Differential Regulation by Heat Stress of Novel Cytochrome P450 Genes from the Dinoflagellate Symbionts of Reef-Building Corals[▽]

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Exposure to heat stress has been recognized as one of the major factors leading to the breakdown of the coral-alga symbiosis and coral bleaching. Here, we describe the presence of three new cytochrome P450 (*CYP*) genes from the reef-building coral endosymbiont *Symbiodinium* (type C3) and changes in their expression during exposure to severe and moderate heat stress conditions. Sequence analysis of the *CYP* C-terminal region and two conserved domains, the "PERF" and "heme-binding" domains, confirmed the separate identities of the *CYP* genes analyzed. In order to explore the effects of different heat stress scenarios, samples of the scleractinian coral *Acropora millepora* were exposed to elevated temperatures incrementally over an 18-h period (rapid thermal stress) and over a 120-h period (gradual thermal stress). After 18 h of gradual heating and incubation at 26°C, the *Symbiodinium CYP* mRNA pool was approximately 30% larger, while a further 6°C increase to a temperature above the average sea temperature (29°C after 72 h) resulted in a 2- to 4-fold increase in *CYP* expression. Both rapid heat stress and gradual heat stress at 32°C resulted in 50% to 90% decreases in *CYP* gene transcript abundance. Consequently, the initial upregulation of expression of *CYP* genes at moderately elevated temperatures (26°C and 29°C) was followed by a decrease in expression under the greater thermal stress conditions at 32°C. These findings indicate that in the coral-alga symbiosis under heat stress conditions there is production of chemical stressors and/or transcriptional factors that regulate the expression of genes, such as the genes encoding cytochrome P450 monooxygenases, that are involved in the first line of an organism's chemical defense.

The cytochrome P450 (CYP) enzymes are a superfamily of heme-thiolate proteins (43). These enzymes are include different forms and are found in all aerobic eukaryotes and some prokaryotes (42). Typically, CYPs oxidize a substrate by incorporation of a single oxygen atom, which defines them as monooxygenase-type enzymes. They catalyze hydroxylation, epoxidation, N, S, and O dealkylation, N oxidation, sulfoxidation, dehalogenation, and a number of other more complex reactions (9, 27, 40). Also, CYP enzymes play a major role in xenobiotic metabolism and in phase I drug metabolism due to the oxidation of exogenous and endogenous chemicals, including drugs and carcinogens (21). As a result of the versatile biosynthetic potential of CYPs, there has been growing interest in using these enzymes in drug development and discovery of novel valuable chemicals, as well as in bioremediation (24, 26, 33, 53, 68).

The role of cytochrome P450s in xenobiotic metabolism and steroidogenesis in marine organisms has been widely recognized. CYP activity has been detected in the tissues of marine Cnidaria, Mollusca, Arthropoda, Annelida, and Echinodermata, and higher levels have usually been observed in hepatic organs and tissues carrying out steroidogenesis, as described by Rewitz et al. (48). In the phylum Cnidaria, the presence of *CYP* genes has been demonstrated using expressed sequence tag (EST) sequences from freshwater *Hydra* sp. (48), and in the sea

anemone *Nematostella vectensis*, CYPs have been recognized as a "chemical defensome" because of their role in protection against chemical stressors (25). In the scleractinian corals, CYP CO (carbon monoxide) difference spectra have been observed for the species *Favia fragum*, *Siderastrea siderea*, and *Montastraea faveolata* (22, 47). Environmentally induced changes in CYP activity were observed in the coral *Stylophora pistillata* after exposure to hyposaline conditions (18), as well as in *Madracis mirabilis* after exposure to the photosynthesis inhibitor Irgarol (17). Furthermore, inducible *CYP* expression has been demonstrated in the scleractinian coral *Montastraea faveolata* after exposure to benzo[*a*]pyrene and in *Pocillopora damicornis* after exposure to fuel oil (47, 54). The great substrate diversity of CYPs is also thought to be the result of evolutionary adaptation driven by environmental conditions (20). P450 activity involving specific xenobiotic biotransformation processes has also been reported for unicellular green algae (13, 65), while the function of CYP in the marine diatom *Skeletonema costatum* has not been determined yet (74).

