AN ABSTRACT OF THE DISSERTATION OF


Title: Exploring the Cellular Mechanisms of Cnidarian Bleaching in the Sea Anemone Aiptasia pallida

Abstract approved: __________________________

Virginia M. Weis

Many members of the Phylum Cnidaria are mutualistic with unicellular dinoflagellates belonging to the genus Symbiodinium. Corals are the most widely recognized example of these associations due to their key ecological importance in coral reef ecosystems where they serve as the structural and trophic foundation of these rich ecosystems. Coral reefs are severely threatened by human activities worldwide and are at great risk from global climate change, in particular the increase in sea-surface temperatures. Detailed knowledge of how corals respond to stress is scarce. The most serious and immediate response of corals to environmental stress is a process referred to as coral bleaching (a.k.a. cnidarian bleaching). Nevertheless, the cellular and molecular processes by which elevated temperatures elicit the bleaching response are poorly understood. This dissertation deals with this important question by describing two mediators of cnidarian bleaching in the model symbiotic tropical sea anemone Aiptasia pallida (Verril), namely nitric oxide and cyclophilin.

After an introduction to the topic of cnidarian-algal symbioses and cnidarian bleaching (Chapter 1), I present results from a study describing the involvement of nitric oxide (NO) in the anemone A. pallida (Chapter 2). Elevated temperature as well
as oxidative stress induces production of NO and exposure of *A. pallida* to NO induces bleaching at non-stressful temperatures. Co-incubation with an NO scavenger suppresses bleaching. I propose that the host up-regulates NO production in response to elevated oxidative stress and that this situation leads to cytotoxicity and bleaching.

Chapter 3 examines the role of cyclophilin from *A. pallida* in the regulation of the symbiosis. Cyclophilins belong to a highly conserved family peptidyl-prolyl *cis-trans* isomerases (PPIases). Incubation of *A. pallida* with cyclosporin A (CsA), a potent inhibitor of cyclophilin resulted in bleaching and a decrease in tolerance to elevated temperatures. Protein extracts from *A. pallida* exhibited CsA-sensitive PPIase activity. Laser scanning confocal microscopy using superoxide and nitric oxide-sensitive fluorescent dyes on live *A. pallida* revealed that CsA strongly induced the production reactive oxygen species as well as NO. We tested weather the CsA-sensitive isomerase activity is important for maintaining the activity of the antioxidant enzyme superoxide dismutase (SOD). SOD activity of protein extracts was not affected by pre-incubation with CsA *in vitro*. In Chapter 4 I review what is known about the molecular and cellular mechanisms of bleaching and describe a model of bleaching based on the results presented herein as well as studies of non-cnidarian models.
Exploring the Cellular Mechanisms of Cnidarian Bleaching in the Sea Anemone
*Aiptasia Pallida*

by

Santiago F. Perez

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Santiago F. Perez, Author
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CHAPTER 1: INTRODUCTION

Our knowledge of processes such as cell signaling, gene expression, development and differentiation, and the cellular stress response have expanded dramatically due to the application of modern molecular and cellular techniques. These processes take place through complex molecular interactions within as well as between cells. Another level of interaction encompasses the molecular communication that can occur between cells of different species. Studies of these phenomena including the study of pathogenicity, disease and immune function, have in part led the beginnings of modern biology. In contrast, mutualistic symbioses, or the beneficial close interactions between species, have only recently begun to be studied in detail.

Among the mutualisms studied, perhaps the most interesting from the perspective of interspecies interactions at the molecular and cellular level are the endomutualisms in which the smaller symbionts are housed within the confines of the host cell. In these systems, the cellular and molecular machinery of the partners can interact intimately during the events of initial recognition and specificity through maintenance and even during the collapse of the association. The study of endomutualisms has shed light on our understanding of the origin and evolution of the eukaryotic cell as well as our understanding of ecologically important systems such as
plant mycorrhizae and legume nitrogen-fixing bacterial associations. Some ecologically and evolutionarily important mutualisms are relatively poorly studied at the molecular and cellular levels. The system which is focus of this dissertation belongs to this category, the mutualism between members of the phylum Cnidaria and their endosymbiotic dinoflagellate algae.

The Phylum Cnidaria includes mostly marine groups such as jellyfish, hydroids, anemones and corals. Many members of this phylum are mutualistic with unicellular algae, most commonly the photoautotrophic dinoflagellate Symbiodinium spp. The characteristic dinoflagellate pigments give a yellow-brown appearance to the host tissues and for this reason these algae are commonly referred to as zooxanthellae. Corals are perhaps the most widely recognized example of these associations due to their key ecological importance in coral reef ecosystems. Corals form the structural and trophic foundation of coral reef ecosystems. Most coral species are obligate hosts and so without their algal partners their fitness and ability to create coral reef habitats is critically reduced.

Given that coral reef systems are of great economic, biological and aesthetic importance, the need for study of the symbioses upon which they are based is fundamentally justified. Greatly amplifying this need, coral reefs are severely threatened by human activities worldwide and are at great risk from global climate change. Over the last several decades, these stresses have resulted in massive losses
of coral abundance and biodiversity. Yet detailed knowledge of how corals respond to stress is scarce. The most serious and immediate response of corals to environmental stress is a process referred to as coral bleaching (a.k.a. cnidarian bleaching). Bleaching results from the loss of a large percentage of the algae from host tissues (Figure 1.1). This loss of algae is often lethal, particularly in the case of corals (Glynn, 1993, Hoegh-Guldberg, 1999a). Hosts may bleach in response to a variety of stresses; however, elevated temperature and excessive UV radiation, often acting synergistically, are well documented as the dominant causes of coral bleaching (Cook et al., 1990, Gates et al., 1992, Glynn and D'Croz, 1990, Hoegh-Guldberg and Smith, 1989). However, the cellular and molecular processes by which elevated temperatures elicit the bleaching response are poorly understood.

Some corals are more tolerant of hyperthermic bleaching than others and tolerance may depend on previous exposures or acclimation (Fitt and Warner, 1995b). The observed differences in tolerance to elevated temperature also result from differences in the tolerance of the algal species or strains (phylotypes) present (Buddemeier and Fautin, 1993, Perez et al., 2001b, Rowan et al., 1997). Of great debate is the adaptive bleaching hypothesis, which views bleaching as having an adaptive value by allowing the host to rid itself of a stress-susceptible phylotype of algae in order to increase the chances of becoming repopulated with zooxanthellae that are more tolerant of the new environmental regime (Buddemeier and Fautin, 1993). Since we do not fully understand the bleaching mechanism, however, we also do not
know how the differential physiological susceptibility of the algae translates
mechanistically into differential bleaching. Understanding the mechanisms behind
bleaching will provide valuable insights into the adaptability and resilience of these
critically threatened symbioses.
Figure 1.1 Colony of a bleaching hermatypic coral in the Florida keys after a bleaching episode (*Montastrea* sp., summer 1998). Brown patches are tissues that still harbor algae. The white coral skeleton can be seen through tissues devoid of algae. Corals that remain extensively bleached for a prolonged period of time often die, as did this colony.
THE BIOLOGY OF CNIDARIAN-ALGAL SYMBIOSES

Cnidarians have a simple tissue construction, composed of two tissue layers, an outside epidermis and an inner endoderm separated by an acellular mesoglea (Fig. 1.2). The body form present in corals and anemones is the polyp, which has a tubular shape, with an aboral pedal disc attached to the substrate and an oral disc raised by the body column. The oral disc contains a mouth surrounded by numerous stinging tentacles. The mouth is the only body opening and leads to a single digestive cavity, the coelenteron, which is continuous with the inside of the hollow tentacles. The endoderm lines this inside space and glandular digestive cells, gonadal cells and phagocytic cells which, in cnidarian-algal symbioses, become hosts to endosymbiotic algae.

Figure 1.2 Anatomy of a cnidarian-algal symbiosis
Cross-section of tentacle shows location of host cells containing the endosymbiotic algae and relationship with the gastric cavity continuous down the length of the tentacle. The mesoglea is very thin in *A. pallida* and is not detailed in this diagram.

The algae within host cells are coccoid in shape with a thin cell wall and no flagella. The algae are taken up from the environment through phagocytosis (Schwarz *et al.*, 1999, Schwarz *et al.*, 2002). These are then contained inside the host cell within a phagocytically-derived membrane. The different phylotypes of *Symbiodinium* are largely defined by molecular criteria, specifically by analysis of ribosomal RNA sequences. Members of *Symbiodinium* are grouped into several clades of varied resolution depending on the molecular markers used in the phylogenetic inferences (Lajeunesse, 2005, Rowan, 1998, Rowan and Powers, 1991, , 1992, Santos *et al.*, 2002). The taxonomy of the group is made difficult by the of lack useful species-defining morphological characteristics and by the apparent absence of sexual reproduction. Soon after uptake of algae from the environment, there is a poorly understood process specificity through which incompatible phylotypes are lost (Rodriguez-Lanetty *et al.*, 2004, Weis *et al.*, 2001, Wood-Charlson *et al.*, 2006).

Once established, the algae benefit by being in a relatively constant environment and in a refuge from herbivory. These symbioses are also centered on nutrient exchange. The algae receive nutrients such as inorganic nitrogen, phosphate and carbon as byproducts from host catabolism. The algae grow and divide within the host cell which can then contain multiple numbers of algal cells. The host benefits by
obtaining a significant amount of reduced carbon substrates (photosynthate) fixed by the algae. The photosynthate is mostly in the form of glycerol, glucose and some amino acids. This translocated reduced carbon can sometimes account for 100% of the daily respiratory need of the host (Dubinsky, 1994, Gattuso et al., 1993). The tight mutual cycling maximizes retention of nutrients and minimizes losses to the environment. This is of great importance especially in nutrient-poor environments such as the tropical waters where these mutualisms are particularly abundant (Muscatine and D'Elia, 1978). Given the important mutual benefits to the partners of this symbiosis it is no surprise that its collapse in response to environmental stress imposes such detrimental effects on the fitness of the partners.

In this study I examine two cellular processes hypothesized to play important roles in the bleaching mechanism: the role of nitric oxide produced by zooxanthellae (Chapter 2) and the role of the molecular chaperones known as cyclophilins (Chapter 3). These studies were performed on the symbiotic anemone Aiptasia pallida, a species which has been used as a model symbiotic system (Figure 1.3). In Chapter 4 I review what is known about the mechanisms behind cnidarian bleaching and present new view of the breakdown of cnidarian-algal symbiosis as a conserved innate immune response leading to activation of programmed cell death pathways.
Figure 1.3. Close-up of a small symbiotic specimen of *Aiptasia pallida*
Its small size as a newly developed asexually derived polyp makes this anemone useful for laser scanning confocal microscopy, a technique utilized in this dissertation. Diameter of oral disc is approximately 3mm.
REFERENCES


EXPLORING THE CELLULAR MECHANISMS OF CNIDARIAN BLEACHING IN THE SEA ANEMONE *Aiptasia pallida*

CHAPTER 2: NITRIC OXIDE AND CNIDARIAN BLEACHING: AN EVICTION NOTICE MEDIATES THE BREAKDOWN OF SYMBIOSIS

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ABSTRACT

Nitric oxide (NO) is a free radical implicated in numerous cell signaling, physiological and pathophysiological processes of eukaryotic cells. Here we describe the production of NO as part of the cellular stress response of the symbiotic sea anemone *Aiptasia pallida* which hosts dinoflagellates from the genus *Symbiodinium*. We show that exposure to elevated temperatures induces symbiotic anemones to produce high levels of NO leading to the collapse of the symbiosis. These results shed light on the poorly understood cellular mechanism through which elevated sea water temperature causes the release of symbiotic algae from symbiotic cnidarians, a detrimental process known as coral (cnidarian) bleaching. The results presented here show that the host cell is a major source of NO during exposure to elevated temperatures and that this constitutes a cytotoxic response leading to bleaching. These results have important evolutionary implications as the observed NO production in these basal metazoans displays many parallels to the cytotoxic inflammatory response to pathogens, a well understood process in mammalian model systems.
INTRODUCTION

Many cnidarians symbiotic with the intracellular dinoflagellate *Symbiodinium* sp., such as the ecologically important reef-building corals, lose their algal partners in response to a variety of stresses, including exposure to elevated temperature and excessive visible and UV radiation (Gates *et al.*, 1992, Glynn and D'Croz, 1990). This process, known as coral (cnidarian) bleaching, is often lethal to the host and can have devastating ecological effects on the coral reef ecosystem (Glynn, 1996, Hoegh-Guldberg, 1999b). In cnidarian-dinoflagellate symbioses, exposure to elevated temperature can result in inhibition of algal photosynthesis and increased production of reactive oxygen species (ROS) such as superoxide radical (Franklin, 2004, Lesser, 1996a, Lesser *et al.*, 1990, Perez *et al.*, 2001a). The increase in ROS production is hypothesized to increase cellular damage and lead to bleaching, however, the cellular and molecular mechanisms leading to the loss of algae is poorly understood.

Cnidarian bleaching is known to be mediated in part through apoptosis and necrosis leading to the loss of integrity of gastrodermal tissue which contains the algae (Dunn *et al.*, 2004). However, the events that lead to host cell death have not been studied in detail. In other systems, an important mediator of cell death due to oxidative stress is the reactive nitrogen species peroxynitrite which is a reaction product of superoxide and nitric oxide (NO) (Radi, 2000). In animals, NO is produced by a family of enzymes known as nitric oxide synthases (NOS; EC 1. 14. 13. 39). These enzymes catalyze the conversion of arginine, NADPH and O₂ to NO, citrulline
and NADP⁺. Early evidence suggests that NO is produced in symbiotic cnidarians and that it may play a role in bleaching. In the symbiotic anemone *Aiptasia pallida*, an arginine-dependent NOS-like activity has been reported (Trapido-Rosenthal et al., 2001). Further, NO was produced in freshly isolated algae from the coral *Madracis mirabilis* and was implicated in the bleaching process (Trapido-Rosenthal et al., 2005).

We were interested, therefore, in further investigation of NO in symbiotic cnidarians and its potential cellular role in the bleaching process. We visualized *in vivo* production of NO as a function of heat stress in the symbiotic anemone *Aiptasia pallida* (Verrill), using laser scanning confocal microscopy and the NO-sensitive fluorescent dye DAF-FM (Nagano and Yoshimura, 2002). We present evidence in support of a model in which the host cell increases its production of NO as a function of an increased oxidative load during exposure to elevated temperatures. We suggest that this constitutes a cytotoxic response that leads to bleaching.
MATERIALS AND METHODS

Maintenance of Aiptasia pallida and Symbiodinium cultures

Cultures of Aiptasia pallida (Verril) from the Florida Keys were maintained in artificial seawater (Instant Ocean®) at 25°C and 12h/12h light/dark photoperiod with a light irradiance of approximately 70 µmol. Aposymbiotic anemones were obtained by the cold stress technique (Muscatine et al., 1991) and kept in the dark. Algae from the Florida anemones (Symbiodinium sp.) were isolated by homogenizing anemones in a glass tissue grinder followed by repeated centrifugations and subsequent resuspension in sterile sea water. The algae were then placed in 50ml tubes with sterile Guillard’s f/2 enriched seawater culture medium. Symbiodinium bermudense isolated from A. pallida from Bermuda were obtained from Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (culture No. CCMP830). Algal cultures were kept in the same light and temperature conditions as the symbiotic anemones.

