AN ABSTRACT OF THE DISSERTATION OF

Melissa L. deBoer for the degree of Doctor of Philosophy in Zoology presented on August 26, 2004.

Title: Onset of Symbiosis in a Cnidarian-Dinoflagellate Association

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Abstract approved: _____

Virginia M. Weis

Scleractinian corals, like many other cnidarians, engage in intracellular symbiosis with dinoflagellates belonging to the genus *Symbiodinium*. This association makes invaluable structural and energetic contributions to the marine environment and to the coral reef ecosystem in particular. While many aspects of the cnidarian-dinoflagellate relationship have been well studied, nothing is known about the molecular and biochemical mechanisms of recognition and specificity between the coral and algal partners. This thesis focuses upon these aspects of the association during the onset of symbiosis (infection) between the scleractinian coral *Fungia scutaria* and the dinoflagellate, *Symbiodinium* type C1f.

Chapter 2 examines a known "symbiosis" gene for its role during the onset of symbiosis. In other cnidarian-dinoflagellate associations, carbonic anhydrase (CA) has been shown to function within the symbiosis by supplying the dinoflagellate with CO_2 for photosynthesis. This chapter presents bioinformatic analyses of two CA sequences isolated from *F. scutaria* larvae, as well as the expression pattern of CA

over the course of infection. Though a "symbiosis" CA isoform was recovered from adult *F. scutaria*, all attempts to do so in the larvae failed; the sequences isolated from the larvae are more similar to other forms of the enzyme. Furthermore, expression of these larval CA isoforms did not vary significantly over the course of infection.

Chapters 3 and 4 present attempts to uncover novel biochemical and molecular players in the onset of this symbiosis. The soluble proteomes of *F. scutaria* symbiotic and aposymbiotic larvae are compared in chapter 3, using 2-dimensional polyacrylamide gel electrophoresis. The protein profiles proved remarkably similar between infection states. Chapter 4 examines differences between infection states on a molecular level. A cDNA plasmid library, enriched for symbiotic larval ESTs, was constructed using subtractive hybridization. Library screening revealed differences in expression between symbiotic and aposymbiotic larvae. Bioinformatic analyses of some of the differential ESTs are presented; no homologues could be identified in the sequence databases. Though unidentified changes in gene expression are evident in newly symbiotic larvae, few biochemical changes, such as the implementation of the carbon supply mechanism, seem to be occurring at this stage of the symbiosis.

Onset of Symbiosis in a Cnidarian-Dinoflagellate Association

by

Melissa L. deBoer

A DISSERTATION

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented August 26, 2004

Commencement June 2005

Doctor of Philosophy dissertation of Melissa L. deBoer presented on August 26, 2004.

APPROVED:

Redacted for Privacy

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Melissa L. deBoer, Author

ACKNOWLEDGMENTS

Many thanks to my advisor, Dr. Virginia Weis, for her unwavering support, both personally and professionally, over the course of my graduate career. Her patience, encouragement, and excitement about symbiosis were a strong positive force during my years at Oregon State University. Thanks to my fellow lab members, past and present, for creating an exciting and intellectually stimulating work environment. I am very lucky to have received the tutelage of Wendy Reynolds in particular. She donated her valuable time and experience to help me with the molecular techniques used in these experiments. I could not have completed these experiments without the assistance of Dr. Dave Krupp as well, who aided in the yearly collection of larvae at the Hawaii Institute of Marine Biology on Coconut Island, Oahu, HI. My committee; Joey Spatafora, Barb Taylor, Tom Wolpert, and Jean Hall, made it extraordinarily easy to schedule meetings and almost always answered my emails in a timely manner! They also provided very valuable feedback about my work for which I am extremely grateful. Thanks to the Oregon State University Department of Zoology, and in particular Joe Beatty. They provided essential support to me in the form of teaching assistantships and ZORF research funds. Last and most of all, thanks to my family Brian and Camden Searcy for all of their support and encouragement and for providing balance and respite during my pursuit of this degree.

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CHAPTER 1

INTRODUCTION

Symbiotic relationships have historically encompassed all of those associations where two or more interspecific organisms live together in close association, permanently or for an extended period of time, whether the association is parasitism, commensalism, or mutualism. A parasitism occurs when the symbiont is benefited to the detriment of the host, commensalisms benefit one partner while the other is unaffected, and mutualisms refer to associations which are beneficial to both partners and result in increased fitness. In some cases the symbiosis is facultative, the symbionts are able to live in a non-symbiotic state; in other cases it is obligatory and the symbionts cannot persist outside of the association. Symbiont juxtaposition also varies, an ectosymbiont resides outside of the host while an endosymbiont is located within the host, inracellularly or extracellularly.

Endosymbiotic associations are found in all biological kingdoms between a wide variety of organisms. Methanogenic archaebacteria form associations with anaerobic bacteria and ciliates; fungi form associations with photosynthetic algae or cyanobacteria (lichens) as well as with terrestrial plants (mycorrhiza); and bacteria form a multitude of pathogenic (ex: cholera) and mutualistic (ex: the *Rhizobium*-legume symbiosis) associations with other bacteria, protozoans, fungi, plants and animals. Single celled protozoans are exceptionally successful intracellular endosymbionts. Nonphotosynthetic members of this group of eukaryotes cause many serious illnesses in animals such as chagas disease, leishmaniasis, toxoplasmosis, and malaria. Single celled photosynthetic members, such as some dinoflagellates, form mutually beneficial endosymbiotic associations with many marine organisms like the foraminiferans, sponges, flatworms, molluscs, and cnidarians. The cnidarian –

dinoflagellate association is an especially productive one in the marine environment and is the focus of this thesis.

Cnidarian – dinoflagellate symbioses

Endosymbiotic dinoflagellates occur in all three classes of cnidarians: the anthozoans (corals and anemones), hydrozoans, and scyphozoans (jellyfish), though most of the associations occur within the anthozoan class. The relationship is a mutualism and is nutritionally beneficial to both partners. The alga is provided with a stable, high-light habitat and the animal is able to colonize and inhabit nutrient poor, mostly tropical and subtropical, shallow marine environments. Environmentally, the importance of this symbiosis cannot be understated; the existence of the entire coral reef ecosystem depends upon it. When in association with reef-building scleractinian corals, the presence of the symbiont increases the rate of calcium carbonate deposition by the coral, and this calcification process forms the bedrock of the coral reef structure (Goreau 1959; Pearse and Muscatine 1971). The reef ecosystem is threatened when the host-symbiont relationship breaks down. If this happens the host loses most of its dinoflagellates in a process called bleaching, and along with them the benefits of the relationship (reviewed by Douglas 2003).

The host

The Phylum Cnidaria, comprised of diploblastic metazoans, is the closest ancestral group to the triploblastic metazoans. Members are carnivorous, found in both freshwater and marine environments, and have a single opening to the digestive system (gastrovascular cavity) which is surrounded by stinging tentacles. These animals possess two tissue layers, an outer ectodermis and inner endodermis, which are separated by a gelatinous non-living layer called the mesoglea. As adults, most cnidarians are either sessile polyps (ex: sea anemones and corals) or planktonic medusae (ex: jellyfish).

Many cnidarians engage in both sexual and asexual reproduction. Sexually, cnidarians may be gonochoristic and/or hermaphroditic; spawners or brooders. The spawning species most often broadcast eggs and sperm into the water column where fertilization occurs. The resulting embryo becomes a planula larva, a developmental stage which aids in dispersal. The amount of time larvae spend in the water column before settling and metamorphosing into the polyp stage varies anywhere from one week to well over a month (Fadlallah 1983).

The symbiont

Dinoflagellates are predominantly unicellular, flagellated protozoans closely related to the parasitic apicomplexans (ex: *Plasmodium* and *Toxoplasma*) and ciliates (Bhattacharya et al. 2003). Members of this algal group are almost entirely marine, photosynthetic or nonphotosynthetic, and free-living or endosymbiotic (parasitic or mutualistic). Like cnidarians, dinoflagellates engage in both sexual and asexual (vegetative) reproduction. In the free-living state, dinoflagellates posses two flagella, one transverse and one longitudinal, and many are protected by an elaborate outer membranous covering. In symbiosis, many dinoflagellates have an arrested cell-cycle and persist in a vegetative state (Paracer and Ahmadjian 2000); sexual reproduction by symbiotic dinoflagellates in a cnidarian-dinoflagellate association has never been observed. In the vegetative (symbiotic) state, dinoflagellates are bound by a continuous cell wall, composed of cellulose-containing microfibrils, which lies external to the cell's plasma membrane (Taylor 1987).

Most photosynthetic dinoflagellates engaging in endosymbiotic mutualisms with cnidarians belong to the genus *Symbiodinium*, and are commonly referred to as "zooxanthellae". Historically, the classification of zooxanthellae has proven difficult due to the lack of distinguishing morphological characters in the symbiotic state. Until recently, most zooxanthellae have been thought to belong to the species *Symbiodinium microadriaticum* (Freudenthal 1962). Subsequent morphological,

biochemical, physiological, and, most recently, molecular genetic analysis however, have lead to the breakdown of *Symbiodinium* into several different species and clades (Banaszak et al. 1993; Blank and Trench 1985; Carlos 1999; Darius et al. 1998; LaJeunesse 2001; LaJeunesse 2002; Rowan and Powers 1991b; Rowan and Powers 1992; Trench and Blank 1987; vanOppen et al. 2001). Currently the genus *Symbiodinium* consists of at least seven clades (A to G) as well as subclades or strains within each clade.

The symbiosis

In cnidarian-dinoflagellate symbioses, the zooxanthellae most often reside within the cytoplasm of the host's endodermal cells (Smith and Douglas 1987). The number of algae in the host cell cytoplasm varies; in some species the cell will harbor multiple zooxanthellae while in others it will contain just one. *In situ*, symbiotic dinoflagellates lose their flagella and remain in a permanently nonmotile and vegetative state surrounded by a multi-layered cell wall within multiple layers of membrane; the outermost of these membranes, the symbiosome, is host-derived (Smith and Douglas 1987; Wakefiel and Kempf 2001).

While these symbionts exhibit a significantly decreased mitotic rate (Taylor 1987; Wakefield et al. 2000); the photosynthetic rate of *in situ* zooxanthellae rivals that of free-living species (Muscatine and Weis 1992). This uncoupling of photosynthesis from algal cell growth prevents the symbiont from overgrowing the host and enables the transfer of fixed carbon compounds. Photosynthate transfer from symbiont to host, in tropical, shallow-water corals, can be upwards of 90% (Muscatine 1990; Muscatine and Weis 1992). The symbionts excrete mostly glycerol, but also glucose, alanine, and organic acids (glycolate and fumarate) when *in situ* (Muscatine 1980; Trench 1979). In corals, the symbionts also translocate saturated and polyunsaturated fatty acids, intact neutral lipids, and sterols (reviewed by Papina et al. 2003; Smith and Douglas 1987). In turn, the zooxanthellae metabolize host waste products (nitrogen

and phosphorous compounds) and benefit from a steady supply of CO_2 from the host (Falkowski et al. 1993; Muscatine 1980; Muscatine and Weis 1992; Rahav et al. 1989; Weis et al. 1989).

Onset of cnidarian-dinoflagellate symbioses

Transmission

Cnidarians acquire their symbiont in two basic ways. Direct (vertical) acquisition of the symbiont occurs when the adult host transmits its own algal cells to its sexually or asexually produced offspring. During the sexual cycle, vertically transmitted symbionts enter into the oocyte or brooded planula larva, prior to their release into the environment (Smith and Douglas 1987). Several studies have been done which document to varying degrees the structural events and mechanisms of vertical transmission. Many of these studies document algal entry into the oocytes and larvae of brooding octocorals (Benayahu et al. 1988; Benayahu and Schleyer 1998; Benayahu et al. 1992).

More commonly, transmission of the symbiont during the sexual cycle is indirect or "horizontal". In these cases, each new generation of host, usually during the larval or polyp stage, must acquire its algae from the surrounding environment (Colley and Trench 1983; Fitt and Trench 1983; Schwarz et al. 1999; Trench 1979). For example, larvae without zooxanthellae (azooxanthellate), released by the octocoral *Heteroxenia fuscescens*, settle rapidly and metamorphose into azooxanthellate primary polyps. Infection of the polyp with zooxanthellae occurs on the third day of metamorphosis once the mouth has developed (Benayahu et al. 1989).

During horizontal transmission, entry of the symbiont into the host occurs via phagocytosis (Colley and Trench 1983; Fitt and Trench 1983). Most commonly, algal cells enter into the host gastric cavity where they are phagoctyosed by the host's

endodermal layer, although entry through the ectodermal layer has also been documented (Benayahu and Schleyer 1998; Fitt and Trench 1983; Schwarz et al. 1999). If potential symbionts are absent from the environment, this form of symbiont transmission places the cnidarian at some risk for remaining aposymbiotic (devoid of symbionts). However, it may also present the opportunity to form an association with a more suitable symbiont, one different than that hosted by the parent (Buddemeier and Fautin 1993; Lewis and Coffroth 2004; Rowan and Powers 1991b).

Specificity

Once inside the host cytoplasm, some algal strains are digested or expelled, while other strains persist and become resident. With the appearance of molecular systematics and increasing knowledge of zooxanthellae taxonomy, the presence of a range of specificity (for host and symbiont) in cnidarian-dinoflagellate associations is forming (reviewed by Baker 2003). Some cnidarian species appear to host a homogenous symbiont population while others host a variety of symbionts from multiple clades.

A large fraction of scleractinian corals host a complement of symbiont types (Baker 1999). Often, the symbiont types hosted by Caribbean scleractinian corals are arranged in an ecological pattern correlating with depth (Rowan and Knowlton 1995; Rowan et al. 1997; Toller et al. 2001b). And in general, a coral species living at shallow depths (high-light) will contain higher symbiont diversity than the same species living at deeper (low-light) depths (Baker 1999; LaJeunesse 2002). Individual coral colonies hosting multiple symbiont types also display zonation, similar to the depth zonation seen within a species, according to colony topology and light levels (Rowan et al. 1997). Depth zonation is exhibited by symbionts in tropical Pacific corals as well, though the symbionts in these hosts are primarily different strains all belonging to Clade C, a clade with large sub-clade variablility (Baker 1999; LaJeunesse et al. 2003; vanOppen et al. 2001).

Symbiont specificity during the onset of the symbiosis in ontogeny has been the subject of many investigations (Coffroth et al. 2001; Colley and Trench 1983; Kinzie 1974; Schwarz et al. 1999; vanOppen et al. 2001; Weis et al. 2001). A study done on octocorals from the Red Sea has linked mode of symbiont transmission (vertical vs. horizontal) with the type of symbiont hosted by the adult coral (Barneah et al. 2004; but also see vanOppen 2004). Symbionts belonging to Clade A, a stress tolerant clade with low sub-clade variability, were found in corals which engaged in vertical transmission, while Clade C symbionts were found in corals engaging in horizontal transmission. Several studies have experimented with infecting aposymbiotic hosts with heterologous (isolated from a different species) vs. homologous (isolated from the same species) algae (Colley and Trench 1983; Davy et al. 1997; Kinzie 1974). These studies have shown that while some heterologous species of algae can infect non-native hosts, often the growth rate is not equivalent to that of the natural symbiont (reviewed by Trench 1992).

These findings are supported by recent investigations done on scleractinian corals. While infection experiments performed on the Hawaiian scleractinian coral *Fungia scutaria* showed uptake and incorporation of heterologous algal types (Clades A, B, and C types C15 and C31) by the larvae (Rodriquez-Lanetty et al. 2004; Schwarz et al. 1999), evidence indicates that an association formed with the homologous algal type (Clade C-type C1f) is most successful (Rodriquez-Lanetty et al. 2004; Weis et al. 2001). In *Acropora tenius*, a Great Barrier Reef scleractinian coral, the initial uptake of zooxanthellae by the juvenile corals is nonspecific, but those that harbor the homologous strain have a faster growth rate than those with the heterologous strain (Little et al. 2004). Curiously though, by four months after uptake, the heterologous strain dominates in these juveniles.

Host response to the symbiosis

While it has been shown that the establishment of a successful cnidarian-dinoflagellate association often depends upon the identity of each of the partners, how recognition occurs is unknown. Other symbioses including: plant-fungi and legume-nitrogen fixing bacteria mutualisms, viral or pathogenic microbial parasitisms, and intracellular protozoan (apicomplexan) parasitisms, all utilize molecular mechanisms to achieve recognition and specificity in the association (Cossart and Sansonetti 2004; Parniske and Downie 2003; Sibley 2004; Smith and Helenius 2004). However, compared with those systems, research into the molecular and biochemical aspects of the cnidarian-dinoflagellate relationship is just beginning. The studies that have been performed have focused upon symbiosis-related biochemical and molecular changes in the host.

Superoxide dismutase

One example of such a symbiosis-related modification in the host occurs as a result of oxidative stress. Due to photosynthetic activity by the dinoflagellate, the host undergoes a rapid daily transition between a state of hypoxia at night to one of hyperoxia during the day. Yet, due to modified protective mechanisms, the host is extremely tolerant of these otherwise very damaging oxidative conditions. These mechanisms include an increase in the activity of an enzyme, superoxide dismutase (SOD), involved in the breakdown of harmful superoxide radicals in symbiotic vs. aposymbiotic individuals, and the presence of an unusually wide diversity of SOD enzymatic classes in host endodermal tissue, where the symbiont is housed (Dykens and Shick 1982; Richier et al. 2003; Shick and Dykens 1985). The complement of SOD classes in the endodermal tissue includes one class never before found in the animal kingdom (Richier et al. 2003).

Sym32

A second example of cnidarian host response to the symbiosis involves a change in host gene expression. A fasciclin domain protein, *sym32*, belonging to a class of cell adhesion proteins has been shown to be more highly expressed in symbiotic vs.

aposymbiotic *Anthopleura elegantissima* (a temperate sea anemone). The subcellular localization of the sym32 protein in symbiotic and aposymbiotic anemones also differs. In symbiotic anemones sym32 was found in the symbiosome surrounding the symbiont, while in aposymbiotic anemones it was found contained within gastrodermal cell vesicles (Schwarz and Weis 2003). The function of this protein within the symbiosis is currently unknown.

Carbonic anhydrase

A third example involves the enzyme carbonic anhydrase (CA). CA, an ancient enzyme found in all animals, photosynthesizing organisms, prokaryotes, and archaebacteria (Lindskog 1997), functions in catalyzing the reversible hydration of carbon dioxide: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. In seawater, most inorganic carbon is in the form of HCO_3^-, a form that diffuses slowly across membranes and is not readily converted to CO_2 in the absence of enzymatic action at neutral pH (Cook et al. 1986; Kerby and Raven 1985). Yet CO_2 is the form of inorganic carbon utilized by RUBISCO during photosynthesis by the symbiont. Weis et al. (1989) hypothesized that host CA functions within the symbiosis by supplying the zooxanthellae with enough carbon to fix. This would be an essential function during periods of high photosynthetic rates when carbon from host respiration, and the small pool of CO_2 available in the surrounding seawater, would not be enough to fill the demand (Muscatine and Weis 1992).