Cytochrome P450 enzymes have not been found yet in unicellular photosynthetic dinoflagellates belonging to the genus *Symbiodinium*. Based on ribosomal and chloroplast DNA, symbiotic dinoflagellates are grouped into eight phylogenetic clades (clades A to H) that are further separated into distinct subclades (10, 46, 57, 58). These dinoflagellates live in mutualistic symbiosis with many marine organisms, including the reef-building corals. Rapid changes in the external environment are known to destabilize the symbiosis between corals and their dinoflagellate symbionts, leading to loss of the symbionts from the tissues of the coral hosts (29, 73). The breakdown of the symbiosis between corals and their dinoflagellates

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TABLE 1. GenBank accession numbers, designations, functions, and best BLAST (tblastn) hits (E < 1.0×10^{-5}) for *CYP* genes

Gene	GenBank accession no.	Annotation[a]	Most similar species	E value
CYP-1	EH058016	Cytochrome P450c17 (steroid 17-alpha-monooxygenase)	*Anguilla japonica*	2.00E−20
CYP-2	EH036607	Cytochrome P450	Acidobacteria ("*Candidatus* Koribacter versatilis Ellin345")	2.00E−11
CYP-3	FE864302	Cytochrome P450, family 20, subfamily A, polypeptide 1	*Danio rerio*	2.00E−34
CYP-4	EH036898	Cytochrome P450	*Aedes aegypti*	1.00E−07
CYP-5	FE865804	CYP356A1	*Crassostrea gigas*	1.00E−21

[a] In general, P450 proteins have a role in secondary metabolite biosynthesis, transport, and catabolism.

begins with photosynthetic dysfunction (11, 31) due to damage to photosystem II (2, 37, 62, 72). Overreduction of the light reactions leads to production of oxygen radicals (superoxide and singlet oxygen), which can result in damage to proteins and other cellular constituents, followed by breakdown of cell-cell signaling and removal of the symbiont (23). This is manifested at the colony level as a dramatic loss of the brown pigment of the symbiotic dinoflagellate from coral colonies (coral bleaching).

Due to the increase in carbon dioxide emission, climate change scenarios predict that global temperatures will rise at least 2°C by 2050 to 2100 (30). With a continuing global warming trend, mass coral bleaching events are predicted to increase in frequency and severity (29). Corals exposed to heat stress produce reactive oxygen species (ROS), mainly in symbiotic dinoflagellates, which destabilize the functionality of the coral-alga symbiosis (38, 73). The cytochrome P450 hemoproteins are involved in cell detoxification and protection from oxidative stress (25), and therefore we hypothesized that *Symbiodinium* CYP enzymes would be upregulated in response to thermal stress in the reef-building coral *Acropora millepora*. In the present study, we aimed to identify cytochrome P450 monooxygenase genes in the coral dinoflagellates and to evaluate the effect of a range of thermal stress scenarios on the expression of *CYP* genes and their role in the cnidarian-dinoflagellate symbiosis.

MATERIALS AND METHODS

Sequence identity and analysis. EST libraries were created for *Symbiodinium* (type C3) isolated from the coral host *Acropora aspera* after exposure to thermal and nutrient stresses (36). In the *Symbidoinium* EST database, five putative P450 homologues were identified using BLAST searches (http://blast.ncbi.nlm.nih.gov /Blast.cgi). The sequences of three putative *CYP* genes included sequences encoding two conserved regions, a "PERF domain" and a "heme-binding" region (3), and were used for evaluation. The presence of cysteine in the conserved heme-binding region was necessary since cysteine thiolate binds the heme prosthetic group as the fifth ligand. The sequences corresponding to cytochrome P450 genes and accession numbers are shown in Table 1. Sequence analysis was performed using the BLAST search options tblastx and blastx, while translated sequences were analyzed using the tblastn option and blastp. The highest-scoring hits with E-values less than 10^{-5} were used. Sequence alignment was performed using web-based BioManager-ANGIS services (http://bioman5.angis.org.au) with ClustalW (66), and alignments were viewed using GeneDoc software.