Reinfection of Aiptasia pallida

Small (0.5-1 cm oral disc diameter) aposymbiotic anemones previously kept in the dark for approximately 3 months were thoroughly observed under light and epifluorescence microscope to confirm that no residual algae remained. Fully aposymbiotic anemones were placed in a 1:1 solution of MgCl₂ (0.037M) and artificial seawater mix for 10 minutes to relax them (relaxing solution). Once relaxed, they were inoculated with suspensions of either S. bermudense (Clade B) or (Clade A) by
injection into the gastric cavity with a drawn-out glass pipette. The anemones were then maintained in the light until algal populations were restored (about 3 months) before they were used in experiments.

**Confocal microscopy**

For preparation for confocal microscopy, the media of experimental anemones was replaced with relaxing solution of 1:1, 37mM MgCl₂: seawater (35ppt) with 3µl/ml of the NO probe DAF-FM-DA, (Molecular Probes; (Nagano and Yoshimura, 2002). After incubating the anemones for at least 30 min in the dark, the media was removed and a few drops of melted 1% low-melting agarose in relaxing solution cooled to 30ºC was added to immobilize the anemones. After allowing the agarose to gel for 1 minute, 3 ml of relaxing media was added on top of the agarose-embedded anemones. Unstained control anemones were processed as above without adding the probe to the mix. These were used to control for possible host tissue autofluorescence. Cultured algae were processed by incubating 1ml of suspension with 1µl/ml DAF-FM-DA for at least 30 min before observing on a glass slide and cover-slip. The samples were observed under a Zeiss LSM510 Meta microscope (The Center for Gene Research and Biotechnology at Oregon State University) with a 40X/0.8 3.2 mm working distance water objective lens. Excitation was provided by an Argon Laser at 488 nm to excite the DAF-FM probe and a HeNe543 laser to excite chlorophyll autofluorescence. DAF-FM NO-dependent fluorescence was detected using a 510-530 nm filter and chlorophyll autofluorescence using the metadetector at 600-700 nm.
Each excitation wavelength (488 and 543 nm) was used separately on different scans using the multiscan function. Before image scanning, the focal plane of the optical section was adjusted to include the gastrodermal layer of tentacles. All images were obtained with the same software scanning settings including detector gain and laser intensity settings. After visualization, samples which showed no NO-dependent fluorescence were subsequently incubated for 30 min in the NO donor sodium nitroprusside (1mM SNP; Sigma) and fluorescence was measured to confirm successful loading of the dye. Quantification of NO-dependent DAF-FM fluorescence was achieved by first defining the gastrodermal portions as regions of interest and measuring the average pixel intensity value for that region with the LSM 5 software.

*Experiments*

To test the effect of heat stress on NO production, 3-5 small aposymbiotic and symbiotic anemones (5-10 mm long) were each placed in 3 ml of artificial seawater in 5 ml modified Petri dishes. The Petri dishes were modified by fitting them with a glass coverslip adhered to a hole drilled on the bottom. The anemones were pre-incubated overnight at 25°C. After the pre-incubation, the anemones were incubated in either 25°C or 33°C for 24 h on a 12/12 hour light/dark photoperiod. Algal cultures (50ml) were similarly heat-stressed. After the incubation, the anemones and algal cultures were processed for confocal microscopy. To examine the effect of an NOS inhibitor on NO production after heat stress, heat-stressed or control symbiotic anemones were incubated for 10 min in 10mM L-aminoguanidine (L-AG; Sigma).
before processing for confocal microscopy. Aposymbiotic anemones that served as a positive control for host NOS activity were incubated for 5 h in 1µg/ml lipopolysaccharide (LPS; Sigma) before preparation for confocal microscopy. Some LPS-treated anemones were also pre-incubated in LAG as above.

To test the effect of 3-(3', 4'-dichlorphenyl)-1,1–dimethylurea (DCMU; Sigma) on NO production, anemones or cultured algae were incubated in 50µM DCMU for 24 h. Samples were then prepared for confocal microscopy.

Bleaching experiments were carried out by incubating medium-sized (0.5-1 cm oral disc diameter) symbiotic anemones at 25°C with 1.0mM SNP with or without 1mM of the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO; Sigma) or at 33°C with or without 20mM cPTIO for 24 h. Controls were included and each anemone was incubated individually in 5ml Petri dishes. Bleaching was quantified using methods similar to those previously published (Perez et al., 2001a). Briefly, both algae expelled into the media from anemones and those remaining in the host tissues (in hospite algae) were counted. Percent bleaching was calculated as the number of expelled algae / (expelled algae + number of in hospite algae). In a separate experiment, small anemones were either heat-stressed with or without 20mM cPTIO at 33°C for 24 hours or incubated with 1.0mM SNP with or without 20mM cPTIO for three hours. Treated anemones as well as control anemones (also with or without 20mM cPTIO) were then rinsed with seawater and
prepared for confocal microscopy using DAF-FM to measure NO-dependent fluorescence as described above.
RESULTS

*Heat stress triggers production of Nitric Oxide in symbiotic A. pallida*

To determine if NO was differentially produced as a function of heat stress in *A. pallida*, we incubated symbiotic anemones at 33°C for 24h and observed their tentacles using laser scanning confocal microscopy after loading the animal with the NO-sensitive fluorescent probe DAF-FM-DA (Nagano and Yoshimura, 2002). Exposure to elevated temperature resulted in a significant two-fold increase in NO-dependent DAF-FM fluorescence in tentacles of symbiotic anemones (Fig. 2.1B;  $t$-test $P=0.0015$). The fluorescent NO-sensitive signal was found largely in the host gastrodermal cells containing the algae, both in the intact tissue as well as in the host cells released during bleaching (Fig. 2.1A inset). The difference in the fluorescent signal between control and heat-stressed anemones was not due to unequal loading of the dye as a function of temperature treatment since addition of exogenous NO to controls, by incubation with the NO donor sodium nitroprusside (SNP), always resulted in an increase of the NO-dependent fluorescent signal (data not shown). Unstained controls did not show autofluorescence of host tissues.

*The host A. pallida as a source of NO during heat stress*

To examine more closely the source of NO during exposure to elevated temperature, we performed the heat stress experiments on aposymbiotic anemones and cultured *Symbiodinium* from *A. pallida* loaded with DAF-FM. We did not observe
NO production in either aposymbiotic animals or cultured algae in isolation as a function of heat stress. This suggests that the partners in combination are required to cause the production of NO.

Production of NO through NOS-like activity was tested by using the NOS inhibitor L-aminoguanidine (LAG) (Danielisova et al., 2004). To induce NOS-like activity in anemones, we incubated aposymbiotic anemones in LPS (1µg/ml) which is known to cause the upregulation of inducible NOS in vertebrate systems (Ganster, 2000). LPS induced NO production in gastrodermal tissues of aposymbiotic *A. pallida* at similar levels to those observed in heat-stressed symbiotic anemones (Fig. 2.2A). Incubating LPS-treated anemones with LAG (10mM) resulted in strong inhibition of NO production (Fig. 2.2A). To test whether the heat-stress-induced NO production observed in symbiotic anemones was due to a host NOS inhibitable by LAG, we incubated heat-stressed anemones in 10mM LAG and measured NO production. Incubation of heat-stressed symbiotic anemones in the NOS inhibitor also significantly inhibited NO production due to heat stress (Fig. 2.2B; ANOVA $P=0.006$; Tukey HSD $P=0.021$). This suggests that in response to exposure to elevated temperature, symbiotic anemones produce NO through a host regulated NOS-like activity.
Figure 2.1 Heat stress induces fluorescence of the NO-sensitive probe DAF-FM in *A. pallida*.
A) Optical cross-section through tentacle of symbiotic *A. pallida* incubated at ambient temperature of 25° C (left) and after 24 h of heat stress at 33° C (right) with inset showing expelled host cells with algae. The DAF-FM (510-530nm) NO-dependent fluorescent signal is colored yellow; Algal autofluorescence is colored red. B) Quantification of relative fluorescence intensity of NO-sensitive DAF-FM (510-530nm) in tentacles as a function of heat stress (N=3 anemones; bars represent mean ± standard deviation; *t*-test *P*=0.0015)
Figure 2.2 The NOS inhibitor L-aminoguanidine (LAG) inhibits both LPS and heat stress-induced NO production in *A. pallida*.

A) Gray scale rendering of confocal images of DAF-FM-loaded tentacles of aposymbiotic anemones incubated for 5 h in 1µg/ml LPS alone (left) or in LPS with 10mM LAG, a NOS inhibitor (right). B) Quantification of DAF-FM fluorescence as a function of heat stress alone and in the presence of 10mM LAG (N=3 anemones; bars represent mean ± standard deviation). Heat stressed anemones (*) showed a significantly greater DAF-FM fluorescence than control or heat-stressed anemones incubated in LAG (ANOVA *P*=0.006; Tukey HSD *P*=0.021)
Oxidative stress can trigger the production of NO in symbiotic anemones.

In vertebrate systems, oxidative stress is another well known signal for the upregulation of inducible NOS (Mendes et al., 2003). We hypothesized that production of ROS by the algae, a phenomenon known to occur during heat stress, would result in NO production. To test for NO production due to elevated oxidative stress, we incubated symbiotic and aposymbiotic anemones as well as cultured algae in DCMU, an inhibitor of the photosynthetic electron transport known to increase production of ROS and trigger bleaching (Franklin, 2004, Lesser, 1996a). Incubating symbiotic anemones with 50µM DCMU elicited nearly a three-fold increase in NO production in tentacles similar to that seen in heat-stressed anemones while DCMU did not have a significant effect of aposymbiotic anemones (Fig. 2.3; t-test \( P=0.0041 \)). We could not detect production of NO in either cultured algae in the presence of DCMU (data not shown). These results suggest that anemones produce NO in response to conditions known to elicit oxidative stress in the algae.

Nitric oxide induces cnidarian bleaching in A. pallida

To test whether NO plays a role in bleaching, we incubated anemones with the NO donor (SNP) at room temperature and measured the release of algae from host tissues. SNP-derived NO caused a significant increase in bleaching, increasing from less than 1% in controls to 17% at 1mM SNP (Fig. 2.4A; \( t\)-test \( P=0.012 \)). Further, the effect of SNP on bleaching was significantly decreased by co-incubation with the NO scavenger cPTIO (Fig. 2.4A; \( t\)-test \( P=0.0368 \)). In addition, incubating anemones in
20mM cPTIO during exposure to elevated temperatures (33°C) significantly decreased bleaching by 50% (Fig. 2.4B; t-test $P=0.0176$). The NO-dependent DAF-FM fluorescence significantly increased in anemones incubated with 1mM SNP as well as anemones exposed to elevated temperature (Fig. 2.4C; (Two-way ANOVA $P<0.001$; Tukey HSD $P<0.05$). Coincubation of both SNP and heat-stressed anemones with 20mM cPTIO effectively decreased NO-dependent DAF-FM fluorescence to levels found in control anemones (Fig. 2.4C; Two-way ANOVA $P<0.001$; Tukey HSD $P<0.05$).

*A. pallida* hosting different algal clades produce different levels of nitric oxide during heat stress

We inoculated aposymbiotic *A. pallida* from Florida with two differentially heat-tolerant strains of cultured algae and allowed them to establish stable algal populations (Perez *et al.*, 2001a). The heat-tolerant strain was isolated from *A. pallida* from the Florida Keys (Clade A) while the heat-sensitive was isolated from *A. pallida* from Bermuda (Clade B). Heat-stressed anemones hosting subtropical and more heat susceptible Clade B algae showed a two-fold increase in NO-dependent fluorescence than anemones with Clade A algae (Fig. 2.5; t-test $P=0.0035$).
Figure 2.3 DCMU increases NO production in symbiotic *A. pallida*. Quantification of DAF-FM fluorescence of tentacles from aposymbiotic and symbiotic anemones after a 24 hour incubation in 50μM DCMU (N=3 anemones; bars represent mean+standard deviation; *t*-test *P*=0.0041)
Figure 2.4 Nitric oxide mediates bleaching in A. pallida.

A) Bleaching (% expulsion; bars represent mean + standard deviation; N=3) of anemones incubated for 24 h at 25°C with the NO donor SNP (1 mM) with (black bars) or without (white bars) the NO scavenger c-PTIO (1 mM). Control anemones (without SNP) released <1% of their algae. Incubation with SNP resulted in a significant increase in bleaching (t-test P=0.021) while co-incubation with cPTIO significantly decreased this effect (t-test P=0.0368). B) Bleaching (% expulsion; error bars represent mean + standard deviation; N=3) of anemones incubated for 24 h at 25°C (Control) or 33°C, with or without c-PTIO (20 mM). There was a significant difference between the two heat-stress treatments (t-test P=0.0176). C) NO-dependent DAF-FM fluorescence in control, heat-stressed or SNP-treated anemones with or
without 20mM cPTIO. Bars sharing same letter are not significantly different (Two-way ANOVA $P<0.001$; Tukey HSD $P<0.05$).
Figure 2.5 Differential production of NO during heat stress as a function of algal phylotype. Quantification of DAF-FM fluorescence of tentacles of *A. pallida*, symbiotic with two different phylotypes of algae, after 24 hour incubation at 25º control, or elevated temperature, 33ºC (HS; N=3; bars represent standard deviation). Algae from Florida and Bermuda were identified as belonging to Clades A (empty bars) and B (filled bars) respectively. The differential NO signal during heat stress between the two algal phylotypes was significant (*t*-test $P=0.0035$).
DISCUSSION

This study presents two major findings. Host cells of symbiotic *A. pallida* produce NO as a function heat stress, possibly in response to the resulting increase in oxidative load. Secondly, NO produced during exposure to elevated temperature mediates bleaching.

Symbiotic anemones exposed to elevated temperatures produced NO while aposymbiotic anemones do not. There are two possible (but not mutually exclusive) explanations for this result. Either symbiotic algae in the host are responsible for the production of NO or the host produces NO in response to heat-stressed algae. There are several lines of evidence that support the second scenario. First, we did not detect any production of NO in cultured algae exposed to elevated temperatures. The algae, however, could be producing NO during heat stress only during symbiosis, perhaps in response to an unknown host-derived factor. But to date no homologous NOS genes or cDNAs have been discovered in plants or lower eukaryotes including apicomplexans, a sister taxon to dinoflagellates (Chandok *et al.*, 2003, Gardner *et al.*, 2002, Guo *et al.*, 2003). It is possible that NO could be produced by NOS-independent mechanisms such as by the enzyme nitrate reductase, as has been shown in the chlorophyte *Chlamydomonas* (Sakihama *et al.*, 2002). If the symbionts are producing NO through an NOS-independent activity, then using a classic NOS inhibitor such as LAG should not inhibit NO production of heat-stressed symbiotic anemones. However, we were able to drastically inhibit NO production with LAG at
concentrations that also inhibited LPS-induced NO production in aposymbiotic
anemones (Fig. 2.3). In other systems, LPS has been demonstrated to induce the up-
regulation of the inducible form of NOS (iNOS) transcription through NFκB-
dependent signaling as part of the inflammatory response of mammalian phagocytes in
response to bacterial pathogens (Ganster, 2000). This pathway has not been described
in cnidarians. In a recent publication, preparations of expelled as well as freshly
isolated algae from the coral *Madracis mirabilis* showed increased NOS activity
during heat stress, as measured by the conversion of arginine to citrulline (Trapido-
Rosenthal et al., 2005). This apparently algal NOS-like activity may be explained by
host cell contamination which, in our experience, is unavoidable in such preparations.
Expelled material during bleaching also typically contains numerous gastrodermal
cells containing algae (Gates et al., 1992) which we have observed to produce NO as
well (Fig 2.1B inset). Taken together, this evidence suggests that the host is
responding to heat-stressed algae by producing NO.