In 1989 (Weis et al.) presented evidence to support this "CO_2 supply" hypothesis. They showed higher rates of CA activity in animal vs. algal tissue, in symbiotic vs. aposymbiotic species, and in tentacle (zooxanthellate) vs. column (azooxanthellate) tissue, for many different species of tropical cnidarians. CA activity was also shown to decrease as the photosynthetic rate decreased and when CA was specifically inhibited, the photosynthetic rate decreased as well. Results from Western analysis performed on the tropical anemone *Aiptasia pulchella* indicated that the difference in

CA activity rates between aposymbiotic and symbiotic individuals was due to a difference in the amount of animal enzyme present and not algal enzyme (Weis 1991). And a 1993 study (Weis) in *A. pulchella* showed that 1) dissolved inorganic carbon (DIC) in ambient seawater is limiting to zooxanthellae photosynthesis 2) in the presence of a CA inhibitor, photosynthetic rates decreased regardless of the DIC concentration in ambient seawater and 3) CA was localized using colloidal gold immunocytochemistry to the symbiosome membrane, though it did not appear to be intrinsically bound . A later study by Weis and Reynolds (1999) investigated CA in *A. elegantissima.* This study revealed that the amount of gene product, in the form of transcript and protein, was higher in symbiotic anemones than aposymbiotic anemones, giving direct evidence for upregulation of a gene as a function of symbiosis.

Other preliminary investigations, such as a possible role for the signaling molecule nitric oxide in mediating behavioral aspects (tentacle extension) of the symbiotic association in *A. pallida* (Trapido-Rosenthal et al. 2001), a possible role for a D-galactose-binding lectin in mediating the interaction between an octocoral and its symbiont (Jimbo et al. 2000), potential negative regulation of symbiosis in *A. pulchella* by the ADP-ribosylation factor family of proteins (Chen et al. 2004), and expressed sequence tag analysis of symbiotic and aposymbiotic *A. pulchella* (Kuo et al. 2004) are all indicative of the exciting direction research in cnidarian-dinoflagellate symbiosis is heading. The investigations presented in this thesis are also a part of this new focus. In these chapters, we make the first attempt at identifying biochemical and molecular changes occurring during the onset of symbiosis in a cnidarian–dinoflagellate association. The symbiotic association used for these experiments is the sub-tropical scleractinian coral *Fungia scutaria* and its intracellular symbiont *Symbiodinium* type C1f.

The *Fungia scutaria* – *Symbiodinium* symbiosis

The solitary scleractinian coral *Fungia scutaria* is an obligate symbiont; no aposymbiotic adults have ever been observed in the field. Adults are gonochoric broadcast spawners that engage in horizontal transmission. Spawning occurs predictably 3-4 days after the full moon during the summer months. Negatively buoyant azooxanthellate eggs and azooxanthellate sperm are released into the water column, where fertilization occurs. Embryos develop into azooxanthellate planula larvae; the larvae are swimming actively in the water column by 1- 2 days old, develop their mouth by day 3 and are ready to settle by day 7, although they may remain in the water column much longer. These larvae are able to acquire their dinoflagellate symbiont (*Symbiodinium C1f*) as soon as they are able to feed. Symbiont acquisition occurs when the symbiont enters, during feeding, into the gastric cavity of the host where they are phagocytosed by the endodermal lining. During uptake the symbiont becomes permanently enclosed within the host-derived symbiosome and remains, one per endodermal cell, in the host cell cytoplasm.

Thesis chapters

The focus of this thesis is on the formation of the cnidarian-dinoflagellate symbiosis. Specifically, the investigations are aimed at elucidating biochemical and molecular events occurring within the *F. scutaria* larval host during the onset of the association with the dinoflagellate symbiont. Chapter 2 investigates a known cnidarian "symbiosis" gene, carbonic anhydrase, in *F. scutaria* larvae during the onset of symbiosis. This is the first investigation of CA's role during the onset of symbiosis, and the first look at it in a scleractinian coral. Chapter 3 examines the host's soluble protein profiles over the course of symbiosis onset and larval development, looking for biochemical changes occurring in the host as a function of the symbiosis. And chapter 4 examines changes in larval gene expression during the onset of symbiosis, and attempts to identity genes upregulated as a function of the association.

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CHAPTER 2

TWO ATYPICAL CARBONIC ANHYDRASE HOMOLOGUES FROM THE PLANULA LARVA OF THE SCLERACTINIAN CORAL *FUNGIA SCUTARIA.*

Introduction

Carbonic anhydrase is a zinc metalloenzyme that catalyzes the reversible hydration of carbon dioxide: $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$. In seawater (pH 8.2), most inorganic carbon is in the form of bicarbonate (HCO_3^-); a form that diffuses slowly across membranes and is not readily converted to CO_2 in the absence of enzymatic action (Cook et al. 1986; Kerby and Raven 1985). Symbiotic photosynthetic dinoflagellates (zooxanthellae), enclosed within a vacuole inside the gastrodermal cells of a cnidarian host, are cut off by several layers of membrane from the inorganic carbon supply in the surrounding seawater. And while the symbionts are able to fix host metabolic CO_2, their photosynthetic rates are comparable to those of free-living microalgae and often exceed the respiratory rates of the association (Muscatine 1980; Raven 1981). These symbionts must therefore gain access to the inorganic carbon supply in the surrounding seawater (Goreau (1977)a; Goreau (1977)b; McCloskey and Muscatine 1984; Muscatine et al. 1984) and do so with the help of host carbonic anhydrase (CA) (Furla et al. 2000a; Furla et al. 2000b; Weis et al. 1989).

Several studies using sea anemones have examined the role of host CA in solving the symbiont's carbon shortage. In one study, CA activity in *Aiptaisia pulchella* was shown to be elevated in symbiotic vs. non-symbiotic (aposymbiotic) sea anemones and was localized, using colloidal gold immunocytochemistry, to the host-derived vacuolar membrane surrounding the symbiont (Weis 1993). This CA did not appear to be intrinsically bound to the membrane. Weis and Reynolds (1999) showed that the amount of host CA transcript is significantly elevated in symbiotic *Anthopleura*

elegantissima as compared to aposymbiotic individuals. Studies in *Anemonia viridis* showed that inorganic carbon uptake for zooxanthellae photosynthesis is dependent upon an H^+-ATPase and a membrane-bound CA in host ectodermal cells (Furla et al. 2000a). In each of these associations, host CA appears to increase the availability of CO_2 to the symbiont by catalytically releasing it from bicarbonate at or near the site of algal photosynthesis. Thus the symbionts are able to maintain high rates of photosynthesis within their intracellular environment (Weis 1993; Weis and Reynolds 1999; Weis et al. 1989).

To date, all studies examining the role of host "symbiosis" CA within the cnidarian-dinoflagellate associations have examined symbioses in adult animals. Nothing is known about when during the life history of the host this carbon supply mechanism is activated and the relationship of its activation with the onset of symbiosis. We were interested, therefore, in describing CA and its expression during the early life history stages of the solitary Hawaiian scleractinian coral *Fungia scutaria*, which has a tractable early life history that includes onset of symbiosis. Adult *F. scutaria* individuals predictably release gametes into the water column where fertilization occurs. The free-swimming planula larvae that develop contain no symbiont. Acquisition of symbiont (infection) from the surrounding environment becomes possible at approximately 3 days after fertilization, upon development of a mouth, when the planulae begin to feed (Krupp 1983; Schwarz et al. 1999). During feeding, the symbiont is drawn into the gastric cavity where phagocytosis by the host gastrodermal lining occurs (Schwarz et al. 1999). Each individual symbiont is then housed within a host gastrodermal cell, contained within a host-derived vacuolar membrane or symbiosome (Wakefield and Kempf 2001). The planulae swim in the water column for a variable length of time (1-2 weeks) before they settle and undergo metamorphosis.

The initial focus upon CA's role during the onset of symbiosis in a scleractinian coral

expanded when we identified two novel and unusually short CA sequences from the larvae. In addition to expression analysis, we also performed bioinformatic analyses on these sequences. These analyses are the first to be done on any cnidarian CA sequence.

Methods

Collection

Several dozen adult *F. scutaria* were collected from reefs surrounding Coconut Island, Oahu, HI in July of 1998 and August of 2000. Maintenance and monitoring of the corals were performed as in Schwarz et al. (1999). When spawning occurred, eggs were collected in plastic bowls and diluted generously with 3-4L of 0.2 μm filtered seawater (FSW). Sperm from several males was mixed together in an approximate 1:10 dilution with FSW. Eggs were fertilized by gently squirting the dilute sperm mixture into the bowl using a turkey baster. The fertilized eggs were transferred to covered running seawater tables and left overnight with occasional gentle mixing. Approximately 12-18 h after fertilization and once each day thereafter, the water in each bowl of developing larvae was changed using 60 μm screen and fresh FSW (Schwarz et al. 1999).

Infection

Larval development was monitored daily using light microscopy. At approximately 3 days post-fertilization, once the larvae had developed a mouth, they were exposed to freshly isolated symbionts (A.K.A FIZ) from adult *F. scutaria* and a brine shrimp preparation (Schwarz, et al 1999). Prior to infection, larvae from all bowls were combined into a single population then redistributed into the original bowls. Larvae were then concentrated into a smaller volume (~1-1.5L) of filtered seawater. Half of the bowls were exposed to FIZ + shrimp (~15mL of the FIZ preparation was gently poured into each bowl and mixed followed by ~5mL of the brine shrimp preparation)

and half of the bowls were exposed to shrimp only (~5mL shrimp preparation + ~15mL filtered seawater). Larvae were allowed to incubate for 3 h before the water was changed and the larval concentration was reduced to previous levels. Twenty four hours after the infection procedure, infection rates were measured. Using a light microscope, percent infection was determined by taking a subsample of 100 larvae from each bowl and quantifying the proportion of larvae containing at least one zooxanthella. Three subsamples were counted from each bowl of larvae.

Sampling, RNA isolation and cDNA synthesis

Symbiotic and aposymbiotic larvae were sampled each day (2000) or every other day (1998) following infection until the onset of settlement. A 50mL conical tube was filled with seawater containing larvae and centrifuged for 10 sec at ~8000 g. The pelleted larvae were transferred to a 1.5mL microfuge tube and centrifuged briefly at ~12,000 g in a tabletop microfuge. The seawater was quickly drawn off the larval pellet and the tube dropped into a dry ice- methanol bath. The frozen samples were held at -50°C and shipped to Oregon State University on dry ice where they were held at -80°C until processing. After separating host and algal tissue via centrifugation, RNA was extracted from the larvae and quantities were determined spectrophotometrically at A_{260} (Weis and Reynolds 1999). cDNA was synthesized from 1μg total RNA using a first-strand cDNA synthesis kit (Gibco BRL).

PCR amplification, cloning, and sequencing

Degenerate forward (FWCA: 5'-CARTTYCAYTTYCAYTGGGG-3') and reverse primers (RVCA: 5'-GGNGGNGTNGTNAGNGANCC-3' and FRDP3: 5'-RCTGGNCKSYAGTTGTCCAC-3') designed from a consensus of α-CA sequences were used in attempts to amplify an internal piece of *F. scutaria* CA (FCA) sequence from 5 day aposymbiotic larval cDNA samples. PCR reactions consisted of 1.25μl each of a forward and reverse degenerate primer, 1μl cDNA from 5 day aposymbiotic larvae, 2.5μl 10x PCR buffer (Promega), 2μl 25mM MgCl (Promega), 0.25μl Promega

Taq DNA Polymerase, 2µl 5mM DNTPs, and 14.75µl sterile water. Cycling parameters were: 94°C for 3 minutes, 40 cycles of 94°C - 45 sec, 56°C -1 minute for the FWCA/RVCA primer pair (58°C for FWCA/FRDP3), 74°C -1 minute, followed by an 8 minute extension phase at 74°C. Two µl of the PCR reaction were ligated into a plasmid vector (Invitrogen's TA cloning kit). INVαF' One Shot competent cells were transformed and plated on LB/ampicillin plates spread with Xgal and IPTG, then grown overnight at 37°C. 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^{TM} PCR Centrifugal Filter Device) amplified using the vector primers M13F & M13R and plasmid DNA from overnight cultures of individual colonies purified with a standard small-scale protocol (Qiagen plasmid spin miniprep kit). 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.

Rapid amplification of cDNA ends (RACE)

Primers specific to the *F. scutaria* cDNA piece, which was obtained using FWCA and RVCA, were designed and used in nested RACE reactions to obtain the 3' and 5' ends of the *F. scutaria* CA cDNA. The sense primer: "FUNCA-F" 5'-AGAATTTGCGCGGTTCTGAG-3' starts at nucleotide 497 and the antisense primers: "FUNCA-R" 5'-CGTCGAGGCGTTCGGATACT-3' and "FUNCA2-R" 5'-CTCAGAACCGCGCAAATCT-3' start at nucleotides 597 and 516 respectively (Fig 1). The 3' end of larval *F. scutaria* CA was amplified in nested PCR reactions using the degenerate primer FWCA, the specific primer FUNCA-F and the reverse oligo (dT) primer 1-REV (Weis and Reynolds 1999). The first reaction mixture consisted of 1.25µl 1-REV, 1.25µl FWCA, 1.25µl cDNA from 5 day old aposymbiotic *F. scutaria* larvae, 2.5µl 10x PCR buffer (Promega), 2µl 25mM MgCl (Promega), .25µl Promega

Taq DNA Polymerase, 2µl 5mM DNTPs, and 14.5µl sterile water. Cycling parameters for the first reaction were: 94°C for 3 minutes, 40 cycles of 94°C -1 min, 57°C -1 minute, 74°C -1 minute, followed by an 8 minute extension phase at 74°C. The nested PCR reaction mixture consisted of .5µl 1-REV, 3µl FUNCA-F, 1.5µl of a 1:100 dilution of the first reaction PCR product, 2.5µl 10x PCR Buffer, 2µl 25mM MgCl, 2µl 5mM DNTPs, .25µl Taq, and 13.25µl sterile water. Except for a 60°C annealing temperature, cycling parameters for the nested reaction were identical to the first reaction. The 5' end was amplified using FUNCA-R, FUNCA2-R and the FirstChoice™ RLM-RACE Kit (Ambion) according to the manufacturer's instructions. Both ends were cloned and sequenced as described above.

Sequence analysis

Deduced amino acid sequences were obtained using NCBI's ORF Finder (www.ncbi.nlm.nih.gov/gorf/gorf.html) and were analyzed for conserved motifs using ScanProsite at ExPASy (Sigrist et al. 2002). A search for membrane-spanning domains was performed using TMpred at the ExPASy site as well (Hofmann and Stoffel 1993). Related amino acid sequences were obtained from the GenBank database using the BLAST algorithm (Altschul et al. 1990). Sequences were aligned using ClustalX (Thompson et al. 1997) with a gap opening penalty of 35.00 and a gap extension penalty of 0.75 (pairwise alignment) and a gap opening penalty of 15.00 and a gap extension penalty of 0.30 (multiple alignments). All alignments were masked in BioEdit (Hall 1999) prior to tree building. Similarity matrix PAM250 was used for all identity/similarity calculations and calculations included all insertion/deletions, although 3' end overhangs were masked. Phylogenic tree construction was performed using both parsimony (heuristic search) and distance methods in PHYLIP (default parameters except for randomizing the input order, restarted 10 times) (Felsenstein 1993). Both methods produced similar trees. Treeview16 was used for drawing consensus trees (Page 1996). The secondary and tertiary structure of the sequence was predicted and compared to known crystal structures by the Protein Structure Prediction

Server (PSIPRED) accessed from the University College London Website (Jones 1999a; Jones 1999b; McGuffin et al. 2000; McGuffin and Jones 2003). The highest hit from that search was used in the structural alignment/viewer program Cn3D at NCBI (Wang et al. 2002).

Real-time quantitative PCR

Real-time quantitative PCR (QPCR) was performed using the Prism 7700 Sequence Detector (Perkin Elmer/Applied Biosystems Division) and SYBR© Green master mix. Specific primers FUNCA-F and FUNCA-R were used in all QPCR reactions. Identical reactions amplifying actin (forward: 5'-CTG ATG GAC AGG TCA TCA CCA T-3', reverse: 5'-CTC GTG GAT ACC AGC AGA TTC C-3') were run concurrently and were used as a reference point for expression level. PCR reactions consisted of: 1.0µl symbiotic or aposymbiotic larval cDNA, 6.0µl sterile water, 1.5µl gene specific forward primer, 1.5µl gene specific reverse primer, and 10.0µl SYBR® green PCR master mix. The default cycling parameters for the ABI PRISM® 7700 Sequence Detection System were used in all reactions. CA expression level was normalized to actin expression and is reported as: 1/ (CA cycle# at mid-log phase amplification – actin cycle# at mid-log phase amplification). Replicate number is the number of PCR reactions performed on larvae of a specific age and infection state and collected in a specific year. All PCR products from preliminary runs performed were checked for the presence of primer dimers using agarose gel electrophoresis. Statistical significance was evaluated using the unpaired t test with two-tail P value (GraphPad InStat version 3.05 for Windows 95/NT, GraphPad Software, San Diego California USA, www.graphpad.com).

Results

F. scutaria CA sequences

An internal piece of CA sequence was obtained from 5-day aposymbiotic larvae using the degenerate primers FWCA and RVCA (Fig 1). Attempts made using the FWCA and FRDP3 primer pair were successful in adult corals but unsuccessful in the larvae. The partial CA sequence obtained from adult coral using FWCA and FRDP3 is included in the multiple sequence alignment (Fig 2). FRDP3 was designed to the conserved amino acid sequence VDNyRPa present towards the end of the alignment.

Two different *F. scutaria* CA 3' ends (FCA-a & FCA-b) were obtained from aposymbiotic larvae using nested RACE reactions (Fig 1). The complete FCA-a sequence consists of a 792-nucleotide cDNA with a 603 bp open reading frame. The start codon is 39 nucleotides downstream from the 5' end. A likely promoter site is located 36 bp upstream from the start codon; the poly-A signal, AATAAA, occurs 28 nucleotides downstream from the in-frame stop codon and 14 bp upstream of the start of the poly-A tail. The deduced amino acid sequence is 201 amino acids in length.

