, p < 0.001) when ROS level was lower than 2.0 fluorescence units 10^{-6} cells and reached maximum PSII inactivation (100%) at the levels higher than 2.0 (Fig. 7).

Exploration of the cause of PSII inactivation

The possible effect of host tissue on PSII inactivation was examined by treating FIS from *Galaxea fasicularis*, as an external control, with host tissue slurries obtained from *Montipora aequituberculata*. After 30 min of isolation, PSII inactivation was always high (>80%; n = 24) in the FIS from *M. aequituberculata*, and was always low in FIS from *G. fasicularis* (<10%; n = 32). However, when FIS from *G. fasicularis* were treated with various concentrations of *M. aequituberculata* tissue slurry (0.1 to 0.7 mg ml^{-1} as measured by total soluble protein), the level of PSII inactivation of these samples was variable. Examining from 32 replicates, 8 samples of tissue-slurry-treated FIS from *G. fasicularis* exhibited >80% PSII inactivation, 16 samples exhibited levels from 20 to 80%, and 8 samples exhibited PSII inactivation <20%. The variation in PSII inactivation between samples was not correlated with the concentration of tissue slurry used (r = −0.076, p > 0.05, n = 32).

Some coral-associated bacteria might release enzymes which quickly inactivate the PSII activity of isolated *Symbiodinium* (Sussman et al. 2009). Therefore, an antibiotic cocktail developed by Polne-Fuller (1991) was supplemented into 250 ml seawater in a crystallization dish to check the retention of PSII function in the
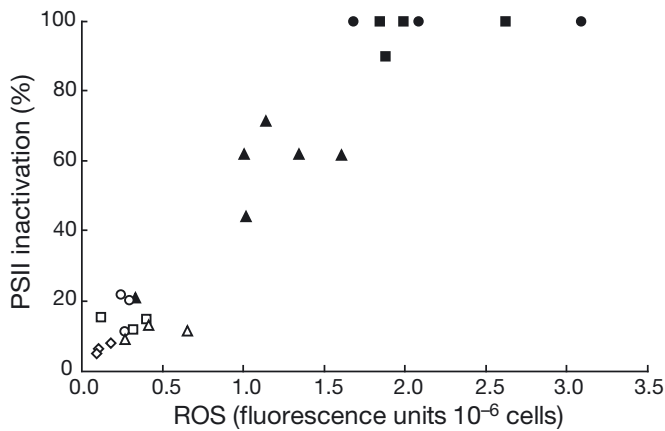
Fig. 7. ROS production and PSII inactivation in isolated *Symbiodinium*. For these comparisons, *Symbiodinium* were isolated from *Acropora humilis* (◇), *Porites lutea* (□), *Acropora nana* (△), *Stylophora pistillata* (○), *Montipora stellata* (▲), *M. digitata* (■), and *M. efflorescens* (●)

FIS from sterile *Montipora* spp. However, the coral tissue of *Montipora aequituberculata* disintegrated overnight and left a patch of *Symbiodinium* on the bottom of the crystallization dish. When *Symbiodinium* were instead obtained from *M. aequituberculata* via tissue necrosis, using Polne-Fuller's antibiotic cocktail in an overnight procedure, the released algae retained PSII function (mean ± SD F_v/F_m = 0.650 ± 0.021, n = 6).

DISCUSSION

The data in this study demonstrate that isolating *Symbiodinium* from corals into seawater, by standard methods, can induce physiological failure such as inactivation of PSII, cell-wall Na^+ channel dysfunction, ROS production, plastid membrane disruption, and degradation of PCP and protein. Moreover, these effects were coral host species-specific, with FIS from *Montipora* spp. invariably demonstrating the greatest damage. These findings show that care should be taken when using FIS to study the physiology of coral–*Symbiodinium* associations, especially when comparing different species of coral.

Previously, loss of PSII activity in FIS from *Montipora digitata* was suggested to be a consequence of vertical symbiont transmission in *Montipora* spp. (Bhagooli & Hidaka 2003). Vertical transmission through oocytes potentially creates lineages of *Symbiodinium* that never experience seawater environments (but cf. Hirose et al. 2001), and therefore lose the capability to survive outside of hosts. Horizontal transmission, in contrast, implies the existence of free-living *Symbiodinium* that must remain adapted to

seawater. In this study, however, some corals with vertical symbiont transmission (*S. pistillata*, *Pocillopora damicornis*, and *Porites lutea*) yielded FIS that retained PSII function, implying that sensitivity of FIS to seawater is not an inevitable consequence of vertical symbiont transmission.

In our study, all FIS from *Montipora* spp. exhibited severe inactivation of PSII, whereas FIS from other coral genera did not (Table 1). This observation suggests that *Montipora* spp. might harbor a distinct lineage of *Symbiodinium* that does not tolerate isolation into seawater. Indeed, previous studies of *Symbiodinium* diversity at other locations implied that *Montipora* spp. harbor a unique lineage of *Symbiodinium* (LaJeunesse et al. 2004a,b, van Oppen 2004), identified as ITS-2 type C21 (LaJeunesse et al. 2004b). However, LaJeunesse et al. (2010) reported *Symbiodinium* ITS-2 type C15 in *Montipora* spp. in Thailand, which was the predominant ITS-2 type in our *Montipora* spp. from Taiwan. Additionally, some colonies of *M. aequituberculata*, *M. efflorescence*, and *M. stellata* in our study harbored *Symbiodinium* ITS-2 type D1a. Both these ITS-2 types (C15 and D1a) are tolerant to high temperatures (LaJeunesse et al. 2003, Fabricius et al. 2004, Fisher 2006), which is consistent with the fact that our corals were collected near the effluent from a power plant (1.5 to 2°C higher than the ambient water in Kenting reefs; Meng et al. 2008). This diversity of *Symbiodinium* in *Montipora* spp. argues against the hypothesis of a distinct, seawater-sensitive lineage of *Symbiodinium* in these corals. Indeed, the ITS-2 type C15 symbionts from *Porites lutea* and ITS-2 type D1a symbionts from *Galaxea fasicularis* were observed to be relatively insensitive to isolation in seawater.