Heat stress inhibits photosynthesis while stimulating ROS production in the
algae (Lesser, 1996a). Inducing oxidative stress in the algae by blocking the electron
transfer from photosystem II with the use of DCMU (Franklin, 2004, Lesser, 1996a)
also led to the production of NO in symbiotic anemones only but not in cultured algae
or aposymbiotic anemones. Like LPS, oxidative stress is known to lead to up-
regulation of iNOS through the activation of the transcription factor NFκB (Mendes et
al., 2003). Interestingly, *Plasmodium berghei*, an apicomplexan parasite (a sister
taxon of dinoflagellates) induces a similar response of upregulation of NOS and oxidative stress, leading to host cell death when it infects the epithelial cells of the host mosquito *Anopheles stephensi* (Kumar et al., 2004). We therefore hypothesize that ROS is acting as a signal to induce the host cell production of NO during heat stress in symbiotic *A. pallida* (Fig. 2.6).

Our results indicate that NO is an important mediator of bleaching in *A. pallida* and therefore NO could be acting as a cytotoxic compound during bleaching (Lamattina et al., 2003). Incubating anemones with the NO donor SNP resulted in bleaching while co-incubation with the NO scavenger cPTIO decreased the rate of bleaching in both heat-stressed anemones as well as anemones incubated in SNP. Production of NO during heat stress was assayed after 24 hours of exposure to elevated temperatures, but it is likely that production of NO began at an earlier time point after stress to elicit bleaching given that, by 24 hours of exposure to elevated temperatures, symbiotic *A. pallida* had already expelled algae. We hypothesize that the production of NO could have deleterious effects in conjunction with increased oxidative stress brought about by the effect of elevated temperature on the photosynthetic apparatus of the algae.

NO can react with superoxide to form the reactive nitrogen species (RNS) peroxynitrite (ONOO⁻) which can act in several deleterious ways. It can 1) undergo decomposition reactions to produce the highly reactive hydroxyl radical (Beckman *et
al., 1990, Radi, 2000); 2) inactivate several steps in the mitochondrial electron transport chain and ATP synthesis, thereby increasing the production of ROS (Radi, 2000); 3) inactivate Mn-SOD leading to a rise in the steady state levels of ROS; and 4) influence homeostasis by increasing the permeability of the mitochondrial membrane (Radi, 2000). This last mechanism is critical since it is known to cause the release of potent pro-apoptotic molecules such as cytochrome c from mitochondria. We hypothesize that the negative effect of high levels of NO during bleaching is in part due to its conversion to cytotoxic peroxynitrite concomitant with the increase in oxidative stress (Fig. 2.6).

Some coral-algal associations are more tolerant of hyperthermic bleaching than others (Fitt and Warner, 1995a). The observed differences in tolerance to elevated temperature may result from the differential tolerance of the algal strain (phylotype) present (Buddemeier and Fautin, 1993, Perez et al., 2001a, Rowan et al., 1997). Since we do not fully understand the bleaching mechanism, we also do not know how the differential susceptibility of the algae translates mechanistically into differential bleaching. Could differential host production of NO in response to differences in the algal tolerance explain this variation in bleaching? To test this question, we inoculated aposymbiotic A. pallida from Florida with two differentially heat-tolerant strains of cultured algae and allowed them to establish stable algal populations (Perez et al., 2001a). One strain was isolated from A. pallida from the Florida Keys (Clade A) while the other was isolated from A. pallida from Bermuda (Clade B). Heat-
stressed anemones hosting subtropical and more heat susceptible Clade B algae produced greater amounts of NO than anemones with Clade A algae (Fig. 2.5). Based on our model, we hypothesize that the differential susceptibility to heat stress of algal photosynthesis also leads to differential levels of oxidative stress and therefore triggers a differential production of NO by the host. The differential production of NO explains the correlation between algal photosynthetic susceptibility to elevated temperature and bleaching rates (Perez et al., 2001a). Based on our model, we hypothesize that the differential susceptibility to heat stress of algal photosynthesis also leads to differential levels of oxidative stress and therefore triggers differential production of NO by the host.
Figure 2.6 Proposed model for the role of nitric oxide during cnidarian bleaching. The host cell responds to algal-derived ROS (including superoxide and hydrogen peroxide) by producing NO through signaling leading to the up-regulation of NOS. This signaling could involve the transcription factor NFkB, and important mediator of NOS transcription in other systems. The reaction of superoxide with NO produces the reactive nitrogen species peroxynitrite (ONOO⁻) with additive deleterious effects.
The results of this study show that, upon exposure to elevated temperature, symbiotic *A. pallida* produce NO through an NOS-like system and that this process leads to bleaching. We propose that bleaching is an innate animal response to algal dysfunction which, in turn, has been linked to temperature stress. This response is similar to that described in some mammalian systems exposed to pathogens together with oxidative stress and therefore suggests that there is a remarkable degree of conservation of these cellular processes. The cytotoxic effect of NO during bleaching is likely dependent in part on the levels of oxidative stress and the production of damaging reactive nitrogen species such as peroxynitrite (Fig. 5). This hypothesized detrimental interaction between NO and superoxide provides additional insight into the well-described importance of the superoxide-detoxifying enzyme superoxide dismutase in cnidarian-algal symbioses (Richier *et al*., 2005, Richier *et al*., 2003).

Another example of conservation of cellular mechanisms that spans large taxonomic distances is the production of NO in *A. pallida* upon exposure to LPS. This mechanism may explain bleaching in corals exposed to *Vibrio* sp. and bacterial SOD as a virulence factor in that process (Ben-Haim *et al*., 2003). The regulatory role of NO as a function of oxidative stress may also explain some of the bleaching variability observed in both the laboratory and the field linked to the differential tolerance of algal photosynthesis to elevated temperatures. Nitric oxide is emerging as an important regulator in both parasitic and mutualistic symbioses and this illustrates the
importance of understanding the innate immune response mediating host-symbiont
interactions in these systems.

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EXPLORING THE CELLULAR MECHANISMS OF CNIDARIAN BLEACHING IN THE SEA ANEMONE Aiptasia pallida

CHAPTER 3: CYCLOPHILIN AND THE REGULATION OF SYMBIOSIS IN Aiptasia pallida

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ABSTRACT

The sea anemone *Aiptasia pallida* symbiotic with intracellular dinoflagellates expresses a peptidyl-prolyl *cis-trans* isomerase (PPIase) belonging to the conserved family of cytosolic cyclophilins (ApCypA). Protein extracts from *A. pallida* exhibited PPIase activity. Given the high degree of conservation of ApCypA and the known function in the cellular stress response we hypothesized plays a similar role in the maintenance cnidarian-algal symbiosis. To explore its role, we inhibited the activity of cyclophilin using the inhibitor cyclosporin A (CsA). CsA effectively inhibited the PPIase activity of protein extracts from symbiotic *A. pallida*. CsA also induced the dose-dependent release of symbiotic algae from host tissues (bleaching). Laser scanning confocal microscopy using superoxide and nitric oxide-sensitive fluorescent dyes on live *A. pallida* revealed that CsA strongly induced the production of these known mediators of bleaching. We tested whether the CsA-sensitive isomerase activity is important for maintaining the activity of the anti-oxidant enzyme superoxide dismutase (SOD). SOD activity of protein extracts was not affected by pre-incubation with CsA *in vitro*. These are the first results describing a cyclophilin homolog of a cnidarian species and of the role of cyclophilin in a cnidarian-algal symbiosis.
INTRODUCTION

Many cnidarian species, such as sea anemones and corals, are symbiotic with intracellular dinoflagellates of the genus *Symbiodinium*. To date there is little understanding of the molecular and cellular interactions governing these intimate associations. Comparing the gene expression patterns of symbiotic vs. aposymbiotic (those lacking symbionts) individuals has been a fruitful approach in resolving some of the cellular mechanisms behind these ecologically important symbioses (Barneah *et al.*, 2006, deBoer, 2007, Kuo *et al.*, 2004, Rodriguez-Lanetty *et al.*, 2006, Weis and Levine, 1996). In a recent study using the symbiotic tropical sea anemone *Aiptasia pulchella*, one of the genes that was found highly expressed in symbiotic anemones was identified as a peptidyl-prolyl *cis-trans* isomerase (PPIase) of the cyclophilin family (Kuo *et al.*, 2004). PPIases, also known as rotamases or foldases, catalyze the isomerization of peptide bonds in which one of the adjacent amino acids is proline (Fanghanel and Fischer, 2004, Scholz *et al.*, 1997). This isomerase activity has been hypothesized to be important during the stress response of organisms. For example, cyclophilin mRNA levels increase in response to temperature stress as well as stress induced by pathogens (Chou and Gasser, 1997, Hacker and Fischer, 1993, Sykes *et al.*, 1993). In symbiotic cnidarians, one well known stress response to elevated temperature, UV irradiance and bacterial infections involves the loss of algal symbionts from host tissues. This response, known as cnidarian bleaching, results through mechanisms dependent on host cell death pathways triggered in part by cellular oxidative and nitrosative stress (Dunn *et al.*, 2004, Franklin *et al.*, 2004,
Lesser, 1996a, Lesser, 2006, Perez and Weis, 2006, Perez et al., 2001b). Cnidarian bleaching of scleractinian corals has had deleterious ecological effects on tropical coral reef ecosystems worldwide (Hoegh-Guldberg, 1999a). Given that cyclophilins may be involved in pathways of the heat shock response and that, in this context, their rotamase/chaperone activity may be critically important, we studied the role of cyclophilin in the symbiotic sea anemone *A. pallida* as part of a continued effort to understand the molecular and cellular mechanisms behind cnidarian bleaching.

The cyclophilins constitute a large family of well-conserved paralogous genes some of which are also known as immunophilins due to the fact that these proteins are the targets of the potent immunosuppressive drug cyclosporin A (CsA) (Fruman et al., 1992, Liu et al., 1991). CsA, a non-ribosomally-derived undecapeptide isolated from the fungus *Tolypocladium inflatum*, binds to the active site of cyclophilin and inhibits its PPIase activity (Schreiber, 1991). CsA binds with greatest affinity to the prototypical cytosolic cyclophilin isoform (Fruman et al., 1994, Gothel and Marahiel, 1999). CsA-insensitive PPIases not homologous to the cyclophilins include the FK506 binding protein (FKBP) and the parvalbins (Galat, 2003). The isomerase activity is believed to be important for proper protein folding (Galat, 1993, Kofron et al., 1991). The physiological roles of immunophilins are varied and are beginning to be understood in greater detail. The human genome contains at least sixteen expressed genes encoding cyclophilins (Galat, 2003). Some of these functions even extend beyond their PPIase activity and include cell signaling, inflammation, control
of cell cycle (Barik, 2006). In addition some of the effects of CsA are attributed to non-PPIase functions, such as the inhibition of signaling through the phosphatase calcineurin involved in the control immune response. Several lines of evidence led us to test the function of cyclophilin within the context of cnidarian bleaching.

Yeast deletion- mutants of CypA are more susceptible to extreme heat stress (Dolinski et al., 1997, Sykes et al., 1993). Although mutant yeast strains containing deletions of all of their known cyclophilins are viable at room temperature, and CypA is not essential in a mammalian model (Colgan et al., 2004, Dolinski et al., 1997). In other systems, including bacteria, studies have also demonstrated the requirement of cyclophilin during stress conditions (Barik, 2006). Cyp mRNA levels also increase in plant tissues in response to temperature stress as well as stress induced by infection of pathogens (Chou and Gasser, 1997). In vascular smooth muscle cells, oxidative stress leads to increased expression and secretion of CypA and the PPIase activity of CypA is necessary for inhibiting NO-induced apoptosis and activating extracellular signal-regulated kinase (ERK1/2) (Jin et al., 2000). Therefore cyclophilins are hypothesized to be during the stress response of corals and other symbiotic cnidarians during bleaching as well as serve as a potentially useful markers of environmental stress.

An interesting role of cyclophilins in a symbiotic process is illustrated by the requirement for host cell cyclophilins in the infection process of apicomplexan
parasites (Hoerauf et al., 1997). This is particularly interesting to us given that dinoflagellates are a sister group to the apicomplexa (Wolters, 1991). Expression of cyclophilin A (CypA; cytosolic isoform; E.C.5.2.1.8) in macrophages was critically important in the successful replication of Leishmania major amastigotes (Hoerauf et al., 1997). In addition, CsA also inhibits the growth of Plasmodium falciparum and Toxoplasma gondii (Hoerauf et al., 1997). Other studies suggest cyclophilins play a role during oxidative stress (Boulos et al., 2007, Hong et al., 2002, Jin et al., 2004, Jin et al., 2000, Krauskopf et al., 2003, Liao et al., 2000, Santos et al., 2000). The role of cyclophilin during oxidative stress may be linked to the function of the antioxidant enzyme superoxide dismutase (Lee et al., 1999, Reddy and Suleman, 2004).

Here we describe the A. pallida CypA homolog (ApCypA) and show that CsA inhibits the in vitro isomerase activity of protein extracts from symbiotic A. pallida. In addition we show that incubation with CsA induces production of reactive oxygen species (ROS), nitric oxide (NO) and bleaching. We tested the hypothesis that these effects could be due to an indirect inhibition of activity of the enzyme superoxide dismutase SOD, which is known to have functional importance as an antioxidant in cnidarian-algal symbioses (Lesser and Shick, 1989, Richier et al., 2005, Richier et al., 2003).
MATERIALS AND METHODS

Maintenance of anemones

Cultures of *Aiptasia pallida* from the Florida Keys were maintained in artificial seawater (Instant Ocean®) at 25°C and 12h/12h light/dark photoperiod with a light irradiance of approximately 70 µmol PAR quanta/m²/s. The anemones were fed to satiation twice per week with freshly hatched *Artemia salina* nauplii.

RNA extraction and cDNA synthesis

All anemones used in the experiment were starved for one week to avoid contamination from *Artemia* RNA. Twenty anemones were blotted dry and placed in 1 ml microfuge tubes and immediately placed in liquid nitrogen and stored -80°C. Total RNA was extracted using a modification of the acid pH guanidinium thiocyanate/phenol/chloroform method (Bird, 2005). From the RNA extracted, cDNA was synthesized using the SuperScript™ First-Strand Synthesis system for RT-PCR Kit (Gibco BRL®, Life Technologies) using the supplied oligo(dT) primer to hybridize to the mRNA poly(A) tails. Newly synthesized cDNA was cleaned using Amicon, Microcon PCR system and kept at -20°C.

PCR amplification and cloning of *A. pallida* cyclophilin gene

The 3’ end of the *A. pallida* cyclophilin cDNA was amplified by PCR using a reverse primer which anneals to the poly-A tail the cDNA (PST2: 5’-GCCGAATTCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT-3’) and the forward primer based on the
published cyclophilin sequence from Aiptasia pulchella (GenBank Accession# CK663116): (ApCyp F8: 5’-CTGGACGTGTTGTGATGGAGCT-3’) (Kuo et al., 2004). This amplified product was ligated to pGEM®-T vector (Promega) and transformed into E. coli using MAX Efficiency DH5α™ (Invitrogen). Cells were plated on LB/ampicillin plates spread with xGal and IPTG and grown overnight at 37°C. After screening for transformants, the cloned inserts were PCR-amplified and those colonies with inserts of an expected size were sequenced. White colonies were PCR screened for the correct insert size, using the vector primers M13F and M13R; those containing the correct size were sequenced. All sequencing reactions were performed on column-purified PCR products (Montage PCR centrifugal filter device) amplified using the vector primers M13F & M13R. The 5’ end sequence was amplified using ApCypA reverse primers (ApCyp R7: 5’-AACGTGCTTGTTATCCAGCCAG-3’) with the First-Choice RLM-RACE kit (Ambion) according to the manufacturer’s instructions. Sequencing was performed using the Applied Biosystems Taq DyeDeoxy Terminator cycle sequencing kit, and the reaction product was analyzed on an Applied Biosystems model 373 DNA sequencer. A single contiguous sequence was generated using the Staden Package software, ver. 2003.0.