FCA-b differs from FCA-a only at the 3' end of the cDNA (Fig 1). The complete FCA-b sequence consists of an 849-nucleotide cDNA with a 675 bp open reading frame. The sequence is identical to FCA-a from nucleotides 1 – 717, however at nucleotide 718 (amino acid 194) there is a change in both nucleotide and deduced amino acid sequence. The in-frame stop codon occurs at nucleotide 814 and a polyadenylation signal, AATAA, occurs 8 nucleotides downstream from the stop and 18 nucleotides upstream from the start of the poly-A tail.

Sequence analysis & alignments

Both larval FCAs were subjected to analysis independently, however where the results did not vary between the two they are reported only once. Blast searches of GenBank using both FCA sequences revealed hundreds of significant alignments with α-CAs, a family of related CA enzymes found mostly in animals.

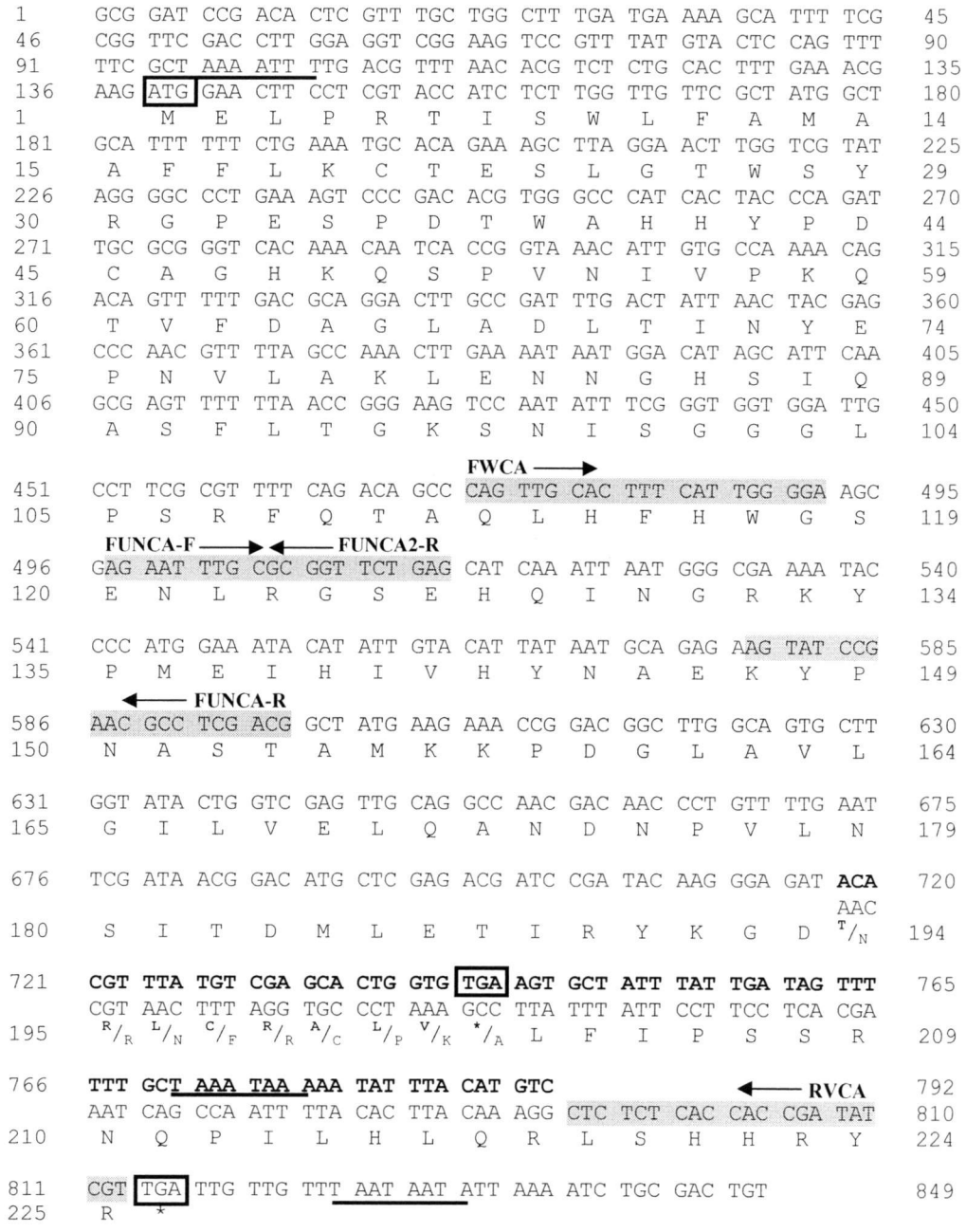

Figure 1. Nucleotide (nt) and predicted amino acid (AA) sequences of *F. scutaria* CA obtained from aposymbiotic larvae. The start codon (ATG) is boxed and the putative promoter site, a TA rich region 36 base pairs (bp) upstream from the start, is underlined. Two different 3' ends, starting at nt 719, were obtained using RACE. The shorter sequence, FCA-a, is in bold. FCA-a is a 792 bp cDNA with a 603 bp open reading frame (ORF); FCA-b is an 849 bp cDNA with a 675 bp ORF. Both stop codons, TGA, are boxed and both polyA signals are underlined. The FCA-a and FCA-b signals occur 14 and 18 bp upstream from the start of the polyA tails (not shown), respectively. Primer sites are shaded, arrows indicate directionality.

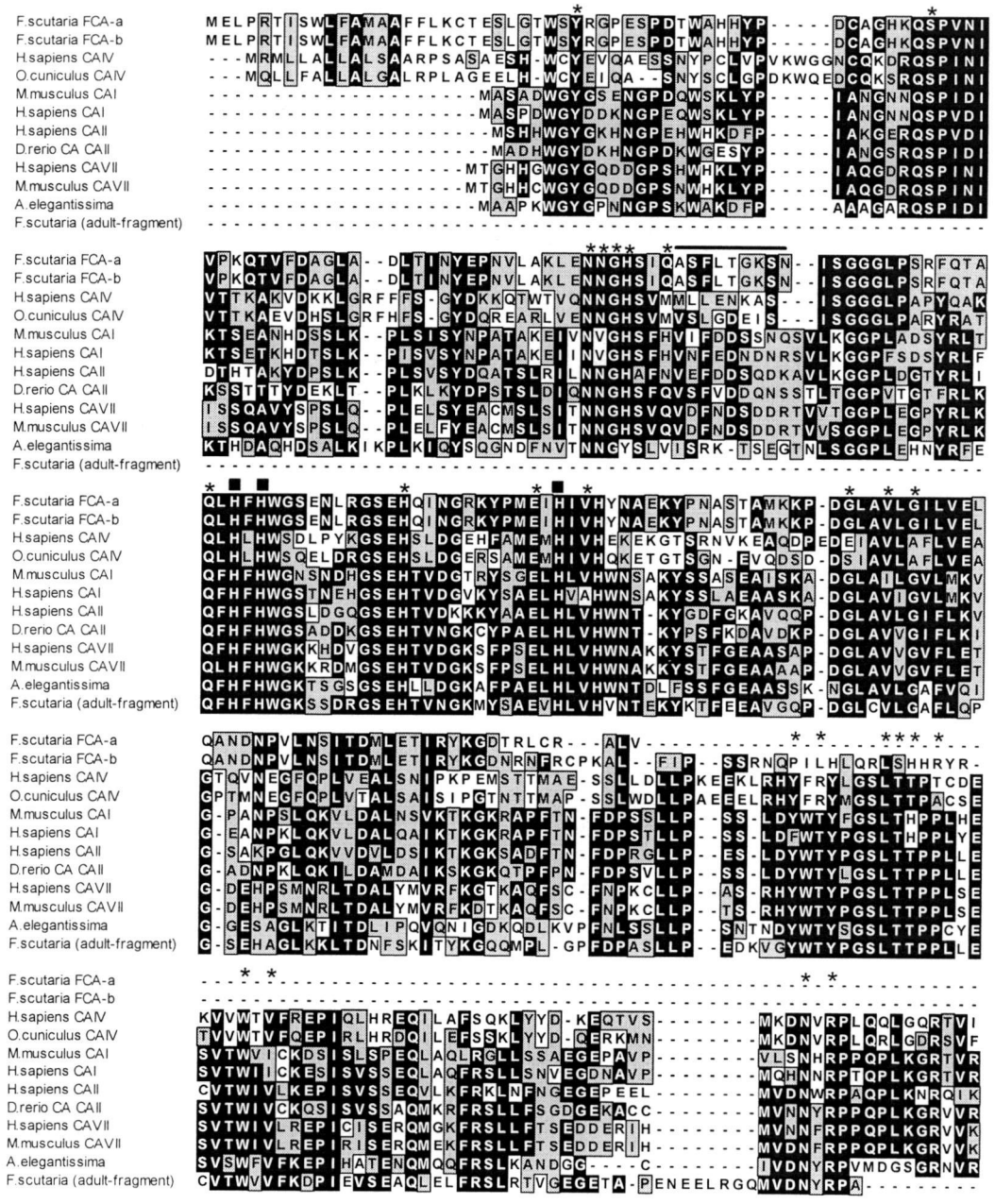

Figure 2. Alignment of α-CA sequences from *F. scutaria*, human, rabbit (*O. cuniculus*), mouse (*M. musculus*), zebrafish (*D. rerio*), and the sea anemone *A. elegantissima*. *F. scutaria* (adult-fragment) is an incomplete α-CA sequence from adult coral. Amino acids (AA) identical in 30% of the sequences are shaded black; conserved AA are gray. Squares mark histidines (H) involved in direct binding of the zinc cofactor; asterisks mark AA participating in the hydrogen bond network surrounding the active site. The black line indicates the conserved P-loop motif found in the FCA sequences. Both FCAs are uncommonly short and so are missing several residues normally involved in the hydrogen bond network. The last few AA from all but the *F. scutaria* sequences have been omitted from the figure.

Most hits from the top 150 significant alignments were to vertebrate CA isozyme group II; other CA groups included CA I, VII, XII, XIII, and XIV (all vertebrate). The only non-vertebrate CA sequences included in the top 150 hits were an α-CA from the hydrothermal-vent vestimentiferan *Riftia pachyptila* and an α-CA sequence from the temperate sea anemone *Anthopleura elegantissima*. The top 10 BLAST hits for both FCA sequences are given in Table 1.

An alignment of the FCA deduced amino acid sequences to a subset of these top 10 BLAST hits (CA I, II, IV and VII from mouse and human as well as the sea anemone sequence) is shown in Figure 2. Both FCA-a and FCA-b are much shorter than the other sequences yet have retained three critical conserved histidine residues (direct-ligands) that bind directly to the zinc cofactor, as well as several amino acids (indirect-ligands) that function in forming a hydrogen bond network around the active site (Fukuzawa et al. 1990; Fukuzawa and Ishida 1991; Hewett-Emmett et al. 1984; Sheridan and Allen 1981). However, several other indirect-ligand sites are missing from the FCAs, due to the truncated nature of the sequences. Conserved motifs present in the FCA deduced amino acid sequences include: two N-glycosylation sites at amino acids 98-101 and 150-153, four Protein Kinase C phosphorylation sites at amino acids 28-30, 94-96, 187-189, and 207-209, two Casein kinase II phosphorylation sites at amino acids 60-63 and 180-183 and one N-myristoylation site at amino acids 102-107. All aforementioned patterns have a high probability of occurrence. Additionally, two highly conserved motifs were found: an ATP/GTP-binding site motif A (P-loop) was identified at amino acids 90-97 and a eukaryotic-type carbonic anhydrase signature was found spanning amino acids 125-141 (Figs 2, 3). The p-loop motif was not found in the other CA sequences included in the alignment. A significant (score > 500) possibility of a 19 amino acid transmembrane helix (score = 1461) was found using the TMpred program (Fig 3). The possible helix runs from amino acids 6 – 24 and displays a strong preference for the inside to outside orientation.

Table 1. Top 10 hits from BLASTp searches of all non-redundant GenBank CDS translations, PDB, SwissProt, PIR and PRF using FCA-a and FCA-b predicted amino acid sequences.

NCBI accession #	Description	E-value	
		FCA-a	FCA-b
954685.1	CA II [Danio rerio]	9e-37	1e-36
005173.1	CA VII [Homo sapien]	1e-34	9e-33
444300.1	CA VII [Mus musculus]	1e-34	7e-33
226204.2	Similar to CA 7 [Rattus norvegicus]	3e-34	2e-32
571185.1	CA [Danio rerio]	3e-34	3e-34
AAP73748.1	Erythrocyte CA [Oncorhynchus mykiss]	7e-34	9e-34
AAR99329.1	Cytoplasmic CA [Oncorhynchus mykiss]	1e-33	4e-33
AAM94169.1	Erythrocyte CA [Lepisosteus osseus]	9e-33	1e-32
035927.1	CA XIV [Mus musculus]	2e-32	5e-33
P00917	CA I [Equus caballus]	1e-31	-
47168790	Extracellular Domain -CA XIV [Mus musculus]	-	2e-31

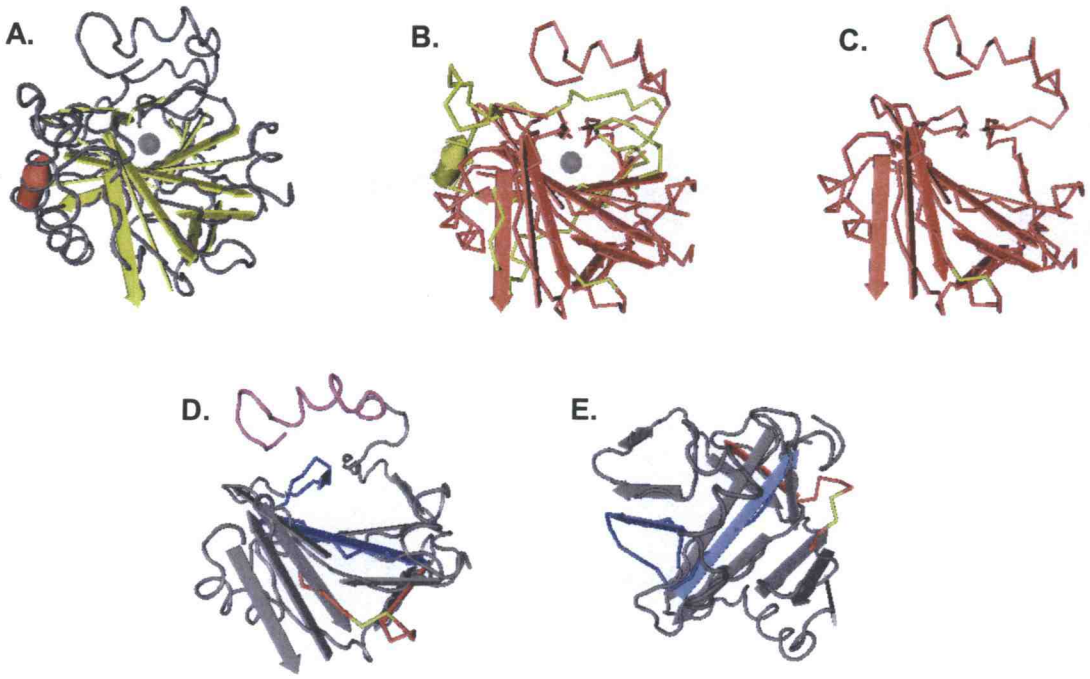

Figure 3. Structural comparison of the FCA predicted amino acid sequences with the known crystal structure of human CAII (hCAII). **A**: The carbon backbone of hCAII; it is dominated by a central, twisted 10 stranded beta sheet which is mostly antiparallel (yellow). A few short helices (red) are present on the outside of the enzyme; only one is visible from this orientation. The zinc cofactor is shown as a gray sphere. **B:** Sequence conserved between FCA-b and hCAII is shown in red; insertions/deletions (indels) and the C-terminus residues absent from the FCA sequence are in yellow. **C:** View of the carbon backbone minus the absent C-terminal residues. The yellow indicates a two amino acid indel (present in hCAII/absent in FCA) in the pairwise alignment. **D:** Conserved domains and motifs present in the FCA sequences: the blue portion highlights the α-CA domain, the red portion (containing the yellow indel) highlights the P-loop motif, and the purple highlights the location of a possible 19 amino acid transmembrane helix. **E:** The structure has been rotated for a clearer view of the CA domain and P-loop motif.

Both FCA deduced amino acid sequences were analyzed using the PSIPRED program to compare the predicted secondary structures to known crystal structures. The results from those two searches were identical. The highest hit was to human CAII (confidence level = high / E-value = 0.001) and the second highest hit was to human CAIV (confidence level = high / E-value = 0.003). The human CAII structure was subsequently used in the structural alignment/viewer program Cn3D to perform structural comparisons with the FCAs (Fig 3). As can be seen in the multiple alignment, although the FCAs are much shorter than other CA sequences, they retain the catalytic domain and the three critical histidine residues that directly bind the zinc cofactor, as well as several residues involved in the hydrogen bond network surrounding the active site. Figure 3 also shows the location and folded structures of the highly conserved CA and P-loop motifs evident in the FCA sequences.

A more extensive multiple alignment was used to generate the phylogenetic tree (Fig 4). The tree was constructed using parsimony analysis and rooted using an α-CA sequence from the cyanobacterium *Nostoc*. The FCA sequences consistently fell closest to the mammalian membrane-bound forms. The two other invertebrate CA sequences included (from the sea anemone *Anthopleura elegantissima* and the vestimentiferan *Riftia pachyptila*) consistently fell a good distance from the FCAs. Both of these CAs are thought to function in symbiosis as inorganic carbon supply enzymes (Cian et al. 2003a; Cian et al. 2003b; Furla et al. 2000a; Weis et al. 1989). A sequence identity matrix generated from this multiple alignment is shown in Table 2.

A second parsimony analysis shown in Figure 5 includes the CA fragment obtained from adult *F. scutaria*. This phylogeny generally mimics that of Figure 4 but with much less bootstrap support most likely due to the masking of all N- and C- terminal ends overhanging the fragment. The *F. scutaria* adult CA fragment falls in with the other two invertebrate "symbiosis" CA sequences.