Experimental design. Coral nubbins (length, 4 to 5 cm) of *A. millepora* harboring the *Symbiodinium* C3 type (35) were collected in May 2009 from the reef flat surrounding Heron Island, Great Barrier Reef, Australia (23°25′S, 152°07′E). Coral nubbins were removed using wire cutters and immediately transported to a seawater facility. Corals nubbins were then attached to nylon threads and suspended in flowthrough seawater aquaria. Samples were acclimated for 48 h in 9 large experimental aquarium tanks (60 liters; 3 tank replicates per treatment) at the winter mean local ambient temperature (23 to 24°C) and at the ambient light levels, which were adjusted using shade cloth to mimic the conditions of the natural reef flat. To examine the influence of temperature stress, coral nubbins were distributed randomly in systems subjected to two experimental temperature regimens (Fig. 1), a gradual thermal stress regimen (temperature increased from 23°C to 32 ± 1°C at a rate of 1°C per ∼10 h over a 120-h period) and a rapid thermal stress regimen (temperature increased from 24°C to 32 ± 1°C at a rate of 1°C per ∼2.5 h over an 18-h period), as well as in a control group (temperature kept at 23 to 24 ± 1°C [ambient temperature]). The gradual thermal stress regimen was relevant to the natural bleaching conditions on the Great Barrier Reef, where the temperature was reported to be between 31 and 32°C over a 5-day period (5, 6, 15), while the rapid thermal stress regimen was more representative of the Heron Island reef flat dynamics observed in 2002, when the temperature oscillated between 25°C and 32°C over an approximately 24-h period and the maximum rate of increase was ∼2°C per h (15). The water temperature was recorded every 2 min using StowAway TidbiT loggers placed in each aquarium (Onset Computer Corporation, Bourne, MA). A subset of coral branches ($n = 4$) was removed from the treatment and control tanks at 0, 18, 72, and 120 h. Corals nubbins were immediately snap-frozen in liquid nitrogen and stored at −80°C before they were processed.

Extraction of total RNA. RNA was separately extracted from 4 coral branches using small fragments (length, 0.5 to 1 cm) of coral nubbins, which were cut with a bone cutter and ground directly in liquid nitrogen. The resulting powder was put in 700 ml of RLT buffer with beta-mercaptoethanol (RNeasy kit; Invitrogen), homogenized with a hand homogenizer (Tissue-Tearor; Biospec Products, Inc.), and centrifuged for 3 min at 13,000 × g at 4°C. The aqueous phase was then used to extract total RNA with an RNeasy kit (Invitrogen) by following the manufacturer's instructions. RNA quantity and integrity were analyzed with an Agilent 2100 bioanalyzer, and 500 ng of high-quality total RNA (integrity number, >7) was subjected to reverse transcription (RT) for cDNA synthesis.

Synthesis of cDNA for qPCR. Reverse transcription was carried out using the QuantiTect reverse transcription procedure (Qiagen, Australia). Briefly, 0.5 μg of purified total RNA was used per reaction mixture and incubated in genomic DNA wipeout buffer at 42°C for 2 min to eliminate any traces of genomic DNA,
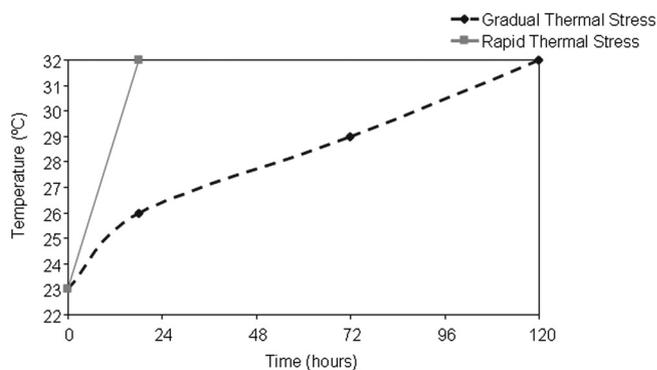

FIG. 1. Stress regimens. Coral nubbins were exposed to two stress regimens, a gradual thermal stress regimen (temperature increased at a rate of 1°C per ∼10 h) and a rapid thermal stress regimen (temperature increased at a rate of 1°C per ∼2.5 h), as well as a control regimen (temperature stable at 23 to 24 ± 1°C [ambient temperature]). The nubbins were exposed to the rapid thermal stress regimen for 18 h and to the gradual thermal stress and control regimens for 120 h.

TABLE 2. Primer sequences, amplicon lengths, and melting temperatures for the primers used for real-time RT-PCR analysis

| Gene | Forward primer | | Reverse primer | | Amplicon length (bp) |
	Sequence (5′–3′)	Melting temp (°C)	Sequence (5′–3′)	Melting temp (°C)	
CYP-1	CCCGAACGTTTCTTGGACTCT	60	TCAGTTTTGGCCAGGGACTCT	60	120
CYP-2	GCATGCTGACGCACAGTGA	59	GCCAAAAGGCATGTGCATCT	60	101
CYP-3	TGTGGCCAGAACCTCAAAGG	60	CTTTCCCGCAAAGCCAAAT	59	101

and this was followed by reverse transcription at the same temperature for 30 min. The cDNA obtained, diluted 1:10 prior to use, was used as a template in the quantitative PCR (qPCR) analysis. In parallel, RNA isolation (52) and cDNA synthesis using the QuantiTect reverse transcription procedure (Qiagen, Australia) were carried out for an *A. millepora* egg-sperm sample (free of algal symbionts).