Sequence and phylogenetic analysis

After vector and adaptor sequences were identified and removed, the resulting 3’ and 5’ sequence ends were aligned and the contiguous sequence obtained was input
into ORF finder (http://www.ncbi.nlm.nih.gov). Identified ORFs were input into translated BLAST search tools (BLASTX and TBLASTX).

The sequences were aligned with CLUSTAL multiple alignment application using the slow-accurate algorithm (BLOSUM matrix, GAP penalty=7, GAP extension penalty=0.5; delay divergent=30%). These non-homologous or highly divergent aligned characters were masked and the final alignment was converted into NEXUS format for use in PAUP. Distance methods where used in PAUP to build Neighbor-Joining trees and to calculate Bootstrap values (1000 replicates).

**Protein extracts**

To obtain a crude protein extract from *A. pallida* for use in the PPIase and SOD enzymatic assays, each of 3 replicate groups of 30 medium anemones each were quickly rinsed in extraction buffer (50mM K$_2$HPO$_4$; 0.1mM EDTA; pH7.8) at 4°C and then homogenized in 25ml of extraction buffer in a glass and Teflon pestle homogenizer on ice. The homogenate was then sonicated for 20 seconds and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was transferred to microfuge tubes and flash frozen in liquid nitrogen. The extracts were stored at -80°C. Protein concentration was measured using a Coomassie (Bradford) assay kit (Pierce, Rockford, IL, USA) (Bradford, 1976). The average final protein concentration of the extracts was approximately 5 mg/ml.
Enzymatic assays

PPIase activity was measured using an in vitro colorimetric assay using protein extracts from symbiotic *A. pallida*. This method employs a synthetic peptide containing a proline which is subsequently cleaved by chymotrypsin when in the trans conformation, releasing the colored nitroanilide derivative (Barrett, 1981, Fischer *et al.*, 1989, Harrison and Stein, 1990, Scholz *et al.*, 1997, Takahashi *et al.*, 1989, Zydowsky *et al.*, 1992). The protein extract was pre-incubated with or without dithiothreitol (DTT; 3.3mM) and with or without CsA (3µM) or boiled for 5 minutes. The CsA negative controls received CsA carrier solvent (100% ethanol). To measure PPIase activity, 20 µl of protein extract, 5 µl of the synthetic peptide substrate succinyl-ala-ala-pro-phe-p-nitroanilide (Calbiochem) dissolved in DMSO (stock 40mM) and 700 µl of reaction buffer (50mM HEPES; 100mMNaCl; 1mM EDTA; pH8.0) were combined in a spectrophotometer cuvette and cooled to 4°C on ice. The cuvette was quickly transferred to the spectrophotometer, the sample was blanked and 5µl of 1:100 of 60 mg/ml chymotrypsin (in 1mM HCl) at 4°C was added to start the reaction. The absorbance of the reaction mix was measured every 5 seconds at 390nm for 1 minute or until the color change reached a plateau. Control blanks (without protein extract) were included. The rate of change in absorbance was
measured on the portion of the curve with the greatest initial maximal rate of change and expressed as Abs$_{390}$/minute.

SOD activity was measured \textit{in vitro} with protein extracts from symbiotic \textit{A. pallida} using a commercial colorimetric SOD microplate assay kit (Dojindo Molecular Technologies, Maryland WST) following the manufacturer instructions (Peskin and Winterbourn, 2000, Ukeda \textit{et al.}, 2002). The assay measures the SOD-inhibitable production of a formazan dye upon reduction of a tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt) with a superoxide anion. SOD activity is expressed as percent inhibition of the maximal reaction obtained with no extract blanks. For each sample, reactions were performed in triplicate and averaged. The concentration of the protein extracts employed in the experimental reactions was that which produced 50% inhibition of the reaction or approximately 1 unit/ml of SOD standard (Sigma-Aldrich, USA) or 20 µl of 0.5mg/ml. The protein extracts were pre-incubated at 4°C for 30 minutes with CsA at 0 (control), 0.1, 1.0 or 100µM, the control receiving CsA carrier solvent (100% ethanol).

\textit{Expulsion of algae (bleaching)}

Bleaching was quantified as previously described (Perez and Weis, 2006). Briefly, hemocytometer counts were made of algae expelled by anemones over 24 hours as
well as algae remaining within host tissues and calculating the percent expelled algae
of the total initial number of algae in the anemones.

**Confocal microscopy**

To measure and visualize production of nitric oxide we employed the NO probe
DAF-FM-DA (4-amino-5-methylamino-2',7'-difluorofluorescein diacetate), as
described in previously published work (Molecular Probes, Eugene, OR, USA)
(Nagano and Yoshimura, 2002, Perez and Weis, 2006). To measure oxidative stress,
anemones were incubated with dihydroethidium (DHEt; absorbance ~355nm). In its
reduced state, cytosolic DHEt exhibits blue fluorescence (~420nm); however, once
this probe is oxidized to ethidium by superoxide anion, it intercalates within the cell's
DNA, staining the nucleus a bright fluorescent red (~625nm) (Carter *et al.*, 1994, Zhao
*et al.*, 2003). To obtain a relative quantification of oxidative state, we measured the
relative fluorescence (after excitation with a 405nm laser) of the reduced and oxidized
ethidium with a 505-550 and 571-625nm band-pass filter respectively and calculated
the ratio of the oxidized ethidium fluorescence to that of the reduced DHEt.

Anemones were placed on 5ml glass-bottom Petri dishes (MatTek, Ashland, MA,
USA) with 3ml of either MFSW alone (plus vehicle solvent for CsA, 100% ethanol) or
with 75µM of the pro-oxidant compound *tert*-butyl hydroperoxide or 1µM CsA and
incubated for 24 hours. After the incubation the media was removed and replaced
with 1ml of relaxing solution (1:1, 37mM MgCl₂: seawater at 35ppt) with 3µl/ml
DAF-FM-DA or DHEt, (Molecular Probes; (Nagano and Yoshimura, 2002).

Anemones were incubated for 30 min in the dark and the media was then removed and a few drops of relaxing solution were added. The samples were observed under a Zeiss LSM510 Meta microscope (The Center for Gene Research and Biotechnology at Oregon State University) with a 40X/0.8 3.2 mm working distance water objective lens. Excitation was provided by an Argon Laser at 488 nm to excite the DAF-FM probe and a HeNe543 laser to excite chlorophyll autofluorescence. DAF-FM NO-dependent fluorescence was detected using a 510-530 nm filter and chlorophyll autofluorescence using the metadetector at 600-700nm. Each excitation wavelength (488 and 543 nm) was used separately on different scans using. Before image scanning, the focal plane of the optical section was adjusted to include the gastrodermal layer of tentacles. All images were obtained with the same software scanning settings including detector gain and laser intensity settings. Quantification of NO-dependent DAF-FM or DHEt/ ethidium fluorescence was achieved by first defining the gastrodermal portions as regions of interest and measuring the average pixel intensity value for that region with the LSM 5 software.
RESULTS

A. pallida CypA

We cloned and sequenced an expressed Cyp gene from *A. pallida* (GenBank Accession# EF208090; ApCypA) homologous to that of *A. pulchella* (GenBank Accession# CK663116). The predicted protein sequence of ApCypA is identical to that of *A. pulchella* (99% identity at the nucleotide level) and 83% identity to the *Homo sapiens* homolog (GenBank Accession# NM_021130) (Fig. 3.1A). The *Aiptasia* sequences have a predicted isoelectric point and molecular weight of pH 9.1 and 17.5 kDa and contain all the amino acids known to be involved in both binding to CsA and in the PPIase activity. These include the invariant R55 known to play a critical catalytic role in the *cis-trans* isomerization reaction (Barik, 2006).

Phylogenetic analysis shows that the ApCypA predicted protein sequence groups well with homologs from higher eukaryotes (Bootstrap value = 87%) and not with those of the apicomplexa, suggesting that the ApCypA sequence is host derived and not from dinoflagellate symbionts (Fig. 3.1B). Polymerase chain reactions using ApCypA-specific primers on genomic DNA prepared from cultured *Symbiodinium* from *A. pallida* sp. as a template did not amplify any product (results not shown).

*Isomerase activity in A. pallida*
Protein extracts from symbiotic *A. pallida* exhibit isomerase activity inhibitable by pre-incubation with 3µM CsA (Fig. 3.2A). CsA inhibited the activity down to background levels (no extract) as well as that of boiled extracts, suggesting that most, if not all, of the PPIase activity is due to a cyclophilin and that this concentration of CsA was enough to inhibit at least 100% of the activity (ANOVA $P \leq 0.001$; SNK multiple comparisons $P \leq 0.05$). We do not know how much of the PPIase activity is partitioned between host and algal sources since the protein extracts contained algal as well as host-derived proteins. The PPIase activity was sensitive to the oxidation state of the enzyme involved since removing the reducing agent DTT from the reaction medium of controls resulted in loss of activity.

*CsA induces bleaching, oxidative stress and NO production*

Incubating anemones at 25°C in CsA (0-1µM) for 24 hours resulted in a dose-dependent increase in the rate of bleaching (Fig. 3.2B). When anemones were incubated at 34°C, CsA had a large synergistic effect on bleaching at 0.1µM but was lethal at the highest concentrations used (0.5 and 1.0µM). This lethality occurred before most algae were released into the medium which explains the measured decrease in the rate of bleaching. These results suggest that the isomerase activity of cyclophilin is important in the maintenance of the symbiosis as well as during the response to heat stress.
Figure 3.1 Sequence and phylogenetic analysis of *Aiptasia pallida* Cyp.  
A) Alignment of the first 120aa of the predicted protein sequence of *A. pallida* (ApCypA), and *Homo sapiens* (GenBank Accession#: EF208090 and NM_021130 respectively). Residues of the human sequence involved in binding of CsA are shaded gray and those involved in PPIase activity are underlined.  
B) Neighbor-Joining phylogenetic reconstruction of selected CypA’s. Species names are followed by GenBank accession numbers in parentheses. The *A. pallida* sequence is outlined. Bootstrap percentages (>50%) are shown enclosed in circles.
Figure 3.2 CsA inhibits peptydyl-prolyl cis-trans isomerase activity in *A. pallida* protein extracts and induces the release of symbiotic algae from host tissues.

A) Results of PPIase colorimetric *in vitro* assay of blanks (no protein extracts), protein extracts pre-incubated with or without dithiothreitol (DTT; 3.3mM); CsA (3µM, +3.3mM DTT) or pre-boiled (+3.3mM DTT). Activity is expressed as change in absorbance at 390nm per minute (bars represent means + standard deviation; N=3 extracts). Bars with asterisk represent significantly different values (ANOVA $P \leq 0.001$; SNK multiple comparisons $P \leq 0.05$). B) Expulsion of symbiotic algae (bleaching) as a function of 24 hour incubation to different concentrations of CsA at 25°C (clear bars) and 34°C (filled bars). Bars represent the mean % expulsion + standard deviation; bars with double daggers (‡) are treatments which resulted in anemone mortality (N=4).
Incubating *A. pallida* with CsA (1 µM) or TBP (75 µM) for 24 hours resulted in an increase in oxidative load as evidenced by an increase in the ratio of the relative fluorescence of oxidized DNA-bound ethidium to that of reduced cytosolic dihydroethidium (Figs. 3.3A and B; ANOVA $P \leq 0.001$; SNK multiple comparisons $P \leq 0.05$). These treatments also resulted in increased relative fluorescence of the NO-sensitive dye DAF-FM (Fig. 3.3C; ANOVA $P \leq 0.001$; SNK multiple comparisons $P \leq 0.05$).

CsA and SOD activity

Incubating protein extracts in up to 100 µM CsA did not result in any measurable inhibition of *in vitro* SOD activity (Fig. 3.4; ANOVA $P=0.019$). This insensitivity is not due to SOD-independent background levels since previous tests showed that the SOD activity in *A. pallida* protein extracts was inhibited by boiling as well as hydrogen peroxide (results not shown).
Figure 3.3 CsA induces oxidative stress and NO production in *A. pallida*.
A) False color images of optical cross-section through tentacles of symbiotic *A. pallida* incubated 24 h in CsA (1µM) or the pro-oxidant TBP (75mM). Top panel: algal chlorophyll autofluorescence (700-750nm); middle panel: fluorescence of
reduced, cytosolic dihydroethidium (DHEt; 505-550nm); lower panel: fluorescence of DNA-bound ethidium (Et; after oxidation of dihydroethidium; 571-625nm). B) Ratio of the relative fluorescence intensity of DHEt to that of Et of tentacles treated as above (N=4 anemones; bars represent mean + standard deviation; bar with asterisk represents a significantly different treatment ANOVA P≤0.001; SNK P≤0.05). C) Quantification of relative fluorescence intensity of NO-sensitive DAF-FM (510-530nm) in tentacles treated as above (N=4 anemones; bars represent mean + standard deviation; ANOVA P≤0.001; SNK P≤0.05). Images of DAF-FM fluorescence not shown.
Figure 3.4 The *in vitro* SOD activity of *A. pallida* protein extracts is insensitive to CsA. Bars represent the mean ± standard deviation of % inhibition of SOD-inhibitable production of colored WST-1 formazan derivative. Protein extracts (+ 3.3mM DTT) were pre-incubated 30min in different concentrations of CsA. A 50% inhibition is approximately equal to 1 unit/ml of SOD activity; N=3.
DISCUSSION

**A. pallida cyclophilin**

This work represents the first description of a cyclophilin from a cnidarian and the first study of its role in a cnidarian-algal symbiosis. The ApCypA sequence is likely of host origin given that the phylogenetic analysis shows that it groups well with other metazoan cyclophilins and not with those from of apicomplexan origin (a close sister group to the dinoflagellates). Sequences from the more basal anthozoan genera (*Aiptasia* and *Nematostella*) do not group with those of the more derived hydrozoan genera (*Hydra, Hydractinia* and *Podocoryne*), which reflects the long evolutionary history since the split of this two cnidarian clades (Bridge *et al.*, 1995).

The predicted isoelectric point and molecular weight matches the molecular weight of the prototypical cytosolic human Cyp18 isoform of 17.7kDa (Gothel and Marahiel, 1999). Given its high degree of conservation and its role in the cellular stress response of other organisms, we studied its function in *A. pallida* using a pharmacological approach.