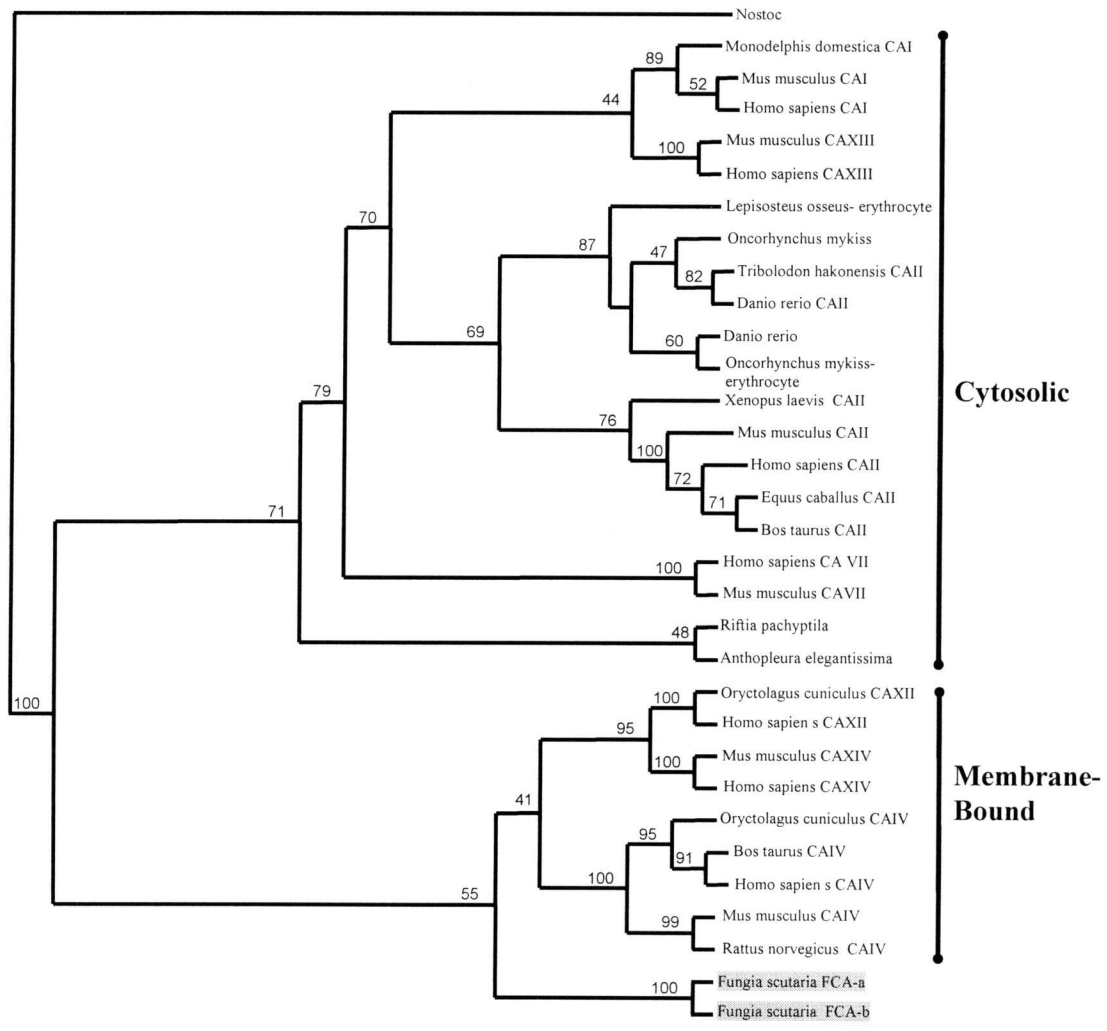

Figure 4. Phylogenetic relationships of vertebrate and invertebrate α-CA sequences inferred from parsimony analysis. The tree is rooted with an α-CA from the cyanobacterium *Nostoc sp.* PCC 7120. Percent of 1000 bootstrap replicates supporting the topology shown is given above each branch. The FCA sequences (highlighted) obtained from aposymbiotic *F. scutaria* larvae are most closely related to membrane-bound forms of vertebrate CAs. The two "symbiosis" CA sequences from the sea anemone *A. elegantissima* and the vestimentiferan *R. pachyptila* form a monophyletic group within the cytosolic CA cluster.

Table 2. Identity matrix calculated from the multiple sequence alignment used in the phylogenetic analysis. 3' end overhang was masked after FCA-b. Sequences include the FCA sequences from *F. scutaria* larvae, CAI from *Mus musculus* (m), CAII from *Homo sapiens* (h) and *Danio rerio* (d), CAIV from *Rattus norvegicus* (r), CAVII, XII, and XIII from *Homo sapiens*, CAXIV from *Mus musculus*, and the "symbiosis" CA from the temperate sea anemone *Anthopleura elegantissima* (A.e.).

Seqs:	FCA-a	FCA-b	mI	hII	dII	rIV	hVII	hXII	hXIII	mXIV	A.e.
FCA-a	1.00	0.87	0.28	0.26	0.31	0.28	0.29	0.28	0.29	0.30	0.25
FCA-b	---	1.00	0.31	0.28	0.34	0.29	0.31	0.29	0.31	0.32	0.27
mI	---	---	1.00	0.61	0.58	0.29	0.52	0.36	0.60	0.30	0.46
hII	---	---	---	1.00	0.64	0.28	0.55	0.32	0.60	0.31	0.46
dII	---	---	---	---	1.00	0.26	0.53	0.32	0.60	0.33	0.44
rIV	---	---	---	---	---	1.00	0.31	0.30	0.29	0.34	0.28
hVII	---	---	---	---	---	---	1.00	0.33	0.50	0.33	0.46
hXII	---	---	---	---	---	---	---	1.00	0.32	0.41	0.30
hXIII	---	---	---	---	---	---	---	---	1.00	0.32	0.45
mXIV	---	---	---	---	---	---	---	---	---	1.00	0.29
A.e.	---	---	---	---	---	---	---	---	---	---	1.00

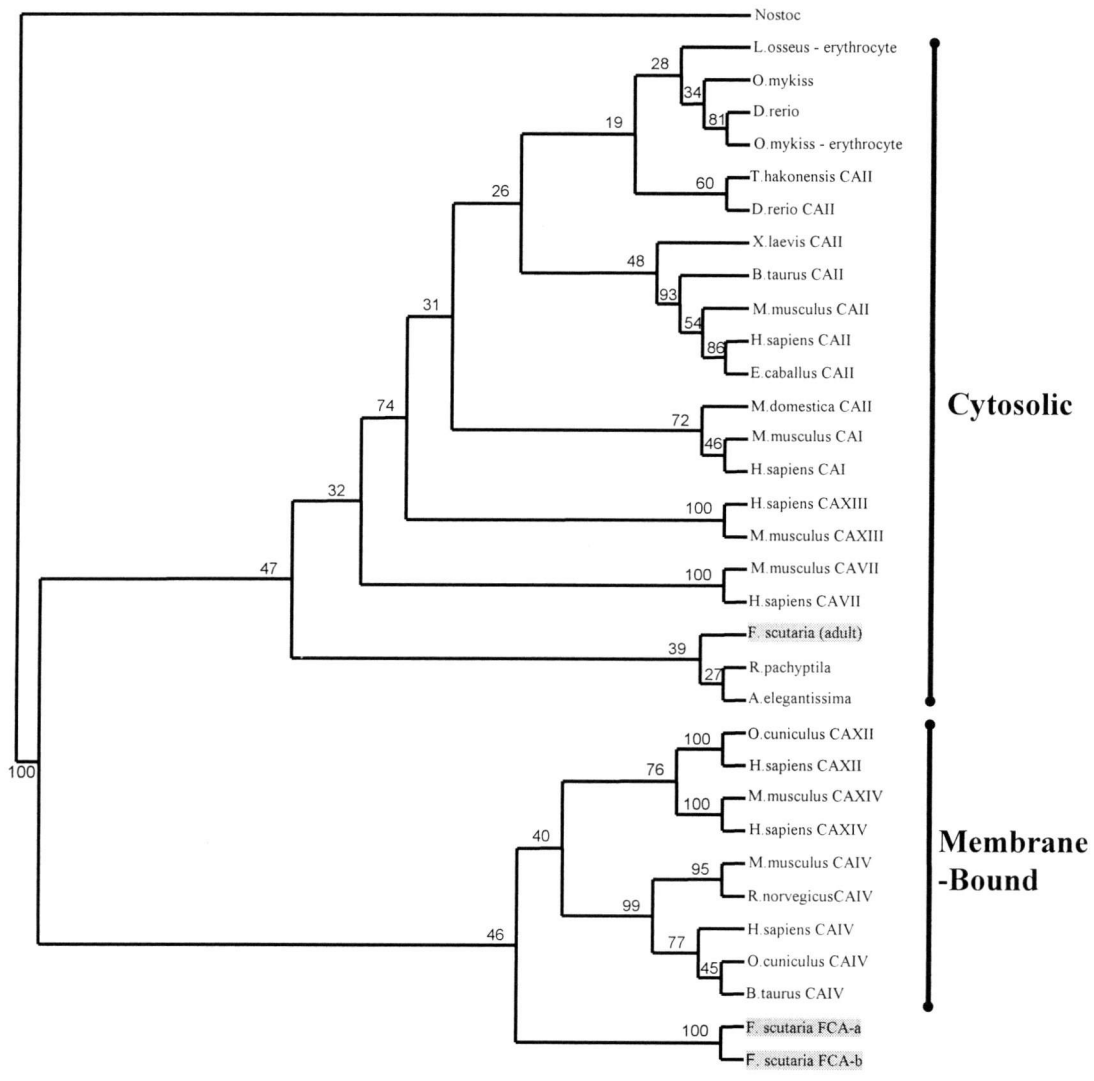

Figure 5. Phylogenetic relationships of vertebrate and invertebrate α-CA sequences inferred from parsimony analysis. Only the portion of the alignment which includes sequence from adult *F. scutaria* was used for the analysis. The tree is rooted with an α-CA from the cyanobacterium *Nostoc sp.* PCC 7120. Percent of 1000 bootstrap replicates supporting the topology shown is given above each branch. The amino acid sequence deduced from the fragment of CA cDNA isolated from adult *F. scutaria* is most closely related to the cytosolic CA sequences from the sea anemone *A. elegantissima* and the vestimentiferan *R. pachyptila*. The FCA sequences isolated from *F. scutaria* larvae are most closely related to membrane-bound forms of the enzyme.

Real-time quantitative PCR

To monitor the amount of CA transcript in *F. scutaria* larvae during the onset of symbiosis, QPCR was performed on cDNA from symbiotic and aposymbiotic larvae, 4 – 7 days old, collected in 1998 and 2000 (Fig 6). CA-specific primers used in the QPCR reactions did not differentiate between FCA-a and FCA-b. No significant difference in CA expression was found between symbiotic and aposymbiotic larvae.

Discussion

The two cDNA sequences isolated from aposymbiotic 5-day-old *F. scutaria* larvae are almost identical, differing only at their 3' ends. Both contain complete open reading frames including start and stop codons, poly-adenylation signals and polyA tails consisting of 200+ adenine residues. The deduced amino acid sequences contain a conserved domain for eukaryotic CA activity and show significant similarity to vertebrate α-CA sequences. Percent residue identities garnered from sequence alignments are well above the similarity threshold *t* of 24.8% (>80 residues) implying structural homology with α-CAs (Sander and Schneider 1991).

Interestingly, both FCA sequences are also short, lacking up to 80 amino acids from the C-terminus. And although the active site appears fully conserved and contains the three critical histidine residues that bind the zinc cofactor, amino acids important to the activity of the enzyme are missing. These missing residues are part of a network of hydrogen bonding amino acids (indirect-ligands) that support the active site structure and which are crucial for catalytic activity (McCall et al. 2000). Zinc affinity has been shown to decrease in CA molecules where a portion of the indirect-ligands have been eliminated (Kiefer et al. 1995). We could find no other example of a similarly sized α-CA in the sequence databases.

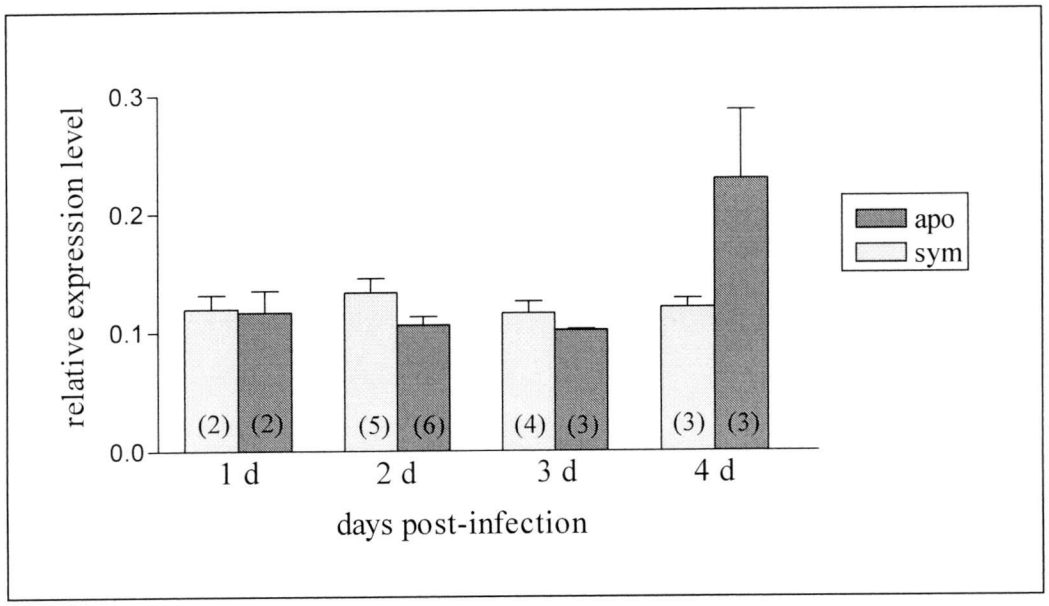

Figure 6. Real-time quantitative PCR was used to monitor the expression level of FCA in symbiotic (sym) and aposymbiotic (apo) larvae. Larvae collected in 1998 were used for the 4 day post-infection time point and larvae collected in 2000 were used for the 1 day and 3 day post-infection time points. The 2 day post-infection time point is a mixture of samples from 1998 and 2000. Expression was evaluated relative to the expression level of the housekeeping gene actin. Relative expression level is equal to the inverse of the difference between the cycle # at mid-log phase amplification of FCA and the cycle # at mid-log phase amplification of actin. Number of replicates is given in parentheses. No significant difference in relative expression level was found between infection states at any time point tested.

Conserved motifs present in the FCA sequences indicate the enzyme may undergo glycosylation during the translational process as well as activation by phosphorylation; common events which occur to many carbonic anhydrases (Narumi and Miyamoto 1974). The presence of a highly conserved ATP/GTP binding site (P-loop) is surprising. This is not a motif ordinarily found in CA and the consensus pattern, [AG]XXXXGK[TS], is not present in any of the CA sequences used to construct the phylogenetic tree. A P-loop interacts with the phosphoryl group of a bound nucleotide and requires a divalent metal ion (like Mg^{2+}) to function. The metal ion forms a complex with the nucleotide which enhances the specificity of the enzyme. P-loops are found in many unrelated protein families including kinases, ras proteins, elongation factors and ATP synthases (Saraste et al. 1990). Of course, biochemical data are needed to determine whether the P-loop motif in this sequence is functional.

The FCA sequences do not clearly belong to any of the currently defined CA isoform groups. They show highest identity to the CA II isozyme in both primary (34% sequence identity with zebrafish) and secondary structure. However, the membrane-bound forms of CA come in at a very close second (32% identity with mouse CA XIV) and in phylogenetic analyses, the FCA sequences consistently fall in with these isozymes. The mechanism of binding to the membrane varies among the membrane-associated forms of CA. Some forms, such as CA IV, utilize a GPI anchor while others such as CA XIV, utilize a membrane spanning domain (Mori et al. 1999; Stams et al. 1996). A GPI anchor motif is not present in the FCA deduced amino acid sequences although a likely transmembrane domain is.

While the FCA sequences do not clearly belong to any particular CA group, they do not group with the known "symbiosis" CA sequences from the vestimentiferan *Riftia pachyptila* or the fellow cnidarian *Anthopleura elegantissima*. The cytosolic CA sequences from these two animals have been linked to a mechanism that functions in

supplying the symbiont with carbon. While a probable match was isolated from adult *F. scutaria*, multiple attempts made to isolate the "symbiosis" isoform from *F. scutaria* larvae were unsuccessful. Furthermore, QPCR results showed no significant change in the levels of the FCA transcript (this study did not distinguish between FCA-a and FCA -b using QPCR) with the onset of symbiosis or with development.

These data do not rule out a role in symbiosis for the FCAs however, and one study by Furla et al. (2000a) showed evidence of H^+-ATPase (P-loop type) and membrane-bound CA involvement in inorganic carbon uptake for endosymbiotic photosynthesis. This study suggests that bicarbonate absorption by ectodermal cells is carried out when H^+ secretion, by the H^+-ATPase, results in the formation of carbonic acid in the surrounding seawater. The carbonic acid is quickly dehydrated into CO_2 by a membrane-bound CA; the CO_2 then passively diffuses into the cell where it is hydrated back into bicarbonate by a cytosolic CA. The fact that FCA shares similarities with other membrane-bound isozymes, including the capability to bind a membrane, and contains a potential P-loop site makes this sequence intriguing.

CA also plays an important role in the process of calcification which results in the formation of the calcium carbonate skeleton formed by many non-symbiotic and symbiotic cnidarians. For example, in the non-symbiotic octocoral *Leptogorgia virgulata,* Lucas and Knapp (1997) show that CA plays a pivotal role in the formation of calcium carbonate spicules regardless of the carbon source, dissolved inorganic carbon (DIC) from the environment or metabolically produced CO_2. In this cnidarian, CA involved in the calcification process has been localized to the spicule vacuole membrane as well as to the inside of electron-dense golgi-derived vesicles (Kingsley and Watabe 1987; Lucas et al. 1996). Furla et al. (2000b) demonstrated that an intracellular CA plays a role in the absorption of metabolic DIC for calcification in the reef-building coral *Stylophora pistillata*, while Marshall and Clode (2003) found evidence for an extracellular CA involved in the calcification pathway in the coral

Galaxea fascicularis. The larvae used in the present study are on the verge of settlement and metamorphosis, at which point they will begin deposition of their calcium carbonate skeleton. There is a good possibility that the FCA sequences obtained from these larvae will function in this deposition process.

In conclusion, we present two α-CA homologues isolated from pre-settlement scleractinian planula larvae. These sequences are atypical in length and seem most closely related to vertebrate membrane-bound forms of the enzyme. Expression of these homologues does not appear to vary with larval symbiotic state or with development. Moreover, the deduced amino acid sequences show relatively low similarity to the other known cnidarian CA sequence, that from *Anthopleura elegantissima*, which functions in endosymbiont carbon supply.