Primer design. The sequences corresponding to cytochrome P450 genes were identified by performing a BLAST search with the *Symbiodinium* (C3) EST database, as shown in Table 1. Specific primers were designed using Primer Express 2.0 software (Applied Biosystems, United States). The designations of the genes used for real-time RT-PCR analysis, GenBank accession numbers, primer sequences, amplicon lengths, and melting temperatures are shown in Tables 1 and 2. Standard PCR amplification was performed using selected primers (Table 2) to confirm the reproducibility of the primers used for *Symbiodinium* (C3) isolated from *A. millepora* and the lack of amplification for coral. The PCR conditions were as follows: an initial step consisting of 94°C for 1 min, followed by 35 cycles of 94°C 20 s, 60°C 20 s, and 72°C 1.5 min and then extension at 72°C for 10 min and storage of samples at 4°C.

Quantitative PCR and gene expression analysis. Quantitative PCR assays were performed using SYBR green PCR master mixture (Applied Biosystems, Warrington, Cheshire, United Kingdom), 384-well plates, a 7900HT fast real-time PCR system (Applied Biosystems, United States), and an Eppendorf 5075 robot (Applied Biosystems, United States). The PCR conditions were as follows: initial denaturation for 10 min at 95°C, followed by 45 cycles of 95°C for 15 s and 60°C for 1 min. At the end of the procedure, a dissociation step was included, which consisted of 95°C for 2 min, 60°C for 15 s, and 95°C for 15 s. The final reaction volume was 10 μl, and the mixture contained 300 nM primers (Table 2). All reactions were carried out with three technical replicates. The levels of expression of targeted cytochrome P450 genes were quantified using geNorm software (http://medgen.ugent.be/~jvdesomp/genorm/) (69). The relative quantification method was used for estimation of the expression of cytochrome P450 genes by using reference genes (Table 3) with the most stable expression patterns and specificity for *Symbiodinium* cultures under different experimental conditions (N. Rosic, M. Pernice, and O. Hoegh-Guldberg, submitted for publication). The expression of each gene was determined from the cycle threshold (C_T) value, which corresponded to the number of cycles required for the fluorescent signal from PCR amplification to reach a fixed threshold (71). C_T values were obtained for a specific threshold of 0.1 and were transformed into quantities using the maximal PCR efficiency for each gene (E = 2). A real-time dissociation curve was used to check primer specificity and the presence of a unique PCR product.

Statistical analyses were done using the Statistica 7.0 software (Statsoft Inc., Tulsa, OK). Data normality was confirmed using the Kolmogorov-Smirnov test, and the influence of time and treatment on gene expression was tested by performing a two-way analysis of variance (ANOVA). The levels of expression of the algal *CYP* genes isolated from thermally stressed corals were compared to control levels of expression at each time point (for coral nubbins not exposed to elevated temperatures) using a *t* test ($n = 4$). Values were considered significantly different if the *P* value was <0.05. In this paper data are expressed as means ± standard deviations.

RESULTS

Sequence analysis of the *Symbiodinium CYP* genes. Five contigs corresponding to *CYP* genes with representative ESTs are shown in Table 1. Multiple-sequence alignment of the three *CYP* contigs with sequences corresponding to the C-terminal region including the conserved "PERF" domain (PXFX) and "heme-binding" region (FXXGXRXCXG) was performed with ClustalW (66). Three *CYP* genes were designated *CYP*-1, *CYP*-2, and *CYP*-3, and an alignment of the sequences encoded by these genes is shown in Fig. 2. The EST sequences of two other contigs, *CYP*-4 and *CYP*-5, corresponded to sequences encoding the N-terminal CYP protein region and therefore were not included in the evaluation described below. The sequence analysis of *CYP*-1, *CYP*-2, and *CYP*-3 confirmed the variability of the sequences of the *CYP* genes. At the protein level, despite considerable sequence differences, two well-conserved domains in the C-terminal region of CYP enzymes, the PERF domain (PXRX) and the absolutely conserved heme-binding region (FXXGXRXCXG), were recognized, and they are indicated in Fig. 2. Variants of the conserved PERF domain were found in the sequences encoded by all *Symbiodinium CYP* genes; there was a PERF region in the sequence encoded by *CYP*-1 and there was a PERM region in the sequence encoded by *CYP*-2, while a PDRF domain was found in the sequence encoded by the *CYP*-3 gene. The heme-binding domain was conserved in all translated *CYP* genes. However, a slight variation in the heme-binding region encoded by the *CYP*-3 gene was noticed; an extra amino acid was found between highly conserved F and G residues (Fig. 2).