**ApCypA structure, putative PPIase activity and CsA-sensitivity**

Protein extracts from symbiotic *A. pallida* exhibited PPIase activity which was inhibited by incubation with CsA. CsA inhibited PPIase activity down to background levels, suggesting that one or more CsA-sensitive cyclophilin isoforms were the main source of activity. It is not known how many such isoforms are expressed in *A.*
*pallida* or in their symbiotic algae. The PPIase activity was also sensitive to the addition of the reducing agent DTT. Previous studies show that Cyp is regulated by redox status through the activity of thioredoxin (Motohashi *et al.*, 2001, Motohashi *et al.*, 2003). Interestingly, the predicted ApCypA protein contains two pairs of cysteine groups (Cys40 & Cys62 and Cys115 & Cys161), a remarkably similar arrangement to the two pairs of redox-sensitive cysteines described in the Cyp of *Arabidopsis thaliana* which further suggests that ApCypA is regulated by redox state *in vivo* (Motohashi *et al.*, 2003). The predicted ApCypA protein sequence also contains the amino acid residues known to be involved in catalysis and binding to substrates as well as to CsA (Barik, 2006). Therefore ApCypA is expected to bind CsA and have PPIase activity. However, it is still unclear if the PPIase activity of cyclophilins (and other PPIases) is functionally important *in vivo.*

**Role of cyclophilin in the stress response**

Incubation of *A. pallida* with CsA resulted in increased production of ROS, NO and expulsion of symbionts. TBP, a pro-oxidant and activator of NFκB transcription factor, also induced NO production, suggesting that ROS are involved in similar signal transduction leading to the up-regulation of nitric oxide synthase (NOS) in *A. pallida* (Lee *et al.*, 2005, Perez and Weis, 2006). Superoxide anion readily reacts with NO to produce the cytotoxic compound peroxynitrite which is hypothesized to mediate cnidarian bleaching in *A. pallida* (Perez and Weis, 2006). Micromolar levels CsA induced bleaching at 25°C which suggests that it effectively induces cellular
changes, such as activation of cell-death pathways, leading to increased rate of algal release. Due to algal photosynthesis, the symbiosis imposes a state of perpetual hyperoxia, requiring the sustained dependence on protective, Cyp-dependent or CsA-sensitive anti-oxidant and anti-apoptotic homeostatic mechanisms (Richier et al., 2005, Richier et al., 2003). In this respect, Cyp’s could play a critical role in the regulation of cnidarian-algal symbiosis. This model explains the abundance of Cyp transcripts in symbiotic A. pulchella (Kuo et al., 2004). The temperature-dependence of CsA-mediated bleaching reflects that the cellular flux of CsA was also sensitive to temperature and/or that elevated temperature imposes additional oxidative stress and increased dependence on protective mechanisms mediated by Cyp (Lesser, 1997). These results bear remarkable resemblance to the cytotoxic effects of CsA in mammalian systems, further suggesting a conserved role of Cyp’s in the cell biology of the stress response.

Effect of CsA on SOD activity and oxidative stress

CsA has been shown to increase NO production and NOS expression through production of ROS and activation of ROS-sensitive signaling pathways (Chen et al., 2002, Lopez-Ongil et al., 1998, Navarro-Antolin et al., 1998, Navarro-Antolin and Lamas, 2001, Navarro-Antolin et al., 2001, Navarro-Antolin et al., 2007, Navarro-Antolin et al., 2000). To date however, the mechanism by which CsA increases ROS is unclear. Given that CsA also inhibits in vitro PPlase activity, it is possible that the production of ROS is mediated through alteration of CsA-sensitive PPlase-dependent
mechanisms. Alternatively CsA may be interfering with non-catalytic, chaperone-like functions of Cyp-substrate binding (Barik, 2006). We hypothesized that the CsA-mediated increase in ROS production was due to inhibition of the antioxidant function of SOD.

Several lines of evidence led us to test the effects of CsA on SOD activity. Host CypA binds to SOD secreted by *Mycobacterium avium* during the infection of epithelial cells (Reddy and Suleman, 2004). Interestingly, pathogen-derived SOD was found to be a virulence factor in the infection of the coral *Oculina patagonica* by *Vibrio shiloi* (Banin et al., 2003). The PPIase activity of human CypA was necessary to prevent apoptotic death of cells expressing a familial amyotrophic lateral sclerosis (FALS)-associated mutant SOD which induces apoptosis of neuronal cells in culture associated by an increase in reactive oxygen species (Lee et al., 1999).

The activity of SOD activity of protein extracts from *A. pallida* was not sensitive to CsA treatment even when incubated at a concentration two orders of magnitude greater than necessary to inhibit PPIase activity. Given that all the measurable PPIase activity was sensitive to CsA (Fig. 1.2A), it is unlikely that CsA-insensitive sources of PPIase activity are acting to maintain SOD activity. It is also possible that the PPIase activity of Cyp may be important *in vivo* during the folding of the nascent protein and not critical for the *in vitro* activity of the mature fully-folded form. The strong effect of CsA on ROS production *in vivo* suggests that the main site of its
effect is in the host cytoplasm and not in the algae which contain a greater abundance and diversity of SODs that effectively catalyze the dismutation of superoxide anions (Lesser, 2006, Richier et al., 2005, Richier et al., 2003). Superoxide produced during exposure of symbiotic Aiptasia pulchella to elevated temperature was identified to derive from host, and not algal sources (Nii and Muscatine, 1997). However, the effects of CsA on algal photosynthetic physiology remains unexplored even though chloroplast Cyp isoforms exist and algal photosystem are also a source of ROS during cnidarian bleaching (Lesser, 2006, Motohashi et al., 2003). Theoretically, CsA could be indirectly inhibiting other anti-oxidant mechanisms or it could be inducing the production of ROS by restricting the ability of CypA to act as a negative regulator of ROS production.

The fluorescent reporter system we employed is sensitive to superoxide anion (Carter et al., 1994, Zhao et al., 2003). One of the major sources of superoxide anion is the NAD(P)H oxidase (NOX) multi-protein complex responsible for the oxidative burst directed against phagocytosed pathogens during the innate immune response. The cnidarian Hydra magnipapillata expresses a NOX 1 alpha homolog (GenBank accession# DN816100). CsA can induce NOX activity but the mechanism involved remains unresolved (Vetter et al., 2003). The control of NOX activity by CypA could be analogous its control of another multiprotein complex: interleukin-2 inducible T-cell tyrosine kinase (ITK) in which CypA acts as a negative regulator through PPIase-dependent conformational changes (Brazin et al., 2002, Colgan et al.,
Interestingly, symbiotic algae of soft corals produce abundant diterpene metabolites known as pseudopterosins which are anti-inflammatory agents known to inhibit the oxidative burst as well as the process of phagocytosis (Look et al., 1986, Moya and Jacobs, 2006, Mydlarz and Jacobs, 2004). This suggests that the regulation of NOX activity, perhaps mediated in part through a PPlase-dependent mechanism of CypA, plays a critical role in the maintenance of cnidarian-algal symbiosis.

Another possible link of CypA as a regulator of ROS production involves the regulation of inorganic carbon supply to the algal photosynthetic apparatus. In the unicellular alga *Chlamydomonas reinhardtii*, low CO₂ conditions leads to the up-regulation of cyclophilin which might then aid in the folding of the carbon-concentrating enzyme carbonic anhydrase (Freskgard et al., 1992, Somanchi and Moroney, 1999). This enzyme is physiologically important for nutrient exchange in invertebrate-algal symbiosis (deBoer et al., 2006, Leggat et al., 2002, Weis, 1993, Weis and Reynolds, 1999). CO₂ limitation is also known to induce oxidative stress and programmed cell death in dinoflagellates due to its indirect effects of the photosynthetic electron transport chain (Vardi et al., 1999).

CsA is also known to exert effects through mechanisms independent inhibition of PPlase activity of CypA. The CsA-CypA complex inhibits the signaling pathways involved in immune functions that lead to gene transcription by blocking the highly
conserved Ca$^{2+}$/ calmodulin (CaM) -dependent serine/threonine protein phosphatase activity of calcineurin (Cn; also known as protein phosphatase 2B; PP2B). In yeast, calcineurin promotes survival during environmental stress (Cyert, 2001, Mulet et al., 2006). However, CsA can induce oxidative stress and apoptosis independent of Cn inhibition (Alvarez-Arroyo et al., 2002, Hong et al., 2002). Other effects of CsA are independent of binding to CypA. Some of the antiparasitic effects of CsA against the intracellular apicomplexan pathogens Plasmodium Toxoplasma and Cryptosporidium are mediated by inhibition of the conserved P-glycoprotein, a member of ATP-binding cassette transporter family that provides multidrug resistance of cancer cells (Bell et al., 2006, Perkins et al., 1999, Perkins et al., 1998, Silverman et al., 1997). These pumps are involved in the transport of lipids across epithelial cell membranes and as such might play an important role in the translocation of algal-derived lipids to the host cytoplasm or to the ectoderm in cnidarian-algal symbioses (Devaux et al., 2006). These are well studied effects of CsA however it is unclear how the inhibition of Cn or P-gp would lead to increased ROS production.

This study represents the first description of highly conserved Cyp from a cnidarian and of the effects of CsA in a marine invertebrate symbiosis and further showcases A. pallida as a valuable model for the study of cnidarian-algal symbioses. Further functional studies will be needed to elucidate the role of Cyp in cnidarian-algal symbioses, but the conserved nature of ApCypA and of the cytotoxic effects of CsA promises a conservation of function and regulation supported by studies using
model systems. Exploring the mechanisms proposed here will shed light into some of the cellular and molecular mechanisms regulating cnidarian-algal symbioses and will provide valuable insight into the adaptability and evolution of these systems which are gravely threatened by stresses stemming from environmental change (Hoegh-Guldberg, 2004).

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CHAPTER 4: THE REGULATION OF CNIDARIAN BLEACHING: IS IT AN INNATE IMMUNE RESPONSE?

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ABSTRACT

Many ecologically important species of cnidarians are symbiotic with intracellular dinoflagellate algae. In the case of corals, this important association allows these creatures to successfully play their important role as support of coral reef ecosystems. Corals are severely threatened by environmental stressors of which elevated temperature is the major cause of the phenomenon known as coral bleaching (cnidarian bleaching). Cnidarian bleaching is the loss of the algal partner from the host tissues and is one of the leading causes of the degradation of coral reefs worldwide. This review focuses on the molecular mechanisms behind this detrimental response.

INTRODUCTION

Coral reefs are exquisitely rich ecosystems which support millions of species, protect nearby coastal marine habitats and supply the economies of many throughout the world. This global scale phenomenon relies on a biological system that functions at the microscopic scale: the interaction between the coral host cell and its mutualistic intracellular dinoflagellate algae of the genus *Symbiodinium*. The critical significance of this minute interaction is dramatically evident when the fine balance between partners is upset by stressful environmental conditions. Stressors such as elevated seawater temperature and increased UV exposure are the main causal agents of what is known as coral bleaching, the condition in which mass amounts of symbiotic algae are expelled from host cells. Widely distributed episodes of coral bleaching have been on
the rise since the first reported global episode in 1979 (Hoegh-Guldberg, 2004). Coral bleaching has had devastating effects on survival, fitness and ecological function of corals (Coles and Brown, 2003, Glynn, 1993, , 1996). Most of the current understanding of coral bleaching is the outcome of numerous studies focused on physiological and ecological questions and these are reviewed elsewhere (Coles and Brown, 2003, Glynn, 1993, , 1996, Hoegh-Guldberg, 1999b, Hoegh-Guldberg, 2004). Based on predictions of global climate models, it is believed that in the next several decades coral bleaching will become more prevalent and will result in the collapse of coral reef ecosystems if reef-building corals fail to acclimate or adapt within this short time frame (Hoegh-Guldberg, 1999a). The accuracy of these predictions will largely depend on our knowledge on the capacity of corals to withstand environmental change. A clear understanding of the cellular bleaching mechanism is therefore needed in order to accurately predict this capacity. Some progress has occurred along this front and we are slowly getting closer to a more complete understanding of the mechanisms of coral bleaching.

CNIDARIAN-ALGAL SYMBIOSES AND CNIDARIAN BLEACHING

Cnidarians are composed of two epithelial tissue layers, the external ectoderm and an internal gastroderm separated from each other by a relatively acellular mesoglea rich in extracellular matrix material (Shimizu et al., 2002). Symbiotic cnidarians house their intracellular algal symbionts within phagocytic nutritive cells of the gastrodermal cell layer. The algae are incorporated through a phagocytic event after which the algae are contained within the host-derived symbiosome (Colley and
Trench, 1985, Fitt and Trench, 1983, Schwarz et al., 1999, Wakefield and Kempf, 2001). Coral bleaching is evidenced by the loss of algal-derived coloration of coral tissues. This process can also occur in other cnidarian taxa symbiotic with intracellular algae and therefore it will be referred to as cnidarian bleaching. The loss of algal symbionts is known to occur in response to a variety of environmental stressors which, in addition to exposure to elevated temperature and excessive visible and UV radiation, include exposure to suboptimal salinity and oxygen levels as well as exposure to heavy metals, pollutants and pathogens (Ben-Haim et al., 2003, Brown, 2000, Coles and Jokiel, 1977, Douglas et al., 1993, Gates et al., 1992, Glynn and D'Croz, 1990, Hoegh-Guldberg, 1999a, Hoegh-Guldberg and Smith, 1989, Kleppel et al., 1989, Lesser, 1996b, Porter et al., 1989). Many of these factors often have a synergistic effect (Banin et al., 2000, Fitt et al., 2001). The fact that such wide range of etiological agents lead to bleaching suggests that a correspondingly diverse set of cellular mechanisms exist or, more parsimoniously, that damage from these conditions converge on relatively few mechanisms. Most studies on the mechanism of cnidarian bleaching have used temperature or UV stress as a trigger of bleaching due to their environmental significance.

One of the best described physiologic effects of elevated temperature and UV irradiance in cnidarian-algal symbioses is damage to the photosynthetic machinery of the algae. This process and the resulting increase in production of reactive oxygen species is the subject of a recent review (Lesser, 2006). He we will focus on the proposed cellular mechanisms linking these proximal physiological effects with the
downstream consequence of algal expulsion, especially those mediated through programmed cell death pathways (Fig. 4.1). The first studies aiming to elucidate the mechanism of bleaching were focused on the histological level.

Figure 4.1 What are the mechanisms for cnidarian bleaching? Diagram representing the unknown molecular link (or links) between the physiological effects of environmental stressors and the cellular response of PCD and loss of cell adhesion associated with cnidarian bleaching.
THE HISTOPATHOLOGY OF CNIDARIAN BLEACHING: CELLULAR AND MOLECULAR IMPLICATIONS

Analysis of tissues of tropical corals and anemones exposed to suboptimal temperatures in both the field and the laboratory revealed that the loss of algae could be attributed to exocytosis of algae from host cells, degeneration of host cells and algae in hospite and release of entire host gastroderm cells containing the algae inside (Fig. 4.2) (Brown et al., 1995, Franklin et al., 2004, Gates et al., 1992, Steen and Muscatine, 1987). These observations have led to several hypotheses, a few which have been tested.

Degradation of algae

Degraded or dying algae have been described in tissues of the coral Stylophora pistillata (Franklin et al., 2006, Franklin et al., 2004) and in Aiptasia pallida (Dunn et al., 2004) during bleaching. This is viewed as evidence of digestion of algae as a way to regulate algal density (Titlyanov et al., 2004, Titlyanov et al., 1997). However, it is unknown if damaged algae are more the result of digestion or from the deleterious activity of reactive oxygen or nitrogen species or other factors endogenously produced during bleaching (Dunn et al., 2004, Franklin et al., 2004, Perez and Weis, 2006). Within the symbiosome, algae avoid digestion through avoidance of fusion with lysosomes, a process believed to be mediated in the symbiotic sea anemone Aiptasia pulchella in part through manipulation of GTPases of the Rab family of endocytic vesicle trafficking proteins (Chen et al., 2004, Chen et al., 2003, Chen et al., 2005, Hohman et al., 1982). Therefore, the function of the
Figure 4.2 Bleaching mechanisms based on histological analysis.
(A) Normal cells. Histological analysis has provided evidence of degradation of algae (B), programmed cell death (PCD) or necrosis of host cell and/or algae (C), exocytosis of algae (D) and detachment of host cells followed by PCD or necrosis of host cells and/or algae. After Gates et al. 1992.
endosome maturation and transport system is inferred to play a central role in the onset, maintenance and collapse of the cnidarian-algal symbiosis. Treatment of algae with the photosynthesis poison DCMU which induces oxidative stress results in loss of the early endosome marker ApRab5 and incorporation of late endosome markers ApRab7 and ApRab11 on the symbiosome membrane, which suggests that loss of algal control of this system brought about by oxidative stress and dysfunction may lead to lysosomal digestion of the algae and bleaching.