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CHAPTER 3

THE SOLUBLE PROTEOME OF THE SCLERACTINIAN CORAL *FUNGIA SCUTARIA* AND THE ONSET OF INTRACELLULAR SYMBIOSIS

Introduction

Proteome analysis has proven to be a useful tool in symbiosis research. Proteins differentially expressed or repressed as a function of the symbiotic state have repeatedly been targeted and identified using 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE) in numerous widely diverse mutualistic and parasitic systems. In the cooperative association between the temperate sea anemone *Anthopleura elegantissima* and the dinoflagellate *Symbiodinium muscatinei*, 2-D PAGE profiles of steady state and newly synthesized anemone proteins from symbiotic and non-symbiotic (aposymbiotic) adults highlighted several proteomic differences between the two (Weis and Levine 1996). As a result, two host "symbiosis proteins" have been identified in that system. In the cooperative association between the squid *Euprymna scolopes* and the marine luminous bacterium *Vibrio fischeri*, 2-D PAGE was used during the onset of the symbiosis to search for symbiont-induced changes in the host light organ soluble proteome during the first 3 days of the symbiosis. Many changes in the protein profile were found at 48 and 96 hours after infection with the symbiont (Lemus and McFall-Ngai 2000). Multiple studies using 2-D PAGE (among other techniques) on the symbiosis between nitrogen-fixing soil bacteria of the *Rhizobiaceae* family and legumes have uncovered a suite of plant genes specifically expressed during the onset of the symbiosis (Gloudemans and Bisseling 1989; Govers et al. 1985; Krause and Broughton 1992; Natera et al. 2000; Saalbach et al. 2002). The onset of pathogenic associations also causes changes in patterns of protein synthesis. The protozoan *Hartmannella vermiformis,* upon contact

with its bacterial parasite *Legionella pneumophila*, induces at least 33 proteins (and represses at least 11) which may be involved in the uptake of the bacteria into the endocytic compartment (Kwaik et al. 1994). Abshire and Neidhardt (1993), using 2-D PAGE, found that 40 parasite proteins were upregulated and 100 proteins were repressed during growth of *Salmonella typhimurium* within a host macrophage.

The intracellular symbiotic association between the Hawaiian solitary coral *Fungia scutaria* and the dinoflagellate *Symbiodinium* type C1f (Rodriquez-Lanetty et al. 2004) is a mutualistic one in which the coral host receives benefit from the photosynthetic capabilities of the symbiont and the symbiont receives nutrients and protection from the host (Muscatine 1980; Trench 1979). *F. scutaria* is a gonochoric broadcast spawner that releases negatively buoyant azooxanthellate (non-symbiotic) eggs into the water column predictably 3-4 days after the full moon during the summer months (Krupp 1983). Fertilization occurs in the water column and zygotes develop into azooxanthellate planula larvae that are swimming actively by 1- 2 days of age, develop a mouth by day 3, and are competent to settle by day 7 although they may persist in the water column for longer. The larvae are able to acquire symbionts as soon as they are able to feed (Krupp 1983; Schwarz et al. 1999). Acquisition occurs when the symbiont enters, during feeding, into the gastric cavity where phagocytosis of the algae by the endodermal lining occurs (Schwarz et al. 1999). Thereafter the symbiont resides within a membrane-bound compartment inside a host endodermal cell. Larvae that fail to acquire symbiont may still settle (Schwarz et al. 1999) and undergo metamorphosis, however an aposymbiotic *F. scutaria* adult has never been observed in the field.

Nothing is known about the molecular or biochemical events that accompany the onset of symbiosis (or even an established symbiosis) between these two partners or indeed any cnidarian - dinoflagellate symbiosis. The goal of this study was to investigate changes in the *F. scutaria* proteome during the onset of symbiosis to isolate and

identify proteins functioning in the process. Using 2-D PAGE, *F. scutaria's* steady-state soluble protein profile was monitored over the course of development (at 1, 2, 5 and 6 days old) and infection (at 2 and 3 days after infection). The results of this study indicate that few very detectable changes in the proteome of the host have occurred as a result of symbiosis by these time points.

Methods

Collection

Several dozen adult *F. scutaria* were collected from reefs surrounding Coconut Island, Oahu, HI in July of 1998 and August of 2000. Maintenance and monitoring of the corals were performed as in Schwarz et al.(1999). When spawning occurred, eggs were collected in plastic bowls and diluted generously with 3-4L of 0.2 μm filtered seawater (FSW). Sperm from several males was mixed together in an approximate 1:10 dilution with FSW. Eggs were fertilized by gently squirting the dilute sperm mixture into the bowl using a turkey baster. The fertilized eggs were transferred to covered running seawater tables and left overnight with occasional gentle mixing. Approximately 12-18 h after fertilization and once each day thereafter, the water in each bowl of developing larvae was changed using 60 μm screen and fresh FSW (Schwarz et al. 1999).

Infection

Larval development was monitored daily using light microscopy. At approximately 3 days post-fertilization, once the larvae had developed a mouth, they were exposed to freshly isolated symbionts (A.K.A zooxanthellae (FIZ)) from adult *F. scutaria* and a brine shrimp preparation (Schwarz, et al 1999). Prior to infection, larvae from all bowls were combined into a single population then redistributed into the original bowls. Larvae were then concentrated into a

smaller volume (~1-1.5L) of filtered seawater. Half of the bowls were exposed to FIZ + shrimp (~15mL of the FIZ preparation was gently poured into each bowl and mixed followed by ~5mL of the brine shrimp preparation) and half of the bowls were exposed to shrimp only (~5mL shrimp preparation + ~15mL filtered seawater). Larvae were allowed to incubate for 3 h before the water was changed and the larval concentration was reduced to previous levels. Twenty four hours after the infection procedure, infection rates were measured. Using a light microscope, percent infection was determined by taking a subsample of 100 larvae from each bowl and quantifying the proportion of larvae containing at least one zooxanthella. Three subsamples were counted from each bowl of larvae.

Sampling

Symbiotic and aposymbiotic larvae were sampled each day (2000) or every other day (1998) following infection until the onset of settlement. A 50mL conical tube was filled with seawater containing larvae and centrifuged for 10 sec at ~8000 g. The pelleted larvae were transferred to a 1.5mL microfuge tube and centrifuged briefly at ~12,000 g in a tabletop microfuge. The seawater was quickly drawn off the larval pellet and the tube dropped into a dry ice- methanol bath. The frozen samples were held at -50°C and shipped to Oregon State University on dry ice where they were held at -80°C until processing.

Protein extraction

Frozen larval pellets were ground on ice in 500µl final volume of a low salt buffer (40mM TRIS/10mM EDTA/pH 7.4 + 0.5µl/mL protease inhibitor cocktail) using a ground glass tissue homogenizer. After grinding, the solution was centrifuged at 10,000 g for 10 min at 4°C to pellet the symbionts; the host supernatant was decanted (pelleted tissue and symbionts discarded) into a fresh tube. This was repeated until a pellet no longer formed during the spin (minimum of 3 repetitions). Protein

concentration was measured on a Beckman DU Series 500 spectrophotometer using the Coomassie Protein Assay Kit (Pierce). Protein extracts were stored at –80°C until use.

2-D PAGE

Thirty to 130 μg per sample of extracted protein were resolved on a 2-dimensional polyacrylamide gel using the Pharmacia Biotech (Amersham Biosciences) Multiphor II 2-D Electrophoresis System and the Immobiline® Drystrip Kit according to the manufacturer's instructions. Proteins were separated in the first dimension using isoelectric focusing on an 18cm pH 3-10 NL precast immobiline® drystrip. The protein was loaded onto the strip during rehydration of the drystrip. Size separation then took place on a precast ExcelGel™ SDS (sodium dodecyl sulfate) XL 12-14% gradient polyacrylamide gel. Gels were stained with silver nitrate using methods modified from Heukeshoven and Dernick (1985), preserved in 10% glycerol, and air dried under a mylar sheet.

Analysis

Gels were examined pairwise by eye for gross differences in protein patterns between symbiotic and aposymbiotic larvae of identical age, using a light box. Due to the lack of differential spots further analysis using image analysis software was deemed unnecessary.

Results

Age-related changes in protein complement

The steady-state soluble protein profile of 1- and 2-day-old larvae is dominated by three large (63.2, 82.2, & 95.5 kDa) basic (pH ≅10) proteins (Figure 1). By 2 days post-infection (5-day-old larvae collected in 1998) and 3 days post-infection (6-day-old larvae collected in 2000) these high molecular weight, basic spots were absent (Fig

2) as were several smaller proteins (32-35 kDa) with pI's ranging from 6-10 (Fig 1). No spot was found that appeared through time, in an age-dependant manner.

Symbiosis-related changes in protein complement

The protein patterns from symbiotic and aposymbiotic larvae proved remarkably similar (Table 1). In fact, no consistent differences (proteins abundant in one infection state and essentially absent in the other) in the protein profiles were found. Only a few proteins appearing differential could be detected in each of the larval samples examined. Most of these spots varied in occurrence between samples, including samples of the same age. Among these differential spots, just one 29.2 kDa spot was consistently present in all symbiotic samples and absent or less abundant from the aposymbiotic ones (Fig 2). Two unsuccessful attempts were made, using N-terminal sequencing, to have this protein spot identified.

Discussion

Many changes in the larval soluble protein profile occurred in the larvae as development progressed. Although an exhaustive search of these age-related profiles was not performed, all of the changes were proteins that disappeared from the profiles as the larvae aged. Some of these changes such as the 63.2, 82.2, and 95.5 kDa proteins found in the 1- and 2-day-old profiles are likely to be yolk proteins. The presence of such proteins in the Cnidaria has been predicted and the lipid content of eggs has been investigated repeatedly in many species (Beams and Kessel 1983; Ben-David-Zaslow and Benayahu 2000; Kessel 1968; Richmond 1987). Recently, molecular evidence of a vitellogenin (≅ 76 kDa), the precursor of yolk protein, was described in the egg of the scleractinian coral *Favites chinensis* (Imagawa et al. 2004). Surprisingly, there were virtually no differences between the profiles of symbiotic and aposymbiotic larvae. These unexpected results suggest that during the first few days of a new symbiosis between the *F. scutaria* larval host and the dinoflagellate symbiont

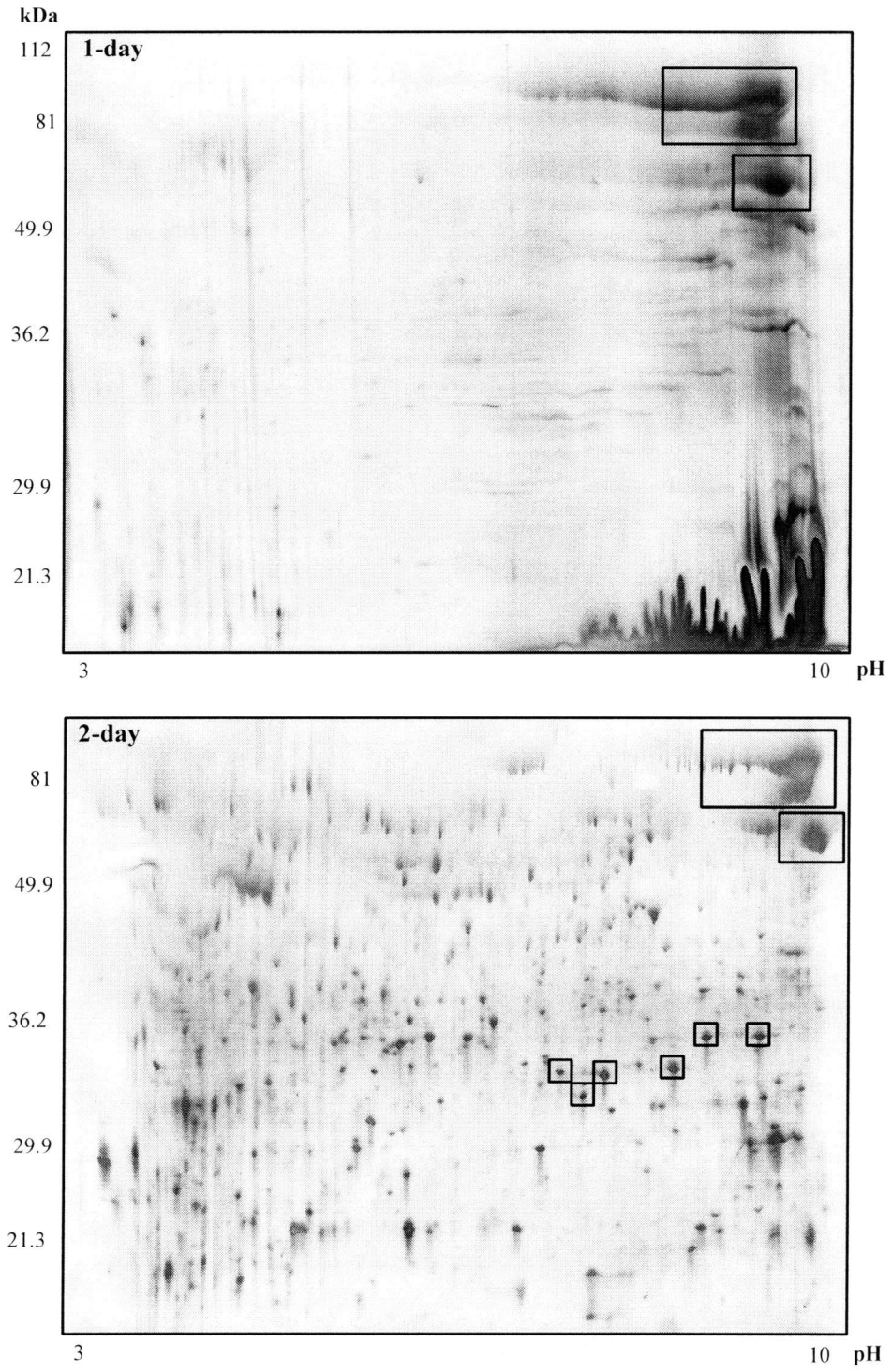

Figure 1. Two dimensional protein profiles from 1- and 2-day old larvae prior to the development of a mouth and therefore before infection. Thirty μg of protein from 1-day-old larvae and 130 μg from 2-day-old larvae were loaded. Boxes indicate proteins absent from profiles of 5- and 6-day-old larvae.

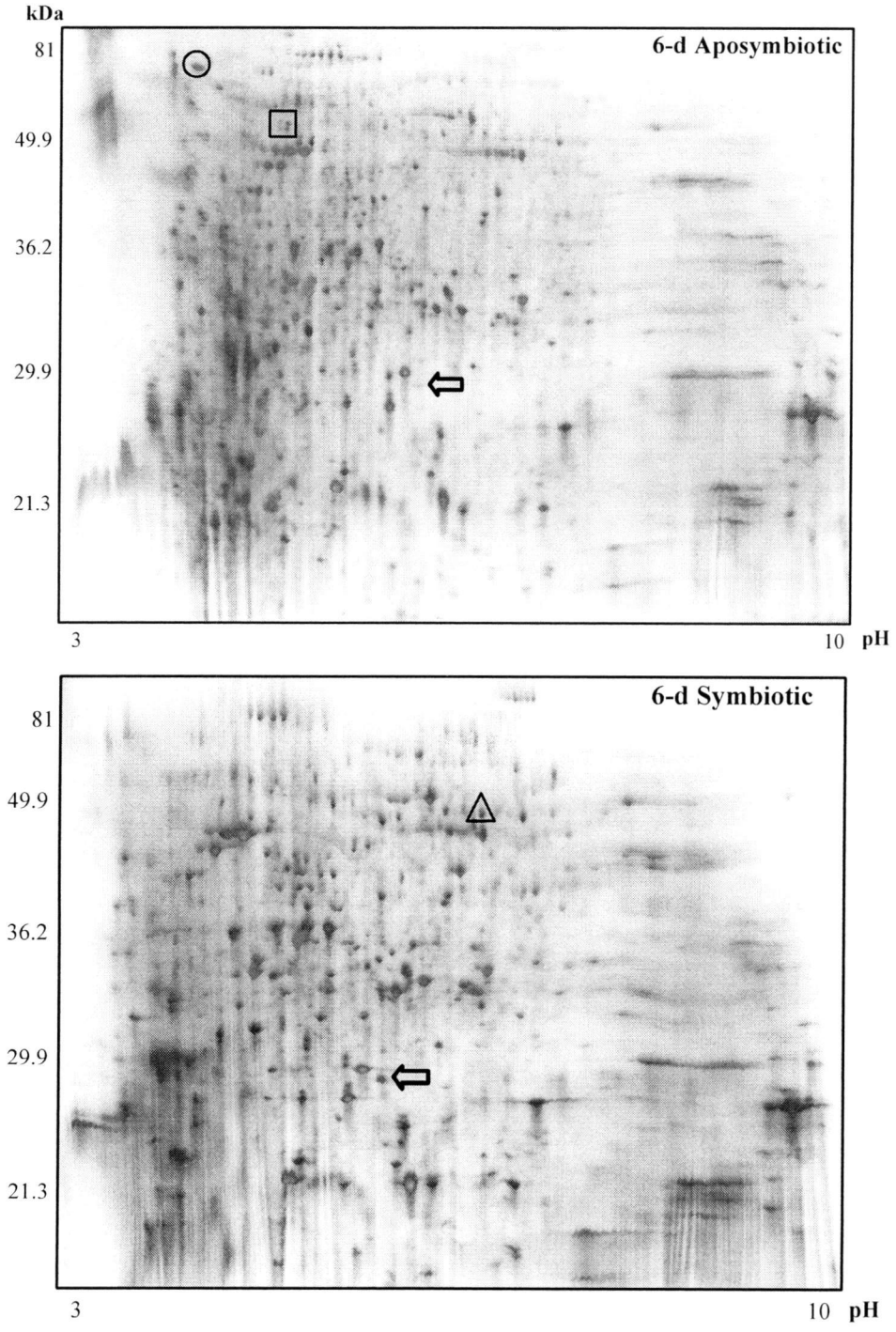

Figure 2. Representative two dimensional protein profiles from 6-day-old symbiotic and aposymbiotic larvae. The symbiotic larvae had a 54% infection rate and each gel was loaded with 50 μg of protein. Differential spots were found within larval samples (square, circle, triangle) but, with one exception, were inconsistent across samples. The arrow indicates a spot consistently differential in all 5- and 6-day-old samples.

Table 1. Tallied results from 2-dimensional gel electrophoresis of soluble proteins from *F. scutaria* planula larvae. Larval age and year of collection are given. Percent infection (inf.) is the number of infected larvae in a given symbiotic (sym) sample; aposymbiotic (apo) larvae were 100% uninfected. Number of differential (diff.) spots refers to variation between a particular pair (1 sym and 1 apo) of gels generated from the same larval sample.