The closest BLAST (tblastn) hits (E value, $<1.0 \times 10^{-5}$) for the deduced protein sequences are shown in Table 2. For the *CYP*-1 gene the C-terminal domain data indicated that there was a metazoan origin, and the closest relationship was the relationship with the cytochrome P450 steroid 17-alpha-hydroxylase/17,20-lyase found in the Japanese eel (*Anguilla japonica*), a frog (*Xenopus laevis*), and the zebrafish (*Danio rerio*). In contrast, the C-terminal domain of the *CYP*-2 gene product exhibited the highest levels of similarity with the C-terminal region of a putative cytochrome P450 in unicellular

TABLE 3. GenBank accession numbers, functions, and primer sequences for housekeeping genes used for real-time RT-PCR analysis

| Gene	Function	Forward primer (5′–3′)	Reverse primer (5′–3′)	GenBank accession no.
Cal	Calmodulin	GGCACCATCACCACCAAAG	TCTGCGTCAACCTCGTTGATC	EH037752
Rp-S4	Ribosomal protein S4	CCGCACAAACTGCGTGAGT	CGCTGCATGACGATCATCTT	EH036413
SAM	*S*-Adenosyl-L-methionine synthetase	GCCTACATTTGCCGACAGATG	AATGGCTTGGCAACACCAAT	EH036622
Tub	Beta-tubulin	TGACGCAGCAGATGTTTGATG	CGACATACGTCCACGGAAGAG	EH037669
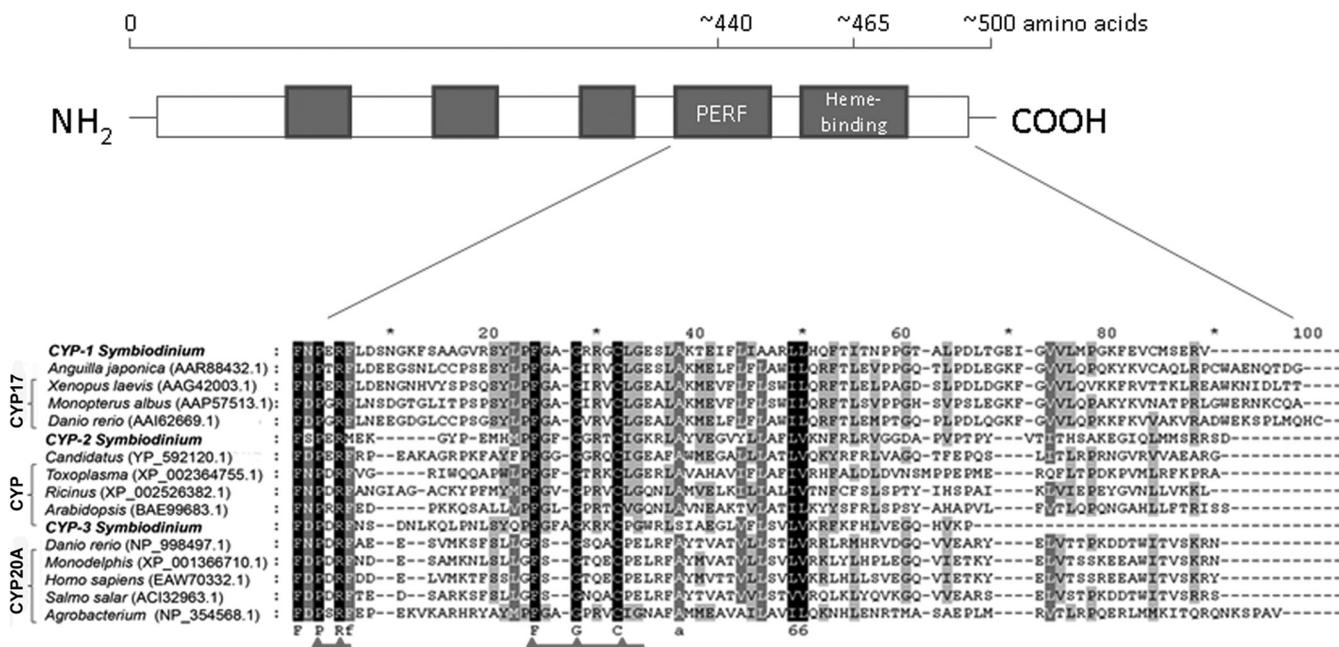
FIG. 2. Diagram of the cytochrome P450 primary structure (48), showing the distribution of the major conserved domains (filled boxes), and multiple-sequence alignment of the *Symbiodinium* CYP C-terminal regions, including the conserved PERF domain (PXRF) and heme-binding region (FXXGXRXCXG), constructed with Clustal W. Alignment was performed using the following pairwise alignment options: Blossum30 protein weight matrix, a gap-opening penalty of 10, and a gap extension penalty of 0.1. Predicted amino acid sequences encoded by the *Symbiodinium* ESTs designated *CYP*-1, *CYP*-2, and *CYP*-3 were aligned with the selected sequences producing the best hits according to a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The identical residues in all sequences are indicated; amino acids conserved in 100% of the sequences are indicated by white letters with a black background, amino acids conserved in >80% of the sequences are indicated by white letters with a gray background, and amino acids conserved in >60% of the sequences are indicated by black letters with a gray background.