Why FIS from only *Montipora* spp. were so sensitive to isolation in seawater is not known. One possibility is that tissues of *Montipora* spp. contain inhibitory factors to which *Symbiodinium* become exposed when the tissues are disrupted. These factors could be host enzymes (Sutton & Hoegh-Guldberg 1990) or, perhaps, bacteria (Sussman et al. 2009). For example, Sussman et al. (2009) described a zinc metalloprotease produced by coral-pathogenic *Vibrio* sp. that quickly inactivated PSII of *Symbiodinium*. For a similar phenomenon to explain our results, the bacterium would have to be specific to *Montipora* spp., as all of our corals were maintained in the same recirculating aquarium and therefore might all have become infected by a generalist pathogen (Ben-Haim et al. 1999, 2003, Kushmaro et al. 2001, Bally & Garrabou 2007, Sussman et al. 2008). A bacterial zinc metalloprotease (Sussman et al. 2009) seems an unlikely explanation for our data because neither a protease inhibitor cocktail nor 50 mM EDTA protected FIS from PSII inactivation (data not presented). Also, metalloproteases would be expected to

act on *Symbiodinium* cell walls, which were not obviously disrupted when PSII was inactivated and internal plastid membranes were disorganized (Fig. 5B).

Symbiodinium experience dramatic environmental change when isolated from their host, and the physiological failure of FIS from *Montipora* spp. represents failure to maintain homeostasis under this challenge. For example, FIS isolated into seawater would experience an environmental increase in [Na^+] from ~60 mM to >500 mM (Goiran et al. 1997). In our experiments, the relatively seawater-insensitive FIS from *Galaxea fasicularis* partially maintained internal Na^+ homeostasis, whereas FIS from *M. aequituberculata* did not. It seems unlikely that an uncontrolled Na^+ shock triggered the physiological failure of FIS from *M. aequituberculata*, because PSII inactivation and ROS production were apparent within 30 min (Table 1, Fig. 6), whereas increased intracellular Na^+ was apparent only after 1 h (Fig. 3). Moreover, isolation of FIS from *Montipora* spp. using ASW with varied [Na^+] (0 to 400 mM) or [Ca^{+2}] (0 to 9 mM) did not protect PSII function (data not shown).

Attempts to demonstrate a host-tissue factor that inactivates PSII in FIS were inconclusive. Clearly, tissue slurries from *Montipora aequituberculata* had some PSII-inhibitory effect on FIS from *Galaxea fasicularis*, but the effect was inconsistent and there was no evidence of a dose-response relationship. This inconsistency contrasted with the situation for FIS from *Montipora* spp., in which PSII was always inactivated. This situation requires further study.

One significant linkage of PSII inactivation with FIS from *Montipora* spp. was ROS production during *Symbiodinium* isolation by standard methods (Figs. 6 & 7). However, the PSII inactivation in the FIS from *Montipora* spp. was completely relieved when the *Symbiodinium* were isolated by necrosis of coral tissue with an antibiotic cocktail (see 'Results: Exploration of the cause of PSII inactivation'). This antibiotic cocktail had no PSII-protective effect when FIS were isolated from hosts mechanically, by tissue blasting. Thus, it appears that these *Symbiodinium* can acclimate to seawater, but only when released from their host slowly. Overall, this study suggests that inactivation of FIS from *Montipora* spp. could be the result of rapid physical disturbance per se, which is known to be associated with ROS bursts in symbiotic or in free-living dinoflagellates (Mydlarz & Jacobs 2004). Dramatic increase in ROS is a general indicator of stress in plant cells (Lamb & Dixon 1997), and is also one of the causative agents in cnidarian bleaching (Jones et al. 1998, Downs et al. 2002, Perez & Weis 2006). ROS may attack almost all intracellular biomolecules, including proteins (Berlett & Stadtman 1997) and membrane lipids (Tchernov et al. 2004) through oxidation reactions, causing dysfunction of general cell activity. Therefore, the instant PSII

inactivation (Fig. 1, Table 1), the Na^+ ion channel dysfunction (Fig. 3), bleaching of protein-associated pigments (Fig. 4), and plastid membrane disruption (Fig. 5) in the FIS from *Montipora* spp. likely resulted from the effect of ROS produced during isolation.

In conclusion, we demonstrated that *Symbiodinium* from *Montipora* spp. do not tolerate rapid isolation into seawater by commonly used methods, as some *Symbiodinium* isolated from other species do. The available evidence implies that this is due to some property of the host corals, and not of the *Symbiodinium* themselves. These findings highlight the importance of host physiology on the physiology of *Symbiodinium*, and argue that physiological data obtained from FIS should be extrapolated to intact associations with caution, especially when comparing different species of coral.

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