Age (days)	Year	# days post-infection	# of larval samples	# Replicate gels	% inf.	# of diff. spots	Approx # spots / gel
1	1998	Pre-inf.	1	2	-	-	100
2	1998	Pre-inf.	1	2	-	-	450
5	1998	2	2	2 sym / 1 apo	85	7	450
				1 sym / 1 apo	74	6	
6	2000	3	2	1 sym / 1 apo	59	4	450
				1 sym / 1 apo	54	4	

Symbiodinium type C1f, translational or posttranslational changes specific to the symbiosis are not occurring in the soluble proteome of the host. This result is in stark contrast to what has been found during the onset of symbiosis in many other cooperative and parasitic associations (Abshire and Neidhardt 1993; Gloudemans and Bisseling 1989; Govers et al. 1985; Krause and Broughton 1992; Kwaik et al. 1994; Lemus and McFall-Ngai 2000; Natera et al. 2000; Saalbach et al. 2002; Weis and Levine 1996)

However, the current study relied on the presence of easily detectable (by naked eye) differences in profiles, as has been found in many other studies (Krause and Broughton 1992; Lemus and McFall-Ngai 2000; Weis and Levine 1996). And although this kind of gross differential display was not evident, the changes may be occurring but may simply be below the limit of detection for this technique; several possibilities present themselves as reasons why. A less than 100% infection rate might lead to a high background of aposymbiotic proteins on symbiotic gels; slow rates of protein turnover could obscure the new "symbiotic" larval profile; or perhaps the types of biochemical changes occurring during the onset of symbiosis in this system are undetectable using 2D-PAGE. For example, instead of seeing an induction of highly expressed proteins, such as those with a structural function, the presence of a symbiont in the host cell may trigger transiently expressed regulatory proteins, such as transcription factors, which could initiate signaling cascades.

Small symbiont population size could also be problematic. In this work we are looking for changes in the whole host proteome, yet at this stage in the association each larva contains an average of 25 algae (Weis et al. 2001). Biochemical changes occurring in these 25 host cells may be obscured by the thousands of larval cells without a symbiont. A disadvantage of this system is the lack of a discreet structure in the larvae used for housing symbionts, such as the squid's light organ in the *E. scolopes / V. fischeri* association. Such a structure could be isolated and examined

apart from the whole animal, which would make detection of proteomic changes easier. Interestingly, recent work on *F. scutaria* larvae has shown that the algae are concentrated in one region of the larval body (Linetty-Rodriguez, Weis - personal communication).

Strategies for overcoming the limitations presented by this particular symbiotic system include significantly increasing the sample size and rates of infection, or using alternate methods of detection and analysis. One technique which might eliminate many of the confounding factors would be to profile only the newly synthesized proteins in the larvae, as the association gets underway. We made several attempts at ^{35}S labeling of newly synthesized proteins in these larvae, but were unsuccessful due mainly to logistics and sample sizes. Another strategy would be to shift the time points chosen for examination. In the *E. scolopes* / *V. fischeri* association, it was found that although the morphogenetic changes in the host occurred early on (within hours of exposure to symbiont), this did not correspond to changes in soluble protein patterns until 2 days post-infection (Lemus and McFall-Ngai 2000). Perhaps a shift in focus to older *F. scutaria* larvae would yield the desired information. However, new work on the soft coral *Heteroxenia fuscescens* suggests that this might not be the case. The onset of symbiosis in *H. fuscescens* occurs in the primary polyp stage, shortly after the non-symbiotic planula larvae settle and undergo metamorphosis. This study followed symbiotic and aposymbiotic polyps until six weeks of age and three weeks into the symbiotic relationship. Comparisons of the soluble protein patterns from symbiotic and aposymbiotic primary polyps were performed weekly. No marked differences between the *H. fuscescens* symbiotic states were detected at any age examined (Orit Barneah, unpublished data).

In conclusion, it appears that 2D-PAGE, a technique that has worked in other systems as a first step in uncovering host biochemical response to the presence of intracellular

foreigners, is not a sensitive tool for the *F. scutaria / Symbiodinium* type C1f association. While the negative results obtained in this study are intriguing, a different approach using alternate techniques must be employed before concluding an absence of host biochemical response to the presence of the algae. With more extensive analysis, a response by the larvae to the intracellular invasion, friendly or otherwise, will most likely be uncovered.

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CHAPTER 4

CHANGES IN HOST GENE EXPRESSION DURING THE ONSET OF INTRACELLULAR SYMBIOSIS IN THE SCLERACTINIAN CORAL *FUNGIA SCUTARIA*

Introduction

Many marine cnidarians engage in symbiosis with dinoflagellates. These associations form the trophic and structural foundation of the coral reef ecosystem. Diploblastic cnidarians house their intracellular symbionts within their endodermal tissue layer and receive the nutritional benefits of a resident primary producer (Muscatine 1980; Trench 1979). The algae benefit from the association as well, receiving nutritional byproducts and protection from the animal host (Smith and Douglas 1987; Trench 1987). How this mutually beneficial association arises varies. Some cnidarian hosts acquire their symbiont from the environment (horizontal transmission) during development while others pass the symbiont along from parent to offspring (vertical transmission) within the confines of the sexual cycle (Douglas 1994; Trench 1979).

In the case of horizontal transmission, uptake of the ingested symbiont occurs via phagocytosis by the endodermal layer, during which process the alga becomes permanently enclosed within a host-derived vesicle (Smith and Douglas 1987; Wakefield and Kempf 2001). While the mechanism of recognition between partners is most likely to occur on a molecular level, and it has been shown that the establishment of the most successful associations depends upon the identity of each partner (Baker 2003; Belda-Baillie et al. 2002; Coffroth et al. 2001; Weis et al. 2001), nothing is known about what cellular and molecular mechanisms are at work after phagocytosis, during the early stages of these symbioses.

Because the cnidarian is without a symbiont during the initial stages of development, horizontal transmission creates an excellent opportunity for uncovering such mechanisms. The pre-symbiotic (azooxanthellate) stage allows, in some systems, for the experimental infection of the cnidarian under controlled conditions. This creates the opportunity to study the association as it proceeds from the initial contact and recognition stages through the establishment of a mature association.

The *Fungia scutaria – Symbiodinium* type C1f (Rodriquez-Lanetty et al. 2004) symbiosis is just such an association. *F. scutaria*, a Hawaiian solitary scleractinian coral, is a gonochoric broadcast spawner that releases negatively buoyant azooxanthellate eggs into the water column predictably 3-4 days after the full moon during the summer months (Krupp 1983). Fertilization occurs in the water column and zygotes develop into azooxanthellate planula larvae that are swimming actively by 1- 2 days of age, develop a mouth by day 3, and are competent to settle by day 7, although they may persist in the water column for longer. The larvae are able to acquire symbionts as soon as they are able to feed (Krupp 1983; Schwarz et al. 1999). Acquisition occurs when the symbiont enters, during feeding, into the gastric cavity where phagocytosis of the algae by the endodermal lining occurs (Schwarz et al. 1999). Thereafter the symbiont resides within a membrane-bound compartment inside a host endodermal cell. Larvae that fail to acquire symbionts may still settle (Schwarz et al. 1999) and undergo metamorphosis, however an aposymbiotic *F. scutaria* adult has never been observed in the field.

Nothing is known about the molecular events that accompany the onset of symbiosis between these two partners. The goal of this study was to investigate changes in *F. scutaria* gene expression during the onset of symbiosis, to isolate and identify genes turned on or upregulated during algal infection. Using subtractive hybridization, symbiosis-specific cDNA sequences were isolated from *F. scutaria* planula larvae newly engaged in symbiosis with their symbionts. These cDNA sequences were

monitored over the first week of infection using real-time quantitative PCR. The results of this study indicate symbiosis-specific changes in gene expression do occur in the larvae with the onset of symbiosis. However, what these sequences encode for is unknown as extensive bioinformatic analyses of the upregulated sequences examined in this study revealed no homologues in the sequence databases.

Methods

Collection

Several dozen adult *F. scutaria* were collected from reefs surrounding Coconut Island, Oahu, HI in July of 1998 and 2003, and August of 2000. Maintenance and monitoring of the corals were performed as in Schwarz et al.(1999). When spawning occurred, eggs were collected in plastic bowls and diluted generously with 3-4L of 0.2 μm filtered seawater (FSW). Sperm from several males were mixed together in an approximate 1:10 dilution with FSW. Eggs were fertilized by gently squirting the dilute sperm mixture into the bowl using a turkey baster. The fertilized eggs were transferred to covered running seawater tables and left overnight with occasional gentle mixing. Approximately 12-18 h after fertilization and once each day thereafter, the water in each bowl of developing larvae was changed using 60 μm screen and fresh FSW (Schwarz et al. 1999).

Infection

Larval development was monitored daily using light microscopy. At approximately 3 days post-fertilization, once the larvae had developed a mouth, they were exposed to freshly isolated symbionts (A.K.A zooxanthellae (FIZ)) from adult *F. scutaria* and a brine shrimp preparation (Schwarz, et al 1999). Prior to infection, larvae from all bowls were combined into a single population then redistributed into the original bowls. Larvae were then concentrated into a smaller volume (~1-1.5L) of filtered seawater. Half of the bowls were exposed to FIZ + shrimp (~15mL of the FIZ

preparation was gently poured into each bowl and mixed followed by ~5mL of the brine shrimp preparation) and half of the bowls were exposed to shrimp only (~5mL shrimp preparation + ~15mL filtered seawater). Larvae were allowed to incubate for 3 h before the water was changed and the larval concentration was reduced to previous levels. Twenty four hours after the infection procedure, infection rates were measured. Using a light microscope, percent infection was determined by taking a subsample of 100 larvae from each bowl and quantifying the proportion of larvae containing at least one zooxanthella. Three subsamples were counted from each bowl of larvae.

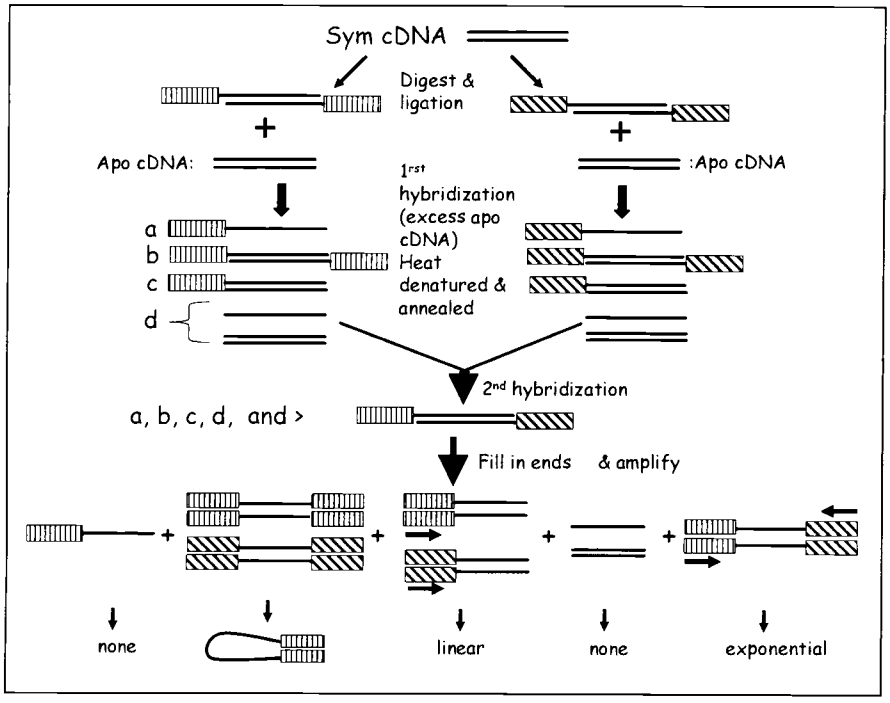
Sampling and RNA isolation

Symbiotic and aposymbiotic larvae were sampled each day (2000 & 2003) or every other day (1998) following infection until the onset of settlement. A 50mL conical tube was filled with seawater containing larvae and centrifuged for 10 sec at ~8000 g. The pelleted larvae were transferred to a 1.5mL microfuge tube and centrifuged briefly at ~12,000g in a tabletop microfuge. The seawater was quickly drawn off the larval pellet and the tube dropped into a dry ice- methanol bath. The frozen samples were held at -50°C and shipped to Oregon State University on dry ice where they were held at -80°C until processing. After separating host and algal tissue via centrifugation, RNA was extracted from the larvae and quantities determined spectrophotometrically at A_{260} (Weis and Reynolds 1999).

Subtracted cDNA plasmid library

With subtractive hybridization one can compare two mRNA populations and isolate genes that are being expressed in one but not the other. The basic molecular premise for the technique is as follows: two populations of mRNA are converted to cDNA and mixed together; those sequences that are present in both populations will hybridize. The hybridized sequences are removed and the remaining unhybridized sequences represent genes that are expressed in only one of the populations.

For this library, the CLONTECH PCR-Select^{TM} cDNA subtraction kit (see the figure below for an illustration of the technique) was used. The library was constructed using 1.45 μg of mRNA from a 3-bowl mixture of 5 day old symbiotic larvae (each with an infection rate ≥ 78%) and 1.24 μg mRNA from a 4-bowl mixture of 5 day old aposymbiotic larvae. The larvae were collected in August 1998. Each mRNA population was converted into cDNA. The cDNA from the population of interest, "tester" cDNA (in this case from symbiotic larvae), was split in half. Each half was exposed to a different adaptor sequence (Adaptor 1 and Adaptor 2R in the figure below) which was ligated to the 5' end of each cDNA strand. Each subpopulation of adapter ligated tester cDNA was hybridized separately to the "driver" (aposymbiotic larvae) cDNA population; sequences derived from the same gene annealed. The two hybridized sets were mixed together and hybridized again. Differentially expressed tester cDNA strands (unhybridized to driver cDNA) that were derived from the same gene annealed, creating double stranded cDNA with different adaptors at either end. The adaptor ends were filled in and, using PCR and adaptor-specific primers, those cDNA sequences were amplified. A secondary PCR amplification followed using nested



primers (a primer that sits 3' from the original primer) to reduce background. Finally, the PCR products were inserted into a vector, cloned, screened, and sequenced. In this way, differentially expressed gene sequences were isolated and identified without physically separating differential sequences from non-differential sequences.

The subtraction was performed according to the manufacturer's instructions with one exception: after the second hybridization, the reaction was diluted with ½ of the recommended amount of dilution buffer. Both the forward and reverse subtractions were done, resulting in a sym-enriched library of expressed sequence tags (ESTs) and an apo-enriched library of ESTs. Both sets of cDNAs were inserted into a T/A cloning vector (Invitrogen).

Primary library screen

Digoxigenin-labeled PCR probes were made using the PCR products from both the sym-enriched library and the apo-enriched library. Each PCR reaction consisted of: 15.6μl sterile water, 2.5μl 10X PCR buffer (Promega), 2μl 25mM MgCl (Promega), 1μl Adaptor 1 nested primer (CLONTECH), 1μl Adaptor 2R nested primer (CLONTECH), 0.25μl Taq DNA Polymerase (Promega), 1μl dNTPs (2mM dATP, dCTP, dGTP and 1.3mM dTTP), 0.65μl dig-11-dUTP, and 1μl of a 1:10 dilution of the primary subtractive PCR product as template. The cycling parameters were: 94°C (1min) denaturing step; 15 cycles of 94°C (30sec), 68°C (30sec), 72°C (1:30sec); and a 5 min extension step at 72°C. The PCR probes were precipitated overnight at -20°C in 1/10 volume 4M LiCl and 2.5 volumes 100% ETOH. They were then centrifuged at 14,000g for 30min at 4°C. Pellets were washed with 70% cold ETOH, centrifuged for 15min, air dried, and resuspended in 20μl sterile water. Probe concentrations were determined using mock hybridizations.

Fifty μl of Max Efficiency DH5α competent cells (Invitrogen) were transformed with 2μl of the sym-enriched cDNA library (according to the manufacturer's instructions).

The cells were plated on LB/ampicillin plates spread with Xgal and IPTG, and grown overnight at 37°C. One hundred and seventy six white colonies were picked and swirled in 200µl LB with 100µg/ml ampicillin and grown overnight at 37°C with shaking. One µl of each overnight culture was aliquoted to a sterile PCR tube and amplified using either nested primers 1 & 2R (colonies 1-60) supplied with the library kit, or vector primers M13F & M13R (colonies 61-176). Each PCR reaction was diluted 1:1 with water and heat denatured. One µl was spotted onto each of two positively charged nylon membranes. PCR amplified actin (amplified from aposymbiotic *F. scutaria* larval cDNA) and sterile water were also dotted on each membrane as controls. Membranes were dried, UV crosslinked, and incubated in preheated hybridization solution (hyb: 10ml 10% blocking reagent), 5ml 20X SSC, 40µl 10% SDS, 200µl 10% N-lauryl sarcosine, 4.76ml water) for 1 hour at 68°C in a rotating hyb oven. The prehybridization solutions were discarded and each membrane was incubated in 5ml hybridization solution with a dig-labeled probe (symbiotic or aposymbiotic) overnight (68°C/rotating). The sym-enriched probe was used at a 1:1000 dilution and the apo-enriched probe was used at a 1:3000 dilution.

The membranes were washed 2 times in 0.3% Tween 20 in Maleic acid buffer (0.1M maleic acid, 0.15M NaCl, pH 7.5) for 5 min, blocked with 2% blocking reagent in Maleic acid buffer for 40 min, and exposed to 1:10,000 anti-DIG-AP conjugate in 2% blocking reagent in Maleic acid buffer for 30 min, following by 2 washes for 20 minutes each. The membranes were then incubated in detection buffer (0.1M TRIS/0.1M NaCl pH 9.5) for 5 min after which they were saturated with CSPD (Boehringer Mannheim) and sandwiched between two sheets of acetate. After incubating for 15 min at 37°C, the membranes were exposed to X-ray film. All membrane incubations were performed on a shaker table. Three identical replicates of the primary screen were performed.

Sequencing & primer design

Six inserts from the sym-enriched library, showing a strongly positive result when probed with the sym-enriched probe and a strongly negative result when probed with the apo-enriched probe, on each of the primary screen replicates, were PCR amplified from glycerol stocks using the vector primers M13F & M13R. The PCR products were washed using Montage™ PCR Centrifugal Filter Device and sequenced on an Applied Biosystems capillary 3100 Genetic Analyzer.