microorganisms (*Acidobacteria* and *Toxoplasma gondii*) and higher plants (*Arabidopsis thaliana* and *Nicotiana tabacum*). A metazoan origin was again indicated for the *CYP*-3 gene product, and the closest connection was the connection with the sequence encoding an "orphan" cytochrome P450 (family 20, subfamily A, polypeptide 1) found in *Homo sapiens*, zebrafish, *Salmo salar*, and a marsupial (*Monodelphis domestica*). Based on data for the C-terminal region, all three *CYP* genes found in symbiotic dinoflagellates contained novel sequences compared to the genes encoding other CYP hemoproteins. In addition, due to symbiosis and the pool of mixed algal and coral transcriptomes, we examined whether three *CYP* genes were present in a dinoflagellate-free coral host using cDNA obtained from an *A. millepora* egg-sperm specimen. Our results confirmed that the *CYP* genes discovered did not have a coral origin (PCR amplification was not successful).

Effect of elevated temperatures on *CYP* gene expression. A real-time dissociation curve confirmed the presence of a specific PCR product in all amplification reactions. The relative quantities of *CYP* genes were normalized using the quantities of two reference genes with the most stable expression pattern as defined by the GeNorm analysis (69). The housekeeping genes used were the ribosomal protein S4 (Rp-S4) and *S*-adenosyl-L-methionine synthetase (SAM) genes, which had an average expression stability value (M) of 0.351 and a pairwise variation for $V2/3$ of 0.137. In Fig. 3, the ratios of the *CYP* gene expression for the treatment to the *CYP* gene expression for the control at different time points are shown for *CYP*-1 (Fig. 3A), *CYP*-2 (Fig. 3B), and *CYP*-3 (Fig. 3C). During gradual thermal stress there was a significant inter-

action between time and treatment for the levels of *CYP*-1, *CYP*-2, and *CYP*-3 expression ($P < 0.001$, two-way ANOVA). However, elevated temperatures affected the transcript abundance of each *Symbiodinium CYP* gene compared to the control at each time point. Increases in *CYP* gene expression were observed after 18 h of incubation at 26°C, and significant increases ($P < 0.01$) were observed for *CYP*-1 (30%) and *CYP*-2 (40%). In contrast, rapid heat stress with the temperature reaching 32°C after 18 h led to significant decreases in the *CYP* mRNA levels ($P < 0.01$) and nearly 50% reductions in *CYP*-1 and *CYP*-2 expression and a ~90% reduction in *CYP*-3 transcript abundance (Fig. 3). Under more moderate heat stress conditions, the *CYP* gene expression significantly increased ($P < 0.01$) after 72 h of incubation at 29°C; the expression of *CYP*-1 and *CYP*-2 increased 2.4-fold (240%), whereas a 4.2-fold (420%) increase was observed for *CYP*-3. Again, gradual extended exposure to heat stress with the temperature reaching 32°C after 120 h resulted in reductions in the *CYP* transcript levels compared to the control levels of approximately 66% for *CYP*-1 and *CYP*-2 transcripts ($P < 0.01$) and of 41% for *CYP*-3 transcripts ($P < 0.05$).