These alterations of Rab5/Rab7 dynamics are similar to what is seen in the persistence of the pathogenic bacterium *Mycobacterium tuberculosis*, which in addition causes alteration in the abundance of phosphoinositides, lipids that interact with and are produced by effectors of Rab GTPases (Gruenberg and van der Goot, 2006). These processes require the secretion of effector molecules from the bacterium. Expression of Rab proteins is under control of innate immune response genes. For example, in macrophages, the cytokines IL-6 and IL-12 regulate the expression of Rab5 and Rab7 respectively during the phagosome maturation process (Bhattacharya et al., 2006). This cytokine-induced expression of Rab5 and Rab7 was shown to be mediated by ERK and p38 MAPK signaling respectively (Bhattacharya et al., 2006). It was also shown that infection of cells with the intracellular bacterial pathogen *Salmonella* was reduced after treatment with IL-12 while a large number of bacteria proliferated in IL-6 treated cells. This suggests that bacterial killing was due to Rab7-mediated targeting to the lysosomes, while survival was due to recruitment of Rab5 and inhibition of fusion with lysosomes (Bhattacharya et al., 2006).
How symbiotic algae mediate this control, presumably through secretion of effector molecules and how oxidative stress affects the regulation of Rab GTPases has not been described. Loss of Rab5 from membranes is activated through rho GDP dissociation inhibitor (GDI) and p38 mitogen activated protein kinase (p38 MAPK) (Cavalli et al., 2001). Oxidative stress is a known activator of p38 MAPK suggesting this signaling could provide a way for the host to regain control of endosome traffic leading to maturation of the symbiosome and lysosomal digestion of the defective algae.

Another important component of endosome dynamics is the interaction of these vesicles with the cytoskeleton and motor proteins. Interaction between Rab7 and its effector protein Rab7-interacting lysosomal protein (RILP) have been shown to play a role in mediating endosome movement, a mechanism exploited by intracellular *Salmonella enterica* (Gruenberg and van der Goot, 2006, Harrison et al., 2004, Jordens et al., 2001). Analysis of tissues of undergoing bleaching showed cytoskeleton-dependent movement of symbiosomes from basal locations to more apical locations of the host cell, indicative of preparation for exocytosis (Fang et al., 1998a, Steen and Muscatine, 1987).

**Exocytosis**

During bleaching, algal cells free of host cell material makeup part of the released algae which suggest that they could be released through exocytosis (Brown et al., 1995, Steen and Muscatine, 1987). Studies on the coral *Acropora grandis* showed
that bleaching depended on the function of the cytoskeleton and on an intracellular calcium signal, both elements known to regulate exocytosis of secretive vesicles (Fang et al., 1998b). Incubating corals with antagonists of the cytoskeleton, the motor proteins myosin and dynein and the calcium-dependent signaling molecule calmodulin abrogated the release of algae after exposure of corals to elevated temperatures (Fang et al., 1998b). Most basic studies of exocytosis have focused mostly on the process of secretion and the delivery of plasma membrane components and not the process of elimination of late phagocytic vesicles containing indigestible material (Gotthardt et al., 2002). However, lysosomes fuse with the cell membrane through a calcium-regulated process mediated by the lysosomal protein synaptotagmin VII (Syt VII) (Reddy et al., 2001, Tucker and Chapman, 2002). In macrophages, late phagosomes can undergo GTP, syntaxin and microtubule regulated exocytosis which results in the egestion of phagocytosed particles and the release of free radicals that accumulate during their digestion (Damiani and Colombo, 2003, Di et al., 2002). Therefore production of ROS during bleaching as well as alterations of intracellular calcium may be involved in signaling both the digestion and the exocytosis of algae.

Dysfunction of cell-adhesion

The most commonly observed mode of algal loss is the detachment of entire host gastrodermal cells containing the algae as well as host nuclei. This mechanism is seen after exposure to elevated temperature as well as to cold shock. It is therefore believed that host cell detachment is the prevalent cellular-level mechanism for bleaching (Gates et al., 1992, Sawyer and Muscatine, 2001). It was suggested that
changes in temperature could cause a series of membrane thermotropic effects and
effects on protein folding which may trigger loss of cell adhesion through a passive
influx of Ca\textsuperscript{2+} which could in turn affect the function of the cytoskeleton and cell
adhesion molecules (Gates et al., 1992, Muscatine et al., 1991). However, studies
using electron paramagnetic resonance of the tropical sea anemone *A. pulchella* and
the coral *Pocillopora damicornis*, did not find evidence for membrane phase transition
changes as a function of temperature (Sawyer and Muscatine, 2001). In addition other
non-thermal stressors can also induce bleaching and a rise in intracellular Ca\textsuperscript{2+} could
result from other sources such as mitochondria, endoplasmic reticulum or calcium-
storage proteins. However, calcium was not observed to play a significant role during
bleaching of *A. pulchella* since incubation with calcium channel blockers, calmodulin
antagonists, Ca\textsuperscript{2+} -ATPase inhibitors or a calcium chelator did not inhibit bleaching
due to cold shock and incubation with calcium ionophore ionomycin could not induce
bleaching even though it caused an increase in intracellular calcium levels of isolated
host cells. Cold shock of isolated host cells did not trigger measurable changes in
Ca\textsuperscript{2+} flux. On the other hand, an increase in intracellular calcium level was observed
during exposure of the coral *Acropora grandis* to elevated temperature and this rise in
intracellular calcium was required for bleaching (Fang et al., 1997, Huang et al.,
1998). These results suggest that calcium is not involved in bleaching as a result of
cold shock but does play a role during the hyperthermic response. It is also likely that
the use of isolated host cells to study Ca\textsuperscript{2+} dynamics may introduce artifacts related to
the viability of these cells. The role of intracellular calcium as an upstream mediator
of bleaching remains to be explored in more detail and should provide valuable
information given its extensive role in regulation of cellular functions including programmed cell death. Also the regulation of cell-cell and cell-matrix adhesion through alterations of the cytoskeleton function remains to be elucidated in more detail.

Another potential mechanism of host-cell detachment is the modulation of the components of the extracellular matrix (ECM). The epithelial cell layers of cnidarians are bound to the basal lamina composed of ECM components such as laminin, type IV collagen, fibronectin and heparan sulfate proteoglycans (Sarras et al., 1991a, Sarras et al., 1991b, Sarras et al., 2002, Yan et al., 1995). In addition to its role as physical support, the ECM plays important roles in signaling and regulation of tissue/cell behavior such as differentiation, proliferation, migration and cell death. This function is mediated in part through the activity of matrix degrading enzymes known as matrix metalloproteinases (Chakraborti et al., 2003, Somerville et al., 2003). In Hydra, MMPs mediate morphogenic processes such as tissue turn-over (Sarras et al., 2002, Yan et al., 2000, Yan et al., 1995). Interestingly various MMPs are known to be activated as well as upregulated by nitrosative and oxidative stress through signaling via MAPK pathways such as ERK, JNK and p38 (Chakraborti et al., 2004, Chakraborti et al., 2003, Mandal et al., 2005, Mandal et al., 2004, Nelson and Melendez, 2004, Siwik and Colucci, 2004, Yoo et al., 2002). The role of MMPs and the ECM during cnidarian bleaching remains unstudied.
Host-cell detachment could also be a secondary outcome due to a loss of tissue integrity after the death of a subpopulation of cells. The role of programmed cell death in cnidarian bleaching due to elevated temperature has been the focus of recent studies. Histological analysis of tissues from the symbiotic sea anemone Aiptasia pallida undergoing bleaching uncovered cell morphologies characteristic of apoptosis or necrosis in both host and algal cells (Dunn et al., 2002). In the following section I describe the process of programmed cell death and necrosis and outline the molecular regulation behind these processes.

PROGRAMMED CELL DEATH

Cells die through several distinct sets of processes which were originally defined based on the morphology of the dying cell. Sometimes this process can occur through an orderly or programmed series of steps and depend on the activation of proteins with specific roles in cell death. This programmed form of cell death manifests itself through two very complex and well regulated processes: Apoptosis (Type I cell death) or autophagy (Type II cell death). In contrast, the term oncosis defines the process of cell death which does not occur through such programs and instead results in the morphological manifestation know as necrosis.

Apoptosis vs. necrosis

Apoptosis is associated with a set of hallmark morphological changes of the cell (Kerr et al., 1972). During the early stages of apoptosis cells detach from neighboring cells and extracellular matrix, become more rounded and decrease in total
volume. The nuclear chromatin condenses and aggregates into dense particles known as pycnotic bodies and the nucleus becomes irregular and fragments. The endoplasmic reticulum swells and becomes vacuolated. The plasma membrane forms blebbs which separate from the dying cell carrying cellular contents including nuclear fragments forming what are called apoptotic bodies. A significant result of this organized breakdown of the cell is that the cellular contents are not released freely into the extracellular space but are packaged within membranes to be later disposed of though engulfment and lysosomal breakdown by phagocytic cells. This is in contrast to what happens through oncosis during which the organelles swell and disintegrate and the cell swells eventually resulting in the rupture of the plasma membrane and leakage of cellular material, a process also known as necrosis. The morphology of apoptotic cells is also defined by characteristic changes occurring at the molecular level. The nuclear chromatin condensation is accompanied by its internucleosomal fragmentation mediated by endonucleases, a process which results in the phenomenon of DNA-laddering of extracted DNA ran on agarose gels. At the plasma membrane, cell-cell and cell-matrix junctions are broken down. In addition, changes in the composition of plasma membrane components take place such as the externalization of phosphatydilserine. Many of the morphological and molecular characteristics of apoptotic PCD and necrosis have been observed in cnidarians including \textit{A. pallida} (Cikala \textit{et al.}, 1999, David, 2005, Dunn \textit{et al.}, 2002, Kuznetsov \textit{et al.}, 2002, Miller \textit{et al.}, 2000, Seipp \textit{et al.}, 2001, Technau \textit{et al.}, 2003). Similar morphological evidence in plants as well as single-cell eukaryotes such as yeast and algae has suggested an early
origin of mechanisms of PCD (Debrabant et al., 2003, Franklin and Berges, 2004, Madeo et al., 1999).

**Autophagy**

Compared to apoptosis, much less is known about the process of autophagy. Through autophagy, cells degrade their proteins and recycle cellular organelles as a survival response to cellular starvation. Cells undergoing autophagy display pronounced remodeling of internal membrane compartments through the engulfing of organelles by endosomal vesicles (autophagosomes) which then fuse with lysosomes to complete the breakdown of organelle components. Cells undergoing PCD frequently display markers of both apoptosis and autophagy (Motyl et al., 2006). Autophagy can lead to the death of the cell and appears to be co-regulated with apoptosis; for example: inhibition of caspase 8 with the caspase inhibitor zVAD or with RNAi-mediated knockdown results in degradation of the antioxidant enzyme catalase, accumulation of ROS and autophagy of damaged mitochondria (Vandenabeele et al., 2006). Autophagy could be involved in the degradation of algae within symbiosomes during bleaching and evidence of autophagy has recently been reported in cnidarians (Chera et al., 2006, Galliot, 2006).

**Mediators of PCD**

Mammalian apoptosis is an energy-dependent process, particularly during the initiation of the apoptotic program but it is not considered to rely much on protein synthesis given that much of the regulation and execution of the apoptotic occurs post-
translationaly (Jacobson et al., 1993, Jacobson et al., 1994). However transcriptome changes do occur in preparation for apoptosis (Johnson et al., 2004). In contrast, apoptosis in yeast appears to rely more on protein synthesis given that cyclohexamide can prevent cell death in response to apoptotic triggers (Madeo et al., 1999). I next describe the molecules involved in apoptosis and their regulation.

The study of the molecular players of apoptosis began with the discovery of the gene *ced-3* responsible for the programmed death of cells during the developmental process of the nematode *Caenorhabditis elegans*. It was determined that this gene was responsible for proteolytic breakdown of the dying cell and, together with its mammalian homolog (interleukin 1β converting enzyme (ICE); caspase-1) belonged to a group of enzymes which played a central role in apoptosis. These enzymes, the cysteine aspartate-specific proteinases (caspases), a family of cysteine proteases, are post-translationaly activated from their pro-enzyme (procaspase) form in response to apoptotic signals (Nicholson, 1999). The activation of caspases due to apoptotic stimuli is a highly regulated process which, once started, self-amplifies through positive feedback mechanisms committing the cell to an irreversible path of orderly self-destruction. These activation events are generally mediated by initiator caspases (e.g. caspase-8 and caspase-9) which have as downstream targets the executioner caspases (e.g. caspase 3, 6 and 7) which ultimately process various molecular targets for activation or destruction. The caspase-driven apoptotic program is well conserved across a wide phylogenetic spectrum.
Hydra polyps express proteins homologous to caspase-3 family (similar to ced-3) and protein extracts display caspase-3-specific activity (Cikala et al., 1999). In A. pallida, a caspase homolog was discovered with structural characteristics of both initiator and executioner caspases (Dunn et al., 2006). Caspase related protease in yeast (Madeo et al., 2002a, Madeo et al., 2002b). However, no homologs of animal caspases have been identified in plants although evidence of true caspase-like activity (cysteine aspartate-specific endoprotease activity) with roles in cell death has been described (Woltering et al., 2002). A likely candidate group for this plant caspase-like activity are the metacaspases due to their relatedness to caspases and given that the yeast metacaspase YCA1 has caspase-like activity and is involved in cell death in response to stress (Khan et al., 2005, Silva et al., 2005, Woltering et al., 2002).

Interestingly, one of the genes upregulated in response to exposure to reactive nitrogen species (RNS; e.g. peroxynitrite) and reactive oxygen species (ROS; e.g. superoxide) in the free-living dinoflagellate Pyrocystis lunula encodes a metacaspase, (Okamoto and Hastings, 2003). This finding strongly suggests that the PCD observed in symbiotic algae during bleaching may be mediated through metacaspases perhaps also upregulated by RNS and ROS produced upon exposure to elevated temperature (Dunn et al., 2004, Perez and Weis, 2006).

Another important group of proteins with roles in apoptosis are the Bcl-2 family proteins. Some of these are anti-apoptotic such as Bcl-2 and Bcl-xL. Others are pro-apoptotic such as Bak, Bax, Bad, Bik, Bid and Bim. A gene encoding a Bcl-2
family protein was cloned and sequenced in *A. pallida* (Dunn *et al.*, 2006). Many Bcl-2 proteins modulate the intrinsic PCD pathway at the mitochondria (see below).

**PCD pathways**

PCD is triggered by physiological signals or stress stimuli through the activation of different but converging and very complex pathways. Here I summarize the major components. Upon receiving such stimuli the cell must adjust physiologically or undergo either PCD or necrosis. Necrosis is considered a non-physiological form of cell death brought about by massive cellular injury, extreme cellular dysfunction or energy depletion. However the same stimuli that in large doses or duration cause necrosis can trigger the apoptotic program when experienced at lower levels. In *A. pallida*, the frequency of host and algal cells displaying PCD increases after short-term exposure to hyperthermic stress while necrosis becomes more prevalent after continued exposure to such stress (Dunn *et al.*, 2004). It is believed that the switch between PCD and necrosis is dependent on the energy status of the cell.