Forward and reverse primers were designed to amplify 100-200 base pairs (bp) of each of the sequenced inserts (expressed sequence tags). Each primer was 20 bp in length with 45-50% G/C content; primer sequences are as follows: Expressed sequence tag (EST) 1 forward primer: 5' GGA CTT GGA CCA TCT TCT AC 3' and reverse primer: 5' TGC GGA TTA TTG GAG CCA GT 3'; EST 66 forward primer: 5' ATG CCC ATC TGA AGC CAT GA 3' and reverse primer: 5' CAC TTC CGA TCC AAC TCT CT 3'; EST 89 forward primer: 5' GAT AGC CAC AAC ACA CCA GA 3' and reverse primer: 5' GCT GAA CAG GAA GAT TGT GC 3'; EST 135 forward primer: 5' TGG ATT CTG TCC CAG TTG CT 3' and reverse primer: 5' CTG TGT GGG TGC AAA CTG TT 3'; EST 158 forward primer: 5' ACC ATT GGT TCA CCT TCC AA 3' and reverse primer: 5' ACA TGG GAG ACC AGG AGA AA 3'; EST 165 forward primer: 5' GGA GTA GTC TTA CGC ACT AT 3' and reverse primer: 5' ACT CAG GAT GCA GAA CTC CT 3'.

Secondary library screen

A secondary screen for differential expression was performed using each set of primers in multiple real-time quantitative PCR reactions (ABI PRISM® 7700 or ABI PRISM® 7000 Sequence Detection Systems) with symbiotic & aposymbiotic cDNA templates synthesized from 1μg total RNA extracted from larvae of various ages collected in 1998, 2000 or 2003, and SYBR® green PCR master mix (Perkin Elmer). Identical reactions amplifying actin (forward primer: 5' CTG ATG GAC AGG TCA TCA CCA T 3', rev. primer: 5' CTC GTG GAT ACC AGC AGA TTC C 3') were run

concurrently and were used as a reference point for expression level. PCR reactions consisted of: 1.0µl cDNA (aposymbiotic or symbiotic), 6.0µl sterile water, 1.5µl gene specific forward primer, 1.5µl gene specific reverse primer, and 10.0µl SYBR® green PCR master mix. The default cycling parameters for the ABI PRISM® 7700 or the ABI PRISM® 7000 Sequence Detection Systems were used in all reactions. All EST expression levels were normalized to actin expression and are reported as follows: 1/(EST cycle# @ mid-log phase amplification – actin cycle# @ mid-log phase amplification). Replication number is the number of PCR reactions run on larvae of a specific age and infection state and collected in a specific year. All runs performed on the ABI PRISM® 7000 included a dissociation curve to check for the presence of primer dimers and all PCR products from preliminary runs performed on the ABI PRISM® 7700 were checked for primer dimers using agarose gel electrophoresis. Statistical significance was evaluated using the unpaired t test with two-tail P value (GraphPad InStat version 3.05 for Windows 95/NT, GraphPad Software, San Diego California USA, www.graphpad.com).

Sequence analysis

ESTs were translated using NCBI's ORF Finder (http://www.ncbi.nlm.nih.gov/). All reading frames of each EST were subjected to a BLASTp or PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) search using default parameters for primary structure comparisons. Secondary structure of each EST reading frame was predicted using the PSIPRED Protein Structure Prediction Server and each was submitted to GenTHREADER for fold recognition (Jones 1999a; Jones 1999b; McGuffin and Jones 2003). In addition, each translated frame was subjected to a PROSITE scan for the presence of biologically significant protein sequence sites and patterns (motifs) (Falquet et al. 2002; Sigrist et al. 2002)

Results are reported for those reading frames completely or almost completely open and/or for the reading frame that generated the lowest expected error values (E-values)

during database searches. The E-value along with the NCBI accession number or the Protein Data Base identification number are reported for all significant and insignificant hits for a particular reading frame. A confidence score ("certainty" E-value < 0.001; "high" E-value < 0.01; "medium" E-value < 0.1; "low" E-value < 0.5; "guess" E-value >= 0.5) for each threading hit is also reported.

For hits with the best E-value, multiple sequence alignments were constructed between members of that protein family and the EST in question. All alignments were constructed using ClustalX (Thompson et al. 1997). Pairwise alignments were done with a gap opening penalty of 35.00 and a gap extension penalty of 0.75. Multiple alignments were done with a gap opening penalty of 15.00 and a gap extension penalty of 0.30. Similarity matrix PAM250 was used for all identity/similarity calculations and calculations included all insertion/deletions, although overhangs were masked.

Results

Primary and secondary library screens

Several ESTs from the sym-enriched library labeled more strongly with the sym-enriched probe than with the apo-enriched probe. PCR-amplified *F. scutaria* actin cDNA failed to hybridize when probed with the dig-labeled sym-enriched PCR product, indicating a successful subtraction (Fig 1). Six ESTs (#1, 66, 89, 135, 158 and 165) that appeared to display the greatest difference in symbiotic vs. aposymbiotic labeling were chosen for further analysis (Fig 1). Primers were designed to amplify a short piece of each of these sequences for a real-time quantitative PCR secondary screen (Fig 2). The expression patterns generated from the secondary screen were variable across larval age and year of collection (Figs 3a & 3b). However, expression of each of the six ESTs was significantly higher in the symbiotic larvae at some point during the time points tested.

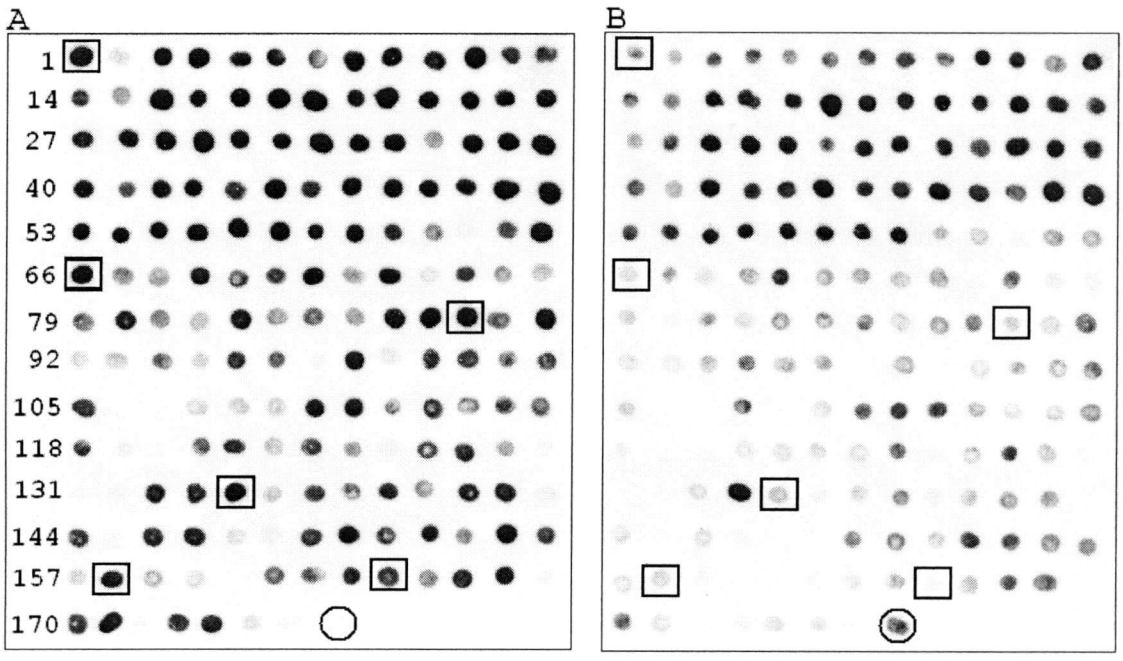


Figure 1. Primary screen for differentially expressed ESTs from the sym-enriched library. PCR amplified ESTs (EST inserts numbered from top left corner) & actin (#177) are spotted in an identical pattern on two membranes. Membrane A was probed with dig-labeled PCR product from the sym-enriched library and membrane B was probed with dig-labeled PCR product from the apo-enriched library. Boxes indicate ESTs (#1, 66, 89, 135, 158 & 165) chosen for secondary screening. The circles indicate PCR amplified actin cDNA on each membrane.

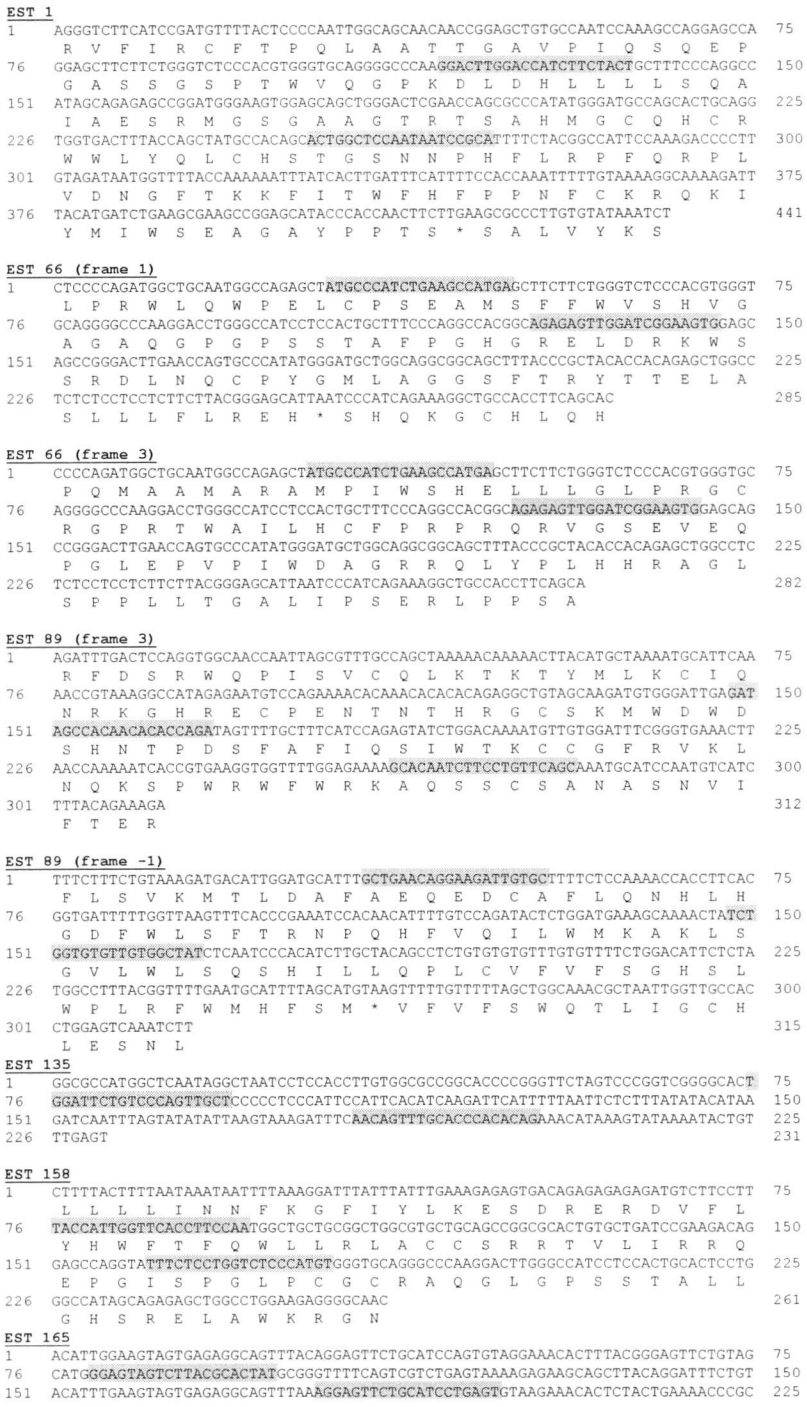


Figure 2. Six ESTs from the sym-enriched library that were differentially expressed in the primary screen. The nucleotide (nt) sequence for each EST is given along with the deduced amino acid sequence for the reading frame(s) that generated the best sequence analysis results. Numbers correspond to the nt sequence. Primer sites, designed to amplify a 100-200 bp internal amplicon for the secondary screen, are highlighted.

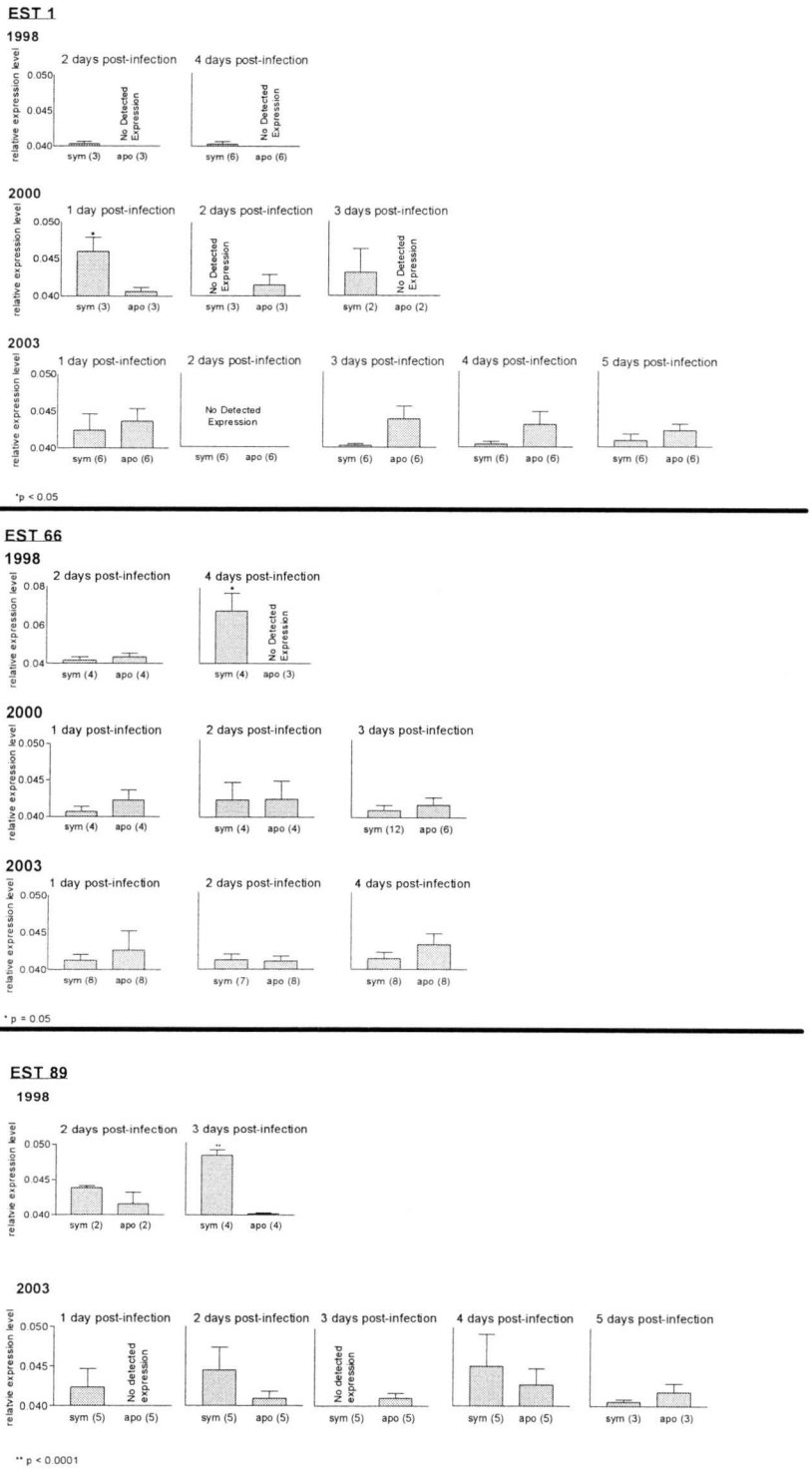


Figure 3a. Secondary screen of ESTs from the sym-enriched library. RT-QPCR results show relative expression of each EST in symbiotic (sym) and aposymbiotic (apo) larvae of various ages collected July 1998, August 2000, and July 2003. Replicate # is given in parentheses. "No detected expression" is reported when no amplification occurred.

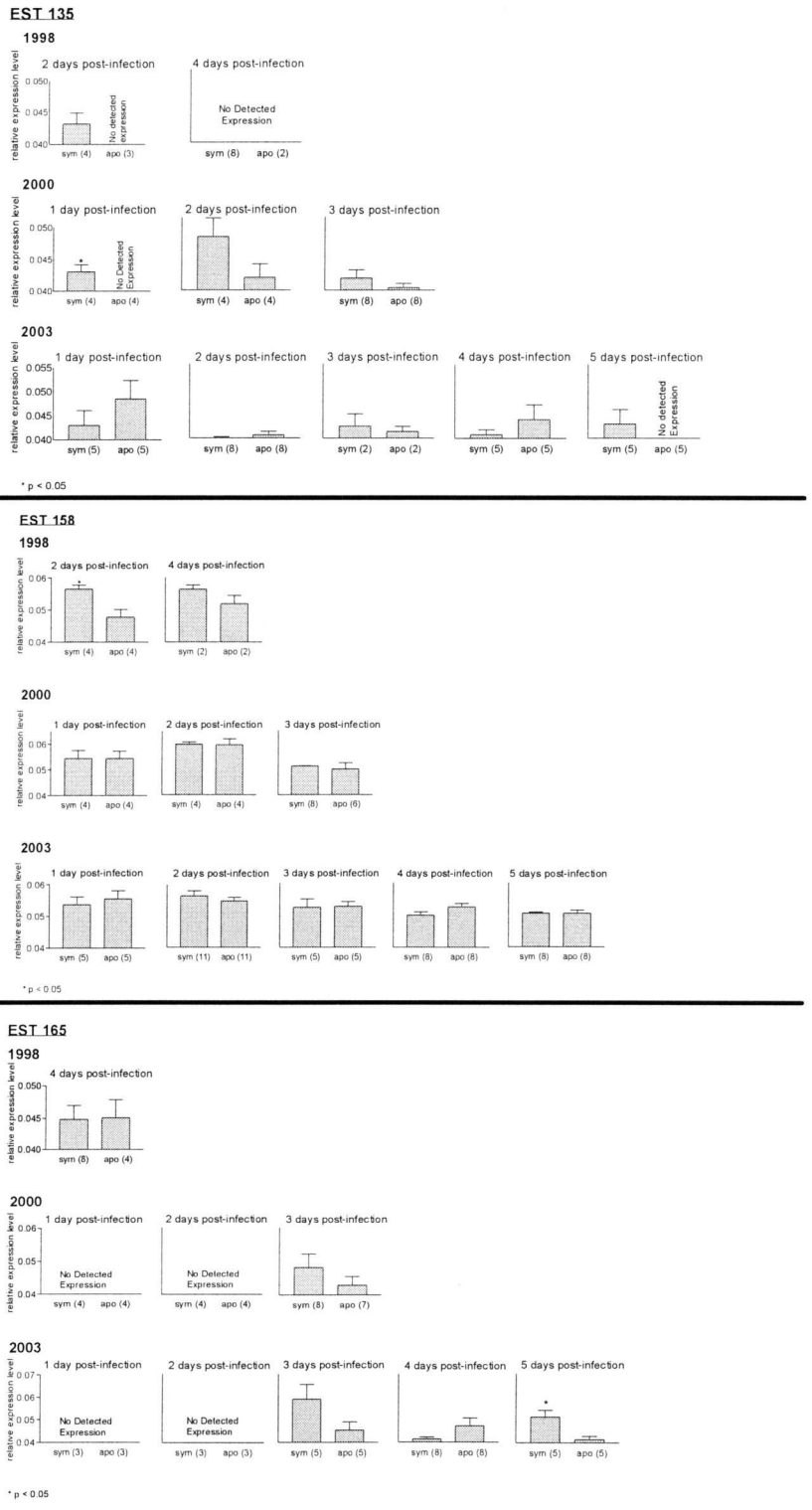


Figure 3b. Secondary screen of ESTs from the sym-enriched library. RT-QPCR results show relative expression of each EST in symbiotic (sym) and aposymbiotic (apo) larvae of various ages collected July 1998, August 2000, and July 2003. Replicate # is given in parentheses. "No detected expression" is reported when no amplification occurred.