DISCUSSION

Elevated sea temperatures have been recognized as one of the major factors leading to coral bleaching (29); consequently, the possible role of new *Symbiodinium* CYP enzymes in the response to heat stress in a coral host was examined in this study. Over the last decade the use of high-throughput sequencing technologies has led to the discovery of new genes,
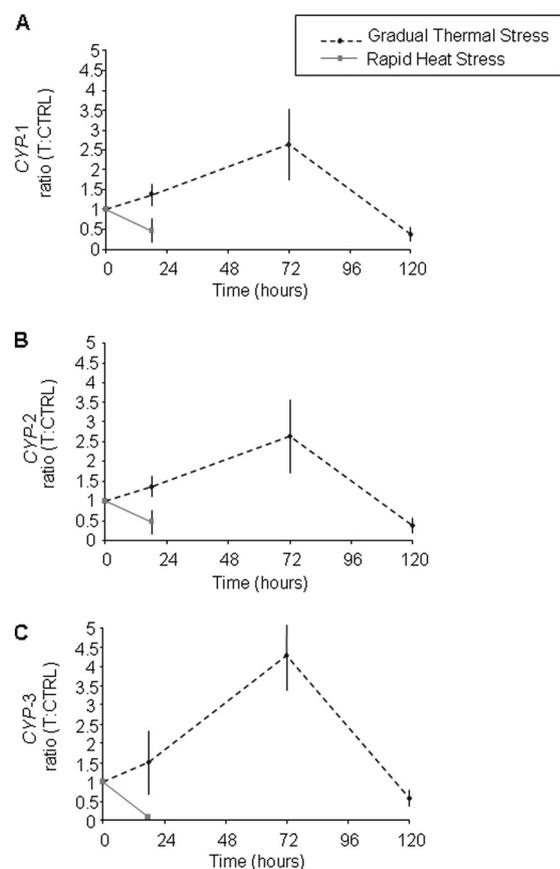
FIG. 3. Regulation of *CYP* gene expression in *Symbiodinium* symbiotic with a coral host (*A. millepora*) exposed to rapid and gradual thermal stress regimens with a maximum temperature of 32°C. The relative quantities were normalized using data for two of the most stable reference genes defined by the GeNorm analysis, the Rp-S4 and SAM genes ($M = 0.351$) with pairwise variations for $V2/3$ of 0.137. The ratio of the CYP gene expression for the treated sample to the CYP gene expression for the control at each time point (T:CTRL) is shown for *CYP*-1 (A), *CYP*-2 (B), and *CYP*-3 (C). All data are the means ± standard deviations of values obtained for four independent biological replicates.

followed by identification of new functions and pathways. Existing EST databases have increased our knowledge about functional genomics and provided a baseline for further functional characterizations and discoveries (64). In these terms, EST sequences were also a helpful tool for obtaining information about enzyme identity and phylogeny (56). In this study, using the *Symbiodinium* (C3) EST database (36), we identified three *CYP* genes that were characterized by sequences encoding a unique C-terminal region and preserved conserved PERF and heme-binding domains. In all of the sequences encoded by the putative *CYP* genes, we recognized an absolutely conserved cysteine in the heme-binding region, confirming the heme-thiolate nature of the enzymes discovered (43). An overview of the CYPs' conserved domains typically found in the majority of membrane-bound CYPs (48) is shown in Fig. 2. The majority of eukaryotic P450 proteins are membrane-bound proteins with molecular masses of approximately 50 kDa and have a C terminus surrounding the cysteine residue and a hydrophobic N-terminal membrane domain (70). The molecular evolution of the CYP superfamily, in contrast to many other protein

families, does not reflect its phylogeny due to numerous genetic changes, including horizontal gene transfer, gene duplications and fusions, and intron loss (12).

The sequence analyses at the protein level showed that the product of the putative *CYP*-1 gene had the greatest similarity to a P450 steroid 17-alpha-monooxygenase (Table 1). This CYP is well known for its role in steroid metabolism, catalyzing both steroid 17-hydroxylation and C17,20-lyase reactions (34, 75). In mammals, cytochrome P450 17α-hydroxylase (CYP17) is a membrane-bound hemoprotein necessary for maintenance of sexual characteristics and is also involved in the stress response (34). The implications of the steroid metabolism in symbiotic dinoflagellates for the steroid signaling pathways between algal symbionts and their coral hosts are not clear and require further study. Identification of the *CYP*-2 gene as a gene encoding a P450 monooxygenase was based on the presence of conserved domains, but there was great sequence variability in the nonconserved regions compared to other P450 family members, suggesting that it may belong to a new CYP family (Fig. 2). Conversely, the *Symbiodinium CYP*-3 gene product showed a high level of sequence similarity with the known cytochrome P450 CYP20A1. Because its biological function is not known, this CYP20A1 protein is the so-called "orphan" P450 (28, 60, 61), and its possible role during heat stress in the coral-alga symbiosis has not been elucidated yet. The high level of similarity of the *CYP*-1 and *CYP*-3 sequences to the sequences of metazoan P450 genes suggests that there might have been host contamination, while our results for aposymbiotic *A. millepora* coral egg-sperm specimens indicated that *CYP* genes discovered did not originate from coral. Further molecular information is needed to explore the possibility of horizontal gene transfer, which has been found, for example, in the *Nematostella* genome for microbial genes involved in the shikimic acid pathway (59).