The two major PCD pathways activated by death stimuli: the receptor-mediated pathway and the intrinsic (Fig. 4.3). In the receptor-mediated pathway, extracellular ligands signal the cell to activate the apoptotic program through these membrane-spanning receptor complexes. The prototypical death receptors include Fas (CD95) and tumor-necrosis factor receptor-1 (TNF-R1) which belong to type I transmembrane proteins and bind to FasL and TNFα respectively. These receptors
belong to a superfamily of death domain containing receptors. When Fas binds FasL, the receptors trimerize and their cytosolic death domains (DD) recruit the adaptor protein FADD (Fas-associated death domain protein) which contains a death effector domain (DED). This domain binds to pro-caspase-8 which then transactivate each other upon trimerization of Fas. This multiprotein complex is known as the death inducing signaling complex or DISC. Active caspase-8 cleaves caspase-3 as well as other executioner caspases which bring about modification of various targets in the breakdown of cell components. Receptor-mediated activation of apoptosis \textit{via} TNF-R1 is mediated through binding with the adaptor protein TNF-R1-associated death domain protein (TRADD) which can recruit FADD to activate apoptosis. Alternatively it can recruit RIP1 and TRAF (TNF receptor-associated factor) which leads to suppression of apoptosis and proliferation or differentiation of cells through activation of the transcription factor NFκB or JNK. Evidence of receptor-mediated regulation of apoptosis in cnidaria is building. In \textit{Hydra}, for example, an expressed TRAF homologue was discovered with a potential role in regulation of apoptosis (Mali and Frank, 2004).
Figure 4.3 Schematic representation of Apoptotic pathways. Inactive pro-caspases are shown in yellow while activated forms are in red.
The intrinsic pathway involves the mitochondria and is therefore also known as the mitochondrial pathway. This death pathway responds to various stress signals such as calcium fluxes and oxidative stress and therefore potentially significant in the mechanism of cnidarian bleaching. This pathway is also activated downstream of the receptor-mediated pathway via caspase-8-mediated activation of tBid and recruitment of Bax to the mitochondria which induces release of cytochrome c (Cyt-c) (Fig.4.3). Bcl-2 family proteins converge on mitochondria where they integrate signals to either prevent or activate the apoptotic program. Apoptotic stimuli cause an increase in the permeability of the mitochondrial outer membrane, causing the release of Cyt-c and other proteins from the intermembrane space. These in turn can activate both caspase-dependent (such as Smac/DIABLO) as well as caspase-independent factors (e.g. apoptosis inducing factor; AIF). Upon release of Cyt-c it combines with a well conserved protein know as apoptotic protease-activating factor 1 (Apaf-1) and this induces an ATP-dependent formation of the multi-protein complex knows as the apoptosome. This complex binds caspase-9 through its caspase recruitment domain (CARD). Homologues of BCL-2 have been discovered in sponges (Wiens et al 2003, 2001). In addition, the mitochondrial pathway is believed to play a role in PCD of yeast as well as plants (Hoeberichts and Woltering, 2003, Pozniakovsky et al., 2005, Priault et al., 1999, Silva et al., 2005, Wissing et al., 2004).
TLRs and the innate immune response

Signaling through Fas/TNF-R has parallel functions with that of Toll-like receptors (TLRs), a family of pattern-recognition receptors involved in the innate immune response. TLRs are also type I transmembrane receptors containing cytosolic death domains. The study of TLRs will undoubtedly provide important information towards our understanding of regulation of specificity and breakdown of cnidarian-algal symbioses. For example, the bacterial cell-wall component lipopolysacharide, a classic pathogen associated molecular pattern (PAMP) which signals through a TLR, induces NO production in *A. pallida* (Perez and Weis, 2006).

How symbiotic algae might exploit these signaling pathways to their advantage remains to be explored. For example, norditerpenes extracted from the symbiotic soft coral *Sinularia* sp. inhibit TNFα production of murine macrophages challenged with LPS (Takaki *et al.*, 2003). Interestingly, the LPS-induced production of NO in *A. pallida* we observed occurs only in aposymbiotic anemones (Perez and Weis, 2006). Also, pseudopterosins produced by symbiotic algae of the soft coral *Pseudopterogorgia elisabethae* are potent anti-inflammatory agents by modulating G-protein or G-protein coupled receptors (Moya and Jacobs, 2006, Mydlarz *et al.*, 2003, Newberger *et al.*, 2006). These observations and studies suggest that the algae can modulate the innate immune response of the host. Loss of this function during the stress response of algae could cause a lift in this hold and precipitate an innate immune response in the host.
CONCLUSION

Our understanding of the molecular mechanisms has expanded in the recent years but, as evident in this review, much work needs to be done. Cnidarian bleaching is clearly not just a simple process of cellular dysfunction directly due to reactive oxygen species (as in necrosis). It involves cellular responses in part mediated by cytoskeletal elements, dynamics of endosomal trafficking and activation of PCD pathways. Central mediators of these processes include intracellular calcium, ROS and RNS, all of which are expected to act in part through signaling systems yet to be fully described in cnidarians. Some of these are expected to negatively regulate fundamental cell processes such as mitochondrial respiration or the cytoskeletal dynamics. Other more subtle effects could impact specific unknown mechanisms through which symbiotic algae attain stable residence within the host cell.

Understanding the role of the innate immune response of the host and how it is modulated by the algae and during the stress response will undoubtedly provide a more complete model of the regulation of cnidarian-algal symbioses. Innate immune mechanisms bring into juxtaposition the processes of 1) initial recognition & uptake of algae, 2) the maintenance of the symbiosis and 3) the breakdown of the symbiosis which have been treated separately in the past, providing a unifying theme.

Major bleaching events are a recurrent and global problem and are major cause of loss of an estimated 30% or reefs (Wilkinson, 2000). It is projected that by the year 2030, 60% of the world’s coral reefs will be lost (Hoegh-Guldberg, 1999b, Hughes et al., 2003, Wilkinson, 2000, Wilkinson and Buddemeier, 1994). What does our
knowledge of the molecular mechanism of cnidarian bleaching tell us about the ability of corals to adapt to the projected increase in sea surface temperatures? The view of cnidarian bleaching as a complex set of processes which are likely highly constrained, suggests that corals will not be able to adapt rapidly enough. However, much of the pathology of bleaching seems to stem from the susceptibility of the algae (e.g. ROS production) to environmental stress. Therefore, to better understand this problem we also need to understand the forces constraining the physiology of the algae.
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APPENDIX A. DIVERSITY OF DINOFLAGELLATE SYMBIONTS IN RED SEA SOFT CORALS: MODE OF SYMBIONT ACQUISITION MATERS

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Diversity of dinoflagellate symbionts in Red Sea soft corals: mode of symbiont acquisition matters

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ABSTRACT: Symbiotic associations are ubiquitous in terrestrial and marine environments and are of great ecological importance. The onset of a symbiotic relationship differs among associations. Symbionts can be vertically transmitted from host parent to offspring or they can be acquired horizontally from the surrounding environment with each new host generation. Cnidarian-algal symbioses, the subject of our study, exhibit both strategies. We investigated the clade identity of symbionts in soft coral hosts (Eilat, Red Sea) in relation to their hosts' mode of symbiont acquisition. We found for the first time that all hosts using horizontal transmission harbored symbionts belonging to Clade C while those with vertical transmission uniquely harbored symbionts from Clade A. The latter, capable of coping with a wide array of environmental conditions, evolved to be optimal vertically transmitted symbionts. The limitation of Clade A symbionts to hosts with vertical transmission suggests a co-evolution of the hosts and symbionts. Clade C symbionts, characterized by large sub-clade variability, are found in corals with horizontal transmission and, most probably, each of its genotypes exhibits a more specialized set of physiological capabilities.

KEY WORDS: Symbiont acquisition · Octocorals · Zooxanthellae · Red Sea

INTRODUCTION

Among the most significant marine mutualisms are those found between members of the phylum Cnidaria, such as corals and anemones, and their photosynthetic dinoflagellate symbionts (Symbiodinium spp., also called zooxanthellae), which together form the trophic and structural foundations of coral reef ecosystems. There is a rich literature on coral systematics and phylogeny, based on skeletal morphology (Veron 1995) and more recently also on molecular markers (Clen et al. 2002). In contrast, classical taxonomic studies of the zooxanthellae are hindered by what seems to be a uniform algal morphology. The advent of molecular tools has resulted in the birth of the field of zooxanthellae molecular phylogenetics and systematics. Zooxanthellae are now known to be taxonomically highly diverse and have been divided into 5 distinct clades (A, B, C, D and E), based on ribosomal RNA gene sequences and RFLP patterns (Rowan & Powers 1991a,b; Rowan 1998, LaJeunesse 2001, Toller et al. 2001). These clades represent subgeneric taxonomic levels, and each contains 1 or more subtypes, as revealed by analysis of the internal transcribed spacer regions (LaJeunesse 2001, Savage et al. 2002).

Symbiotic systems, being based on the relationship of 2 different entities, have always been subject to the study of specificity (see Douglas 1994). Dealing with Cnidarian-algal symbiosis, this subject represents a complex case study, which can be attributed to: (1) the taxonomy of the symbionts being still under investigation; (2) dual mechanism of symbiont acquisition—source of symbionts varies with host taxon; (3) the fact that there are hundreds of host species and unknown numbers of symbiont species. There are different aspects (biotic and abiotic) to the study of specificity and many of them have already been dealt with (see Baker 2003). The use of finer resolution molecular tools and the screening of a wide array of cnidian hosts over a wide biogeographic range (LaJeunesse 2002, LaJeunesse et al. 2003) is doubtless of great importance to our understanding of specificity in this sym-
biosis. Another important factor to consider in studies of host-symbiont specificity is the mode of symbiont acquisition by the host (Rowan 1998).

The onset of symbiosis can occur at a variety of host life history stages, depending on the host species. Symbionts can be transmitted horizontally, where the host's sexual progeny acquire symbionts from the surrounding environment; or vertically, being passed directly from host parent to offspring (Trench 1987, Douglas 1994). Horizontal transmission offers the host the opportunity to recombine with different algal types that are differentially adapted to the existing environmental conditions. There is a risk, however, that a host may fail to establish a partnership, leaving it with severely reduced fitness. In contrast, vertical transmission guarantees that a host is provided with a complement of symbionts. It is still unclear how the mode of symbiont acquisition influences zooxanthellae diversity within a host (Muller-Parker & D’Elia 1997, Rowan 1998).

Soft corals (Octocorallia, Alcyonacea) are a significant component of the coral reefs of the northern Red Sea (Benayahu 1985). The life histories and reproductive biology of many Red Sea soft corals are known, as well as the different modes of algal acquisition by the different species (Benayahu 1997). To date, most studies examining zooxanthellae diversity in the Octocoralia have focused on seawhips (family: Plexauridae) (Coffroth et al. 2001, Santos et al. 2001, LaJeunesse 2002), while no comprehensive data exist on soft coral symbionts. Furthermore, data concerning zooxanthellae diversity in Red Sea anemones are anecdotal (Goulet & Coffroth 1997, Carlos et al. 1999, LaJeunesse 2001). We compared symbionts found in hosts belonging to the 3 most common soft coral families, which exhibit different modes of symbiont acquisition, using restriction fragment length polymorphism (RFLP) of the 18S rRNA gene (Rowan & Powers 1991b). Our results show a novel pattern of symbionts’ clade segregation corresponding to the host’s mode of symbiont acquisition.

MATERIALS AND METHODS

Collection and identification. Of the 3 families Alcyonacea, Nepthiidae and Xenidae, 19 soft coral species were investigated in this study (see Table 1). Samples were collected by SCUBA diving from sites along Eilat’s reefs (northern Red Sea) during August 2001. From each species, 3 colonies were frozen and stored at -20°C until further analysis. Before freezing, a portion of each sample was preserved in 70% ethanol for species identification using the reference collection of the Zoological Museum of Tel Aviv University. When present, mature oocytes were examined under a compound microscope to detect the presence of zooxanthellae, in cases where no previous data were available.

Extraction of DNA. Samples were thawed, transferred to microfuge tubes, and ground with a small plastic pestle in filtered seawater. Homogenates were then centrifuged for 2 min at 2000 rpm (350 x g) to pellet the algae. Animal supernatant was removed and discarded and the algal pellet was resuspended in DNA extraction Buffer (4M NaCl, 50mM EDTA, pH = 8.0). After further centrifugation, the pellet was resuspended in CTAB buffer and incubated for 2 h at 50°C together with Proteinase K. The DNA extraction procedure was continued using a DNAeasy Tissue Kit (IQIAGEN).

PCR amplification and restriction fragment length polymorphism (RFLP) analysis. The 18S rRNA gene was amplified from symbiotic dinoflagellates using the primers RNAF2 5'-TATTGATTGCTTTYCTGCTAC-3' and RNAR2 5'-CAAATWATTTCACCGGATCAC-3', similar to those used by Rowan & Powers (1991a). Amplifications were performed using a DNA thermal cycler (UNO II, BIOMETRA) under the following conditions: 94°C for 45 s, 54°C for 45 s and 72°C for 2 min (31 cycles). RFLP analysis was performed using restriction digest with Taq I (MBI Fermentas) and Dpn II (BioLabs) restriction enzymes for 2.5 h at 65 and 37°C, respectively. Digestion products were separated by electrophoresis in 2% 0.5X Tris-Borate (TBE) agarose gels to generate RFLP patterns, which were compared to the literature to assign each sample to one of the established Symbiodinium 18S rDNA RFLP clades (Rowan & Powers 1991a,b, Banaszak et al. 2000).

DNA sequencing. PCR products were cloned into pDrive cloning vector with IQIAGEN EZ competent cells as a host, using IQIAGEN PCR Cloning Plus kit according to the manufacturer’s instructions. Fledged DNA from individual colonies was purified using QIAprep Spin Miniprep kit (IQIAGEN). The plasmid DNA was sequenced using the ABI PRISM BigDye Terminators v 3.0 Cycle Sequencing Kit (Applied Biosystems). Due to the large size of the insert (1600 bp), each sample was sequenced from both sides using T7 and SP9 primers. Partial sequences of symbiont genotypes derived in this study were submitted to GenBank. Accession numbers for sequences of symbionts isolated from the corals Litophyton arboreum, Rhytisma fusum fulvum, Nephthea sp., Cladiella tubeculoides, Sinularia querciformis, Sarcophyton glaucum, Xenia larvaensis, Paracordiella eburnea, Stereonephthya cuadailabians and Antiqua giawia are AY525018–27, respectively. The symbiont isolated from Heteraxenia fuscescens is available under the accession number AY488089.
In order to sequence the second genotype of symbionts, observed in 5 of the Alcyonid corals, the PCR product of the coral *Sarcophyton glaucum* was cloned as described above. The transformed bacteria colonies were picked and used as template in a PCR reaction, using the vector primers. PCR products were run on an agarose gel to ensure an insert in the right size. Subsequently, the products were digested with *Sac* I restriction enzyme. Three PCR products, which showed the distinct band pattern, were sequenced as described above.