For all three *CYP* genes, similar expression patterns were induced by heat stress. Upregulation of *CYP* mRNA synthesis by moderately elevated temperatures (26°C and 29°C) was followed by a decrease in *CYP* mRNA abundance after the highest temperature (32°C) was reached (Fig. 3). An initial increase in *CYP* gene abundance under moderate heat stress conditions might occur due to the involvement of CYP enzymes in protection of cells from oxidative stress. Together with other oxidative detoxification proteins, members of the cytochrome P450 protein superfamily are involved in an organism's defense against chemical stressors (25). It has been shown that changes in the expression profile of oxidative stress enzymes are likely to occur first in algal symbionts (51). Also, oxidative stress and ROS production that occur first in symbiotic dinoflagellates may lead to initial upregulation of *CYP* mRNA synthesis. However, the negative effects of a very high temperature (32°C) on *CYP* transcript abundance under both rapid and gradual heat stress conditions may be an indication of irreversible cellular changes. Similarly, photosynthesis was greatly impaired in cultured *Symbiodinium* exposed to temperatures above 30°C (32). In addition, a decrease in the *Symbiodinium* cell density in the coral tissue was noted during heat stress treatments, whereas a significant decrease in the number of algal cells was observed after 72 and 120 h (data not shown), indicating that there was coral bleaching. During heat stress-induced bleaching, the rate of cell death increased for both the

cnidarian host and the endosymbiotic algae (19), and the role of the host was proposed to be protection of its algal symbionts from oxidative stress (49).

In other organisms, P450 activity has also been affected by temperature conditions. A positive effect on P450 expression has been observed in mice exposed to heat stress while they are consuming infected food (7), whereas downregulation of the P450 17-alpha hydroxylase and P450 aromatase mRNA levels was observed in hens subjected to hyperthermal conditions (55). Additional changes in the environment, such as hyposalinity conditions (18) and exposure to toxic substances (47, 54), also affected CYP activity in scleractinian corals and other marine organisms. In two unicellular green algae, cytochrome P450 monooxygenase activity was reported during biotransformation of herbicides (65). In plants, the cytochrome P450 enzymes play a major role in one of the nitric oxide (NO) biosynthetic pathways, catalyzing the oxidation of N-hydroxyguanidines and the formation of nitrogen oxides, including NO (39). NO signaling has been found to be involved in the induction of cell death and in interactions with ROS (44), and several studies have reported links between NO synthesis and the breakdown of a cnidarian-alga symbiosis (8, 45, 67). Also, ROS generated during thermal stress in symbiotic dinoflagellates (16, 41) has been related to coral bleaching (38, 50, 73).

Similarities in the changes in expression between *CYP* genes suggest that there are comparable regulation mechanisms and that CYP enzymes may be involved in related oxidative stress metabolic pathways. Previous studies have reported that increases in temperature induced oxidative stress which was related to the first signs of breakdown in the functionality of the coral-alga symbiosis, leading to the coral bleaching phenomenon (38, 73). As thermal stress is one of the major environmental stressors challenging the well-being of the coral reef ecosystem and the stability of the coral-alga symbiosis, it has been predicted that a further increase in the average ocean temperature will lead to mass coral bleaching, followed by a decline in coral cover and changes in the composition of coral species due to various levels of susceptibility and in other coral reef organisms (14). On the other hand, the diversity of symbiotic algae and their ability to change their composition might affect the ability of coral to respond to future increases in temperatures (1, 4, 63). Understanding the mechanisms that regulate the genes encoding widespread enzymes, such as cytochrome P450s, which are involved in the first line of defense in the organisms exposed to various stressors, should bring us a step closer to obtaining a more complete picture of the stress responses in symbiotic dinoflagellates exposed to elevated temperature and to understanding the susceptibility of *Symbiodinium* and the reef-building corals to global climate change.

ACKNOWLEDGMENTS

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