Phylogenetic analysis. Fragments of *ssrRNA* sequences were obtained for the 3' and 5' ends. These sequences were joined to form a partial composite sequence for each species. Sequences were aligned using the multiple sequence alignment program CLUSTAL-W (Thompson et al. 1997). The following reference sequences taken from GenBank for the 18S RNA gene were also included in the alignment: *Symboodium sp.* GenBank accession number AP232526 (Clade A); *Symboodium sp.* GenBank accession number AB016594 (Clade C); and *Cymodoconium simplicum* GenBank accession number G5U41086 (Outgroup). The alignment was checked and adjusted by eye using the sequence manipulation program MacVector. Only the aligned sequence portions which were represented by all sequences included were used in the phylogenetic analysis. This was performed by excluding non-overlapping characters using the mask tool of MacVector. Gaps in the alignments were treated as a fifth character. A total of 1114 characters were included in the mask. The final alignment with the applied mask was converted to a Nexus format file and used with the phylogenetic analysis program PAUP* 4.0. The phylogenetic reconstruction was performed with the Neighbor Joining (NJ) applying the Kimura 2-parameter model, as well as Maximum Parsimony method. The sequence from *Cymodoconium simplicum* (GenBank accession number G5U41086) was used as the outgroup. Bootstrapping was performed using 1000 replications and a bootstrap consensus tree was constructed using the 50% majority rule.

RESULTS

Among the 19 soft coral species studied (Table 1), 16 contained zoanthellae belonging to Clade C, whereas 3 species contained zoanthellae from Clade A (Figs. 1 & 2). All species harboring Clade C algae acquire their symbionts horizontally from the environment, while the species harboring Clade A algae acquire theirs directly from the parent at the oocyte stage. The RFLP patterns of 5 of the alcyonid corals revealed an additional faint band 270 bp in size (Fig 1, Lanes 9 to 13). This band is indicative of the presence of another symbiont genotype. Cloning and sequencing of this genotype (isolated from *Sarcophyton glaucum*) revealed that it belongs to Clade C.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mode of reproduction</th>
<th>Zoanthellae in oocytes</th>
<th>Stage of acquisition</th>
<th>Source</th>
<th>Clade</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cladiella pectinata</td>
<td>Spawning</td>
<td>No</td>
<td>Primary polyp</td>
<td>This study</td>
<td>C</td>
</tr>
<tr>
<td><em>Cladiella tuberculata</em></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>C</td>
</tr>
<tr>
<td>Rhytisma fulvum fulvum*</td>
<td>Surface brooding</td>
<td>No</td>
<td>Primary polyp</td>
<td>Benayahu &amp; Loza (1983)</td>
<td>C</td>
</tr>
<tr>
<td><em>Sarcophyton glaucum</em></td>
<td>Spawning</td>
<td>No</td>
<td>Primary polyp</td>
<td>Benayahu &amp; Loza (1986)</td>
<td>C</td>
</tr>
<tr>
<td><em>Sarcophyton trochelophorum</em></td>
<td>Spawning</td>
<td>No</td>
<td>Primary polyp</td>
<td>Shinkarenko (1981); this study</td>
<td>C</td>
</tr>
<tr>
<td><em>Simularia guttata</em></td>
<td>nd</td>
<td>No</td>
<td>nd</td>
<td>This study</td>
<td>C</td>
</tr>
<tr>
<td><em>Simularia leptalecta</em></td>
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<td>No</td>
<td>Primary polyp</td>
<td>Benayahu et al. (1990)</td>
<td>C</td>
</tr>
<tr>
<td><em>Simularia polyactyla</em></td>
<td>Spawning</td>
<td>No</td>
<td>Primary polyp</td>
<td>Alno &amp; Coll (1989)</td>
<td>C</td>
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<tr>
<td><em>Simularia querciforii</em></td>
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<td>No</td>
<td>nd</td>
<td>This study</td>
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<tr>
<td>Xenididae</td>
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<td></td>
<td></td>
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<tr>
<td><em>Anthelia glauca</em></td>
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<td>No</td>
<td>Embryo</td>
<td>Knagler &amp; Schleyer (1998)</td>
<td>C</td>
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<tr>
<td><em>Heteroxenia fuscescens</em></td>
<td>Brooding</td>
<td>No</td>
<td>Primary polyp</td>
<td>Benayahu (1991)</td>
<td>C</td>
</tr>
<tr>
<td><em>Xenia farinosa</em></td>
<td>Brooding</td>
<td>No</td>
<td>Embryo</td>
<td>Benayahu &amp; Loza (1984); this study</td>
<td>C</td>
</tr>
<tr>
<td><em>Xenia macropilula</em></td>
<td>Brooding</td>
<td>No</td>
<td>Embryo</td>
<td>Benayahu et al. (1992)</td>
<td>C</td>
</tr>
<tr>
<td><em>Xenia umbellata</em></td>
<td>Brooding</td>
<td>No</td>
<td>Embryo</td>
<td>Benayahu (1991)</td>
<td>C</td>
</tr>
<tr>
<td>Nepthiaidae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>Litophyton arboreum</em></td>
<td>Brooding</td>
<td>Yes</td>
<td>Oocërte</td>
<td>Benayahu et al. (1992)</td>
<td>A</td>
</tr>
<tr>
<td><em>Nepthea sp.</em></td>
<td>Brooding</td>
<td>Yes</td>
<td>Oocërte</td>
<td>Benayahu (1997), Lutzky (1997)</td>
<td>A</td>
</tr>
<tr>
<td><em>Parnallia eburnea</em></td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>C</td>
</tr>
<tr>
<td><em>Parnallia thyrsites</em></td>
<td>Brooding</td>
<td>No</td>
<td>Primary polyp</td>
<td>Benayahu (1997); this study</td>
<td>C</td>
</tr>
<tr>
<td><em>Stoeonephthya cuvillii</em></td>
<td>Brooding</td>
<td>Yes</td>
<td>Oocërte</td>
<td>Benayahu (1997); this study</td>
<td>A</td>
</tr>
</tbody>
</table>

Table 1. List of soft corals examined, their modes of reproduction and developmental stage during which symbionts are acquired.

*Partial sequence of 18S rRNA gene of zoanthellae is available. nd = na data*
Fig. 1. Taq I RFLP analysis of 18S rRNA encoding DNA from zooxanthellae of different Red Sea soft corals. Lane 1 *Anthelia glauca*, Lane 2 *Heteroxenia fuscescens*, Lane 3 *Xenia farauensis*, Lane 4 *Xenia macrospiculata*, Lane 5 *Xenia umbellata*, Lane 6 *Cladiella tuberculoides*, Lane 7 *Cladiella pachycladæ*, Lane 8 *Simularia querciformis*, Lane 9 *Simularia polydactyla*, Lane 10 *Simularia leptocladæ*, Lane 11 *Simularia gardineri*, Lane 12 *Sarcophyton glaucum*, Lane 13 *Sarcophyton trochelophorum*, Lane 14 *Rhytisma fulvum fulvum*, Lane 15 *Paralemnalia eburnea*, Lane 16 *Paralemnalia thyrsoidæ*, Lane 17 *Lithophyton arboream*, Lane 18 *Nephthea sp.*, Lane 19 *Stereophyta candiabluensis*, Lane LD 1kb Plus DNA ladder (GibcoBRL). White arrow indicates the 750 bp band.

The mode of sexual reproduction in soft corals (families Alcyoniidae, Nepthidae and Xeniidae) and features such as the onset of symbiosis during development and presence or absence of zooxanthellae in oocytes exhibit a high degree of consistency within genera (Benayahu 1997). Thus, in the few cases in our study where no data are provided for a given species in Table 1, it is likely that the mode of reproduction, presence/absence of zooxanthellae in oocytes and stage of algal acquisition follow the respective patterns of the co-generics.

All the Alcyoniidae corals studied, including spawners and a surface brooder (Table 1), acquired algae from the environment during their primary polyp stage (Table 1) (Benayahu 1997) and possessed zooxanthellae belonging to Clade C (Fig. 1, Lanes 1 to 8) or a combination of 2 genotypes of Clade C (Fig. 1, Lanes 9 to 13). Similarly, all Xeniidae examined possessed Clade C zooxanthellae. The latter display a diversity of brooding mechanisms and differ in the developmental stage during which symbionts are acquired. Planulae of *Heteroxenia fuscescens* lack zooxanthellae when released and acquire symbionts soon after metamorphosis (Benayahu et al. 1989a,b).

In *Anthelia glauca*, zooxanthellae appear in the embryos, which are brooded within the pharyngeal cavity of the polyps (Benayahu & Schleyer 1998, Kruger et al. 1998). Planulae of *Xenia* species develop inside invaginated brooding chambers lined with ectoderm, which are open to the environment and contain zooxanthellae that are phagocytosed by the brood (Achtiv et al. 1992). Hence, planulae of *A. glauca* and...
Xenia species acquire their symbionts horizontally prior to release. Thus, all the studied alocyiniid and xenid soft corals, despite variations in the mode of reproduction and stage of acquisition (see also Table 1), contain symbiotic algae belonging to Clade C.

It is in the Nepthideae that the different modes of symbiont acquisition were found to correlate with the symbiont clade found in adults. Although all 5 nephtheid species studied are brooders, the 2 in the genus Paralemnalia (P. thysoides and P. eburoea), release azooxanthellate planulae (Table 1) that must acquire symbionts horizontally. Like the above-mentioned xenid and alocyiniid species with horizontal transmission, these too contained Clade C zooxanthellae. In contrast, Litophyton arboresum, Nephtyidae sp. and Stereonephthya cumdahilensis have vertical symbiont acquisition, where zooxanthellae are directly transmitted to the sexual progeny at the oocyte stage (Table 1). These 3 species uniquely harbored Clade A zooxanthellae.

**DISCUSSION**

Symbiotic associations between Cnidarian hosts and their symbiotic algae exhibit 2 possible modes of symbiont acquisition: vertical and horizontal. We examined multiple host species in one location and herein provide, for the first time, perspective on the mode of symbiont acquisition and its relation to clade specificity in a variety of soft coral hosts. Previous studies addressing this subject in stony corals have examined a restricted number of species over a broad biogeographic scale (Hidaka & Hirose 2000, Lob et al. 2001, Rodriguez-Lanetty et al. 2001). Recently, Van Oppen (2004) compared the diversity of symbionts in 25 Montipora species (vertical transmission) and Acropora species (horizontal transmission) in Indonesia and the central Great Barrier Reef, Australia. They found that the mode of symbiont acquisition does not affect symbiont diversity (i.e. number of symbiont types within a host) within acropodid corals.

The Gulf of Elat, situated at the northernmost limit of coral reefs distribution, is characterized by extreme environmental conditions such as catastrophic low tides, elevated temperatures and high irradiance (Loya 1986, Achituv & Dubinsky 1990). However, no bleaching events have been reported from this area (Pilcher & Alshuwaiby 2000). Our data indicate that hosts harboring either Clade A or Clade C symbionts co-occur in the same habitats. For example, Litophyton arboresum (Clade A) and Rhytisma fimbrium fimbrium (Clade C) form monospecific carpets on Elat’s reef flats (Benayahu & Loya 1997). Furthermore, molecular analysis of zooxanthellae from Heteroxenia, Similaria, Rhytisma, Stereonephthya and Litophyton, sampled over a depth gradient (1 to 20 m), reveals persistence in clade specificity with depth within a host (O. Benayahu unpubl. data). Therefore, the distribution of symbiont clades in Elat’s soft corals negates the correlation between symbiont clade and depth demonstrated in Caribbean reefs (Rowan & Knowlton 1995, Rowan et al. 1997, LaJeunesse 2001, Toller et al. 2001).

Symbionts belonging to Clade A were only found in hosts that vertically transmit their symbionts. There is evidence from studies performed in the Caribbean that Clade A zooxanthellae are shallow-water specialists (Toller et al. 2001, LaJeunesse 2002), and are relatively stress tolerant or “weedy” (Rowan 1998). It has also been shown that Clade A zooxanthellae are widely tolerant to temperature change (Kinzie et al. 2001) and are the only clade capable of synthesizing mycosporine-like amino acids (MAAs) (Baraszk et al. 2000). Together, these studies suggest that Clade A algae may be well adapted to cope with Elat’s environmental conditions. We suggest that Clade A symbionts, evolved to be optimal vertically transmitted symbionts, that did not fail their host and thus persisted as faithful symbionts for generations. It should be pointed out that data are lacking concerning the survival of Clade A symbionts in the free living state. For example, they might be very successful within the coral tissues but outcompeted by different algal clades while in the water column. The limitation of Clade A symbionts to hosts with vertical transmission suggests a coevolution of the hosts and symbionts. Data on the phylogey of Red Sea soft corals that could be overlaid onto the phylogeny of the symbionts will yield further information on how the observed specificity pattern arose. Were Clade A algae “captured” by a single-nephtheid ancestor that gave rise to the 3 vertically transmitting genera, or did this event occur a multiple of times within the family? No congruence was found between Montipora host and symbiont phylogenies, a fact that might be attributed to occasional lateral transfer of zooxanthellae between species, which might have occurred as a result of interspecific hybridization arising during mass spawning events (Van Oppen et al. 2004). We anticipate a different result in the 3 nephtheid hosts harboring Clade A zooxanthellae. These corals are brooded, from a region characterized by temporal reproductive isolation rather than by mass spawning events (Showalter & Loya 1995), representing the most conservative scenario among cnidarians, in terms of vertical transmission.

Clade C symbionts, characterized by large sub-clade variability (Fig. 2), are found in corals with horizontal transmission and, most probably, each of this clade’s genotypes exhibits a more specialized set of physiological capabilities. Recently, LaJeunesse et al. (2003)
found that the majority of endosymbiotic dinoflagellates in cnidarians in the Great Barrier Reef (Australia) belong to Clade C, which is composed of closely related, yet ecologically and physiologically distinct, types. The observed combination of 2 different genotypes of Clade C in 5 species of alcyonid soft corals further highlights the flexibility of an open system of symbiont acquisition. We hypothesize that free-living stages of Clade C symbionts are prevalent in the reef area and are available for acquisition by juvenile stages of soft coral hosts. Future studies examining distribution and availability of symbionts in the open water and physiological plasticity of different symbionts strains are needed to test these hypotheses.

Based on our results from soft corals and on the recent literature (LaJeunesse et al. 2003, Van Oppen 2004c) in stony corals (Montipora and Porites respectively), we hypothesize that corals which vertically transmit their symbionts will tend to have genetically distinct symbionts, which differ from those found in corals with horizontal transmission. In our study, the distinctive feature is symbiont clade, while in Montipora and Porites, the symbionts were found to belong to a distinct sub-clade. Whether the symbionts belonging to Clade A (from the 3 studied nephtheid corals) represent one type or 3 distinct, yet similar, types is yet to be determined.

Vertical transmission is relatively rare among cnidarians (Trench 1987). Coral hosts with vertical transmission of symbionts are assumed to represent a scenario in which a certain ancestral symbiont was ‘trapped’ and evolved within host tissue. It is likely that these holobionts were exposed to changing environmental conditions during their evolution and their survival therefore indicates their resilience. Is it possible that corals which vertically transmit their symbionts are more resistant to environmental change? Are coral hosts with vertical transmission less susceptible to bleaching? There are some data in support of these hypotheses. Among a variety of Great Barrier Reef stony corals, 2 of the most bleaching resistant species were Montipora digitata and Porites cylindrica, which both transmit symbionts vertically and associate with the unique Symbiodinium strain C15 (LaJeunesse et al. 2003). A combined comparative approach of sampling symbionts from hosts with vertical versus horizontal transmission, and analysis of their physiological capabilities, can answer these questions.

New data are continually emerging on zooxanthellae systematics and the relation thereof to host-symbiont specificity. Our study, by examining the correlation between symbiont taxon and the mode of symbiont acquisition by soft coral hosts, describes another layer of complexity in this symbiotic relationship. The pattern of clade distribution which we describe is exceptional, and raises many questions to be addressed on a global scale.

Looking at other symbiotic systems, such as the aphid-bacteria, in which the phylogeny of symbionts parallels their hosts (Douglas 1994), it is very clear that we are uncovering a multidimensional concept of specificity, rather then a 2-dimensional one.

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