Database search results for each EST are reported individually below. Although some came close, none of the sequence alignments constructed yielded identity/similarity values high enough to imply structural homology (Sander and Schneider 1991); these alignments are therefore not included with the results. Excluding patterns with a high probability of occurrence, no biologically significant motif was found in any reading frame for any of the six ESTs analyzed using PROSITE.

EST 1

Real-time quantitative PCR results for EST 1, from several time points and different years, supported the primary screen. In 1998, EST 1 cDNA amplified in symbiotic larvae at both 2 and 4 days post-infection, but did not amplify in aposymbiotic larvae of the same age (Fig 3a). Samples collected in 2000 support this trend. Though statistically insignificant, the 6, 7 & 8 day old aposymbiotic larvae collected in 2003 had greater amounts of EST 1 than did the symbiotic larvae.

Of the six possible reading frames, one completely open (no stop codons) reading frame was found after translation of the EST 1 cDNA sequence (Fig 2). This predicted amino acid sequence also generated the best blast and threading hits during database searches (Table 1). The 3 best hits (lowest E-values) resulted from a database comparison of secondary structure using genTHREADER and included a bacterial endoglucanase, a beetle (firefly) luciferase, and a human transport protein, Alpha-tocopherol. Multiple sequence alignments of EST 1 with members of the Glycosyl Hydrolase family 5 and the luciferase (Acyl-CoA Synthetase) superfamily generated the highest percentage identity/similarity scores of 11/30 (with the *Thermotoga maritime* endoglucanase) and 10/34 (with an *Arabidopsis thaliana* 4-coumarate: CoA ligase 1 sequence) respectively (data not shown).

Table 1. Results from database searches for sequences similar to EST 1 on both primary (BLASTp) and secondary (GenTHREADER) structural levels.

Search Engine	Conf. level	E-value	Identification	Description	NCBI Accession or PDB #
BLASTp	-	0.63	Putative zinc-alcohol dehydrogenase [*Mycobacterium*]	Threonine dehydrogenase / Zn-dependent dehydrogenases	AAQ12025
Gen-THREADER	Med.	0.070	Endoglucanase (tm1752) *Thermotoga maritime*	Endohydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans	1vjz
	Med.	0.094	Firefly luciferase (oxidoreductase)	Produces green light @ 562 nM.	1lci
	Low	0.119	Human alpha-tocopherol transfer protein bound	Binds alpha-tocopherol and enhances its transfer between membranes	1r5l
	Low	0.120	Arsenite oxidase from *Alcaligenes faecalis*	Detoxification of arsenic	1g8j
	Low	0.133	Imaginal disc growth factor-2 *Drosophila*	Hormone/growth factor	1jnd
	Low	0.134	Hypothetical Protein (Tm1225) *Thermotoga Maritima*		1vkd
	Low	0.138	Anthranilate synthase *Sulfolobus solfataricus*	Tryptophan biosynthesis; first step	1qdl
	Low	0.140	D95a oxidized flavodoxin *Desulfovibrio vulgaris*	Low-potential electron donor to a number of redox enzymes	1akq
	Low	0.141	Extracytoplasmic domain of bovine cation-dependent mannose 6-phosphate receptor *Bos taurus*	Transport of phosphorylated lysosomal enzymes from the Golgi complex and the cell surface to lysosomes	1m6p
	Low	0.160	Endoglucanase cel5a *Bacillus agaradhearens*	Endohydrolysis of 1,4-beta-D-glucosidic linkages in cellulose, lichenin and cereal beta-D-glucans	7a3h

EST 66

Quantitative PCR results show a difference in EST 66 amplification between symbiotic and aposymbiotic 7 day old larvae in 1998 (Fig 3a). At 4 days post-infection EST 66 amplified in the symbiotic larvae and not in the aposymbiotic larvae. In 2000 and 2003, no difference in amplification between symbiotic and aposymbiotic larvae was detected.

Of the six possible reading frames, frame 1, which has a stop codon at the end of the sequence, generated the best BLASTp search hit to a tartrate dehydratase sequence from *Magnetospirillum magnetotacticum* (Table 2). Multiple sequence alignments of the Tartrate Dehydratase/Fumarate Hydratase class I superfamily produced an identity/similarity percentage high of 21/39 between the *M. magnetotacticum* sequence and the EST 66 sequence (data not shown). During searches of known crystal structures, the frame 1 sequence was most similar to glutathione-S-transferase (GST), drawing several hits from plants, animals, bacteria and protozoa (Table 2). Multiple sequence alignments of the GST superfamily produced identity/similarity values of 19/38 (data not shown).

Another reading frame (frame 3) contained no stop codons and generated the best secondary structure database search hit (Fig 2 & Table 2). This hit was to a cobalamine biosynthetic enzyme, cobalt precorrin-4- methyltransferase from *Bacillus megaterium*. Percent identity from a pairwise alignment generated by the threading program was 17%. A BLASTp search with frame 3 generated just one hit to a hypothetical protein from wheat.

EST 89

Significant differences in EST 89 amplification between symbiotic and aposymbiotic larval samples collected 1998 and 2003 were detected using real-time quantitative

Table 2. Primary (BLASTp) and secondary (GenTHREADER) structure database search hits for two different EST 66 reading frames.

	Search Engine	Conf. level	E-value	Identification	NCBI Accession or PDB #
Frame 1	BLASTp	-	0.12	Tartrate dehydratase/Fumarate hydratase *Magnetospirillum magnetotacticum*	ZP_00208260.1
		-	0.66	Tartrate dehydratase alpha subunit/Fumarate hydratase *Azotobacter vinelandii*	ZP_00090226.1
		-	2.8	Zinc transporter *Danio rerio*	NP_001001591
	Gen-THREADER	Low	0.393	Glutathione S-transferase (beta class) *Escherichia Coli*	1a0f
		Low	0.406	Glutathione-S-transferase Human (theta class)	1ljr
		Low	0.409	Glutathione-S-transferase *Plasmodium Falciparum.*	1okt
		Low	0.410	Histocompatibility Antigen Hla-Dm.	1hdm
		Low	0.412	Tricorn Interacting Factor Selenomethionine-F1.	1mt3
		Low	0.419	Beta-Glycosidase From The Hyperthermophile *Thermosphaera Aggregans*	1qvb
		Low	0.5	Glutathione-S-transferase (*Zea mays*) tau class	1aw9
		Low	0.6	Glutathione-S-transferase (*Arabidopsis*) Phi class	1gnw
		Low	0.8	Glutathione-S-transferase (human) omega class	1eem
Frame 3	BLASTp	-	0.46	Hypothetical protein T27D20.1 - *Arabidopsis thaliana.*	T01823
	Gen-THREADER	Low	0.159	Cobalt precorrin-4 methyltransferase, cbif *Bacillus megaterium*	1cbf
		Low	0.273	Molybdopterin biosynthesis moea protein from *Pyrococcus horikosii*	1uz5
		Low	0.295	Arnb aminotransferase *salmonella*	1mdo
		Low	0.312	Formyltetrahydrofolate dehydrogenase *Rattus norvegicus*	1s3i
		Low	0.318	Acyl-homoserinelactone synthase esai *Pantoea stewartii* subsp. Stewartii	1k4j
		Low	0.322	Yeast hypothetical protein, yl85	1f89
		Low	0.333	Ornithine decarboxylase from mouse	7odc
		Low	0.344	Thermostable thymine-DNA glycosylase *Methanobacterium thermoformicicum*	1kea
		Low	0.349	2-Methylcitrate Dehydratase. *E coli*	1szq
		Low	0.353	Cbnr, a lysr family transcriptional regulator *Alcaligenes eutrophus*	1ixc

At 4 days post-infection in 1998, the symbiotic larval samples amplified earlier than the aposymbiotic samples, indicating a greater amount of EST 89 cDNA and therefore increased expression level. At 4 and 5 days old in 2003, the trend is repeated. PCR (Fig 3a).

Translation of the six reading frames yielded one completely open reading frame (Fig 2). The best search hits overall for this reading frame were generated from searches of crystal structure databases (Table 3). Of the threading hits, half were to proteins directly involved in the immune response, and of these hits several were to lectins. Frame -1, while not completely open (Fig 2), generated the best hits overall on both a primary and secondary level. The best blast hit was to an archaebacterial rubisco (E = 1.5) and the best threading hit was to Acetyl-coenzyme A synthetase 1 from yeast (E = 0.075). The second best threading hit was to a lectin. None of the threading hits were to rubisco though there are many rubisco crystal structures in the databases, including one from green algae. Multiple sequence alignments generated identity/similarity scores of 13/28 to a bacterial long-chain-fatty-acid-coA ligase and 12/38 with a cow mannose-binding lectin (data not shown).

EST 135

The primary screen of EST 135 was supported by the real-time QPCR secondary screen in samples collected in 1998 (2 days post-infection) and in 2000. In 2003, no difference in expression was detected until 5 days post-infection when EST 135 could not be amplified in aposymbiotic larvae (Fig 3b). No significant similarity was found in the sequence databases to EST 135's deduced primary structure in any reading frame. The deduced secondary structure of each reading frame was investigated thoroughly yet none yielded obviously better results than the others (data not shown). In addition, each reading frame contained at least one mid-sequence stop codon making the identification of the correct reading frame difficult.

Table 3. Primary (BLASTp) and secondary (GenTHREADER) structure database search hits for two different EST 89 reading frames.

	Search Engine	Conf. level	E-val.	Identification	NCBI Accession or PDB #
Frame 3	BLASTp	-	1.6	MGC68791 protein [*Xenopus laevis*] a Serine/Threonine protein kinase	AAH603 63.1
	Gen-THREADER	Low	0.133	Early activation antigen cd69 [human] (immune function)	1fm5
		Low	0.234	Mannose-binding protein-a. *Rattus norvegicus* (C-type lectin)	1afa
		Low	0.241	Lithostathine-human metal binding protein (C-type lectin)	1qdd
		Low	0.276	Integrin cytoplasmic domain associated protein from human	1k11
		Low	0.326	Bone morphogenetic protein-7 – human (hormone/growth factor)	1m4u
		Low	0.334	Early activation antigen cd69 – human immune system (C-type lectin)	1fm5
		Low	0.346	Cytokyne-binding region of gp130 – human signaling protein (Fibronectin 3)	1bqu
		Low	0.351	Secreted frizzled-related protein 3 – mouse (g-protein coupled receptor)	1ijx
		Low	0.361	Cyclin-dependent kinase inhibitor 3 – human [Defense mechanisms])	1fpz
		Low	0.269	Gal6 (yeast bleomycin hydrolase)	3gcb
Frame -1	BLASTp	-	1.5	Ribulose 1,5-bisphosphate carboxylase Methanosarcina barkeri str. fusaro	ZP_00297638
		-	2.0	Glycosyltransferase *Trichodesmium erythraeum* IMS101	ZP_00327278
		-	3.8	GREB1 protein isoform a - human	NP_055483
	Gen-THREADER	Med	0.075	Yeast acetyl-coenzyme a synthetase-assimilation of ethanol and acetate	1ry2
		Med	0.087	Sap- scavenges nuclear material from damaged cells/calcium-dep.lectin	1sac
		Med	0.096	Abrin-a - a toxin with a galactose-specific lectin domain *Abrus precatorius*	1abr
		Low	0.103	Limulus c-reactive protein – horseshoe crab – probable immunoglobulin	1lim
		Low	0.110	Dfpase - *Loligo vulgaris* a phosphotriesterase	1e1a
		Low	0.126	Phospholipase d family member, *Salmonella typhimurium* - endonuclease	1byr
		Low	0.130	Yeast hal2p- Involved in salt tolerance	1qgx
		Low	0.136	LIVBP – *E.coli*- periplasmic binding protein-dependent transport systems	2liv
		Low	0.146	Human prot l-isoaspartate o-methyltransf,-repair/degradation of proteins	1i1n
		Low	0.152	His - *Thermotoga maritime* Histidine biosynthesis	1h1c

EST 158

This sequence is unique in that it appears to be expressed constantly at equal levels in symbiotic and aposymbiotic larvae at all time points tested, except in 5 day old larvae collected in 1998. On this day, EST 158 amplifies in the symbiotic larvae significantly sooner than in the aposymbiotic larvae, suggesting higher levels of expression of EST 158 in the symbiotic larvae at that point in time (Fig 3b).

The only completely open reading frame (frame 3) generated the overall best hits on both primary and secondary levels. Of these hits, the best was to a human protein kinase and was generated from a BLASTp search (Table 4). A multiple sequence alignment of members of the Serine/Threonine and Tyrosine Protein Kinase superfamily and EST 158 generated a percent identity/similarity high of 18/37 to a fungal sequence related to oncogenic serine/threonine kinases (data not shown).

EST 165

The secondary screen of EST 165 showed significantly higher levels of this sequence in symbiotic larvae, 5 days post-infection, collected in 2003 (Fig 3b). None of the six EST 165 reading frames were open and, except for frame 3, none generated any hits from BLASTp searches. Most of the secondary structure hits generated by frame 1 were to cytokines (Table 5). The 3 top hits belong to the Interleukin 6 (IL6) subfamily of cytokines; an alignment of members of this subfamily and EST 165 showed an identity/similarity percentage high of 15/ 45 to a horse IL6 sequence (data not shown). The best overall hit on a secondary level came from frame -2 and was to a D-amino acid aminotransferase from a thermophilic *Bacillus* species.

Table 4. Results from database searches for sequences similar to EST 158 on both primary (BLASTp) and secondary (GenTHREADER) structural levels.

Search Engine	Conf. level	E-value	Identification	Description	NCBI Accession or PDB #
BLASTp	-	0.055	KIAA0641 protein [*Homo sapiens*]	Serine/threonine & tyrosine prot kinases Apoptosis-associated	BAA31616.2
		2.50	3-phosphoinositide dependent protein kinase-1 [*Mus musculus*]	PDK1 & Serine/Threonine kinases-apoptosis associated	AAD38505.1
Gen-THREADER	Low	0.210	Ypt/rab-gap domain of gyp1p - yeast	Stimulates GTPase activity of YPT1 – endocytosis/exocytosis	1fkmA0
	Low	0.233	Lipase *treptomyces exfoliates*	Platelet- activating factor acetylhydrolases	1jfrA0
	Low	0.258	tRNA nucleotidyltransferase *Archaeoglobus fulgidus*	Synthesis of the tRNA CCA terminus	1uetA0
	Low	0.315	Fha domain from human chk2 kinase	Serine/threonine-protein kinase chk2 Controls cell cycle checkpoint	1gxcA0
	Low	0.329	Carbon monoxide dehydrogenase *Pseudomonas carboxydovorans*		1qj2C0
	Low	0.341	Human lxr beta hormone receptor	Nuclear hormone receptor	1pq6B0
	Low	0.396	Firefly luciferase *Photinus pyralis*	Produces green light at 562 nM	1lci00
	Low	0.411	Thyroid hormone receptor beta human	Nuclear hormone receptor	1bsxA0
	Low	0.437	Alpha-amylase ii *Thermoactinomyces vulgaris r-47*	Hydrolase	1bvzA0
	Low	0.437	Human Vitamin d nuclear receptor	Nuclear hormone receptor	1db1A0

Table 5. Primary (BLASTp) and secondary (GenTHREADER) structure database search hits for three different EST 165 reading frames.

	Search Engine	Conf. level	E-val.	Identification	NCBI Accession or PDB #
Frame 1	Gen-THREADER	Guess	0.561	Viral interleukin-6 receptor beta chain (cytokine)	1i1r
		Guess	0.636	Human oncostatin m (cytokine)	1evs
		Guess	0.654	Human interleukin-6 (cytokine)	1alu
		Guess	0.740	Nk-Lysin From *Sus scrofa* – lipid metabolism / xenobiotic metabolism / defense response to fungi	1nkl
		Guess	0.776	Human interleukin-19 (cytokine)	1n1f
		Guess	0.780	Human obesity protein, leptin (cytokine)	1ax8
		Guess	0.789	Pi3-kinase p85 alpha subunit *Bos taurus*	1qad
Frame 3	BLASTp	-	2.4	Hypothetical protein b2983 *E.coli* -A polysaccharide biosynthesis protein	E65084
		-	2.4	Hypothetical protein yghQ *E.coli* -A polysaccharide biosynthesis protein	NP_755591
		-	2.5	Putative serine protease *E.coli*	YP_026192
Frame -2	Gen-THREADER	Low	0.266	D-amino acid aminotransferase *Thermophilic bacillus* D-alanine + 2-oxoglutarate = pyruvate + D-glutamate	1a0g
		Low	0.323	L-fuculose-1-phosphate aldolase *[E.coli]*- Fucose metabolism	2fua
		Low	0.387	Human eif4gii - eukaryotic initiation factor 4gii	1hu3
		Low	0.441	Core protein p3 of rice dwarf virus (rdv) - outer shell capsid protein	1uf2

Discussion

This study demonstrates differential expression of ESTs from a symbiosis-enriched library, in symbiotic versus aposymbiotic larvae. Expression was variable among years and samples but in all cases at least 1 symbiotic larval population displayed significantly higher expression than the aposymbiotic population. Though significant, the level of this increased expression was extremely small; there was often just a one-cycle amplification difference between the two larval types.

Ambiguity arose when attempting to identify the differentially expressed sequences using bioinformatics. Of the six ESTs chosen for analysis, none showed clear homology to any known sequence in the databases, at either the amino acid level or the folded structure level. Neither did any of the sequences possess an amino acid motif to suggest protein function. However, of the results that were obtained, some interesting trends are apparent. Database search hits for five of the ESTs (#1, 66, 89, 158, & 165) include members of protein families which are either clearly involved in the onset of symbiosis and/or host defense against pathogen attack, or could theoretically function within the established symbiotic association.

The two top search hits for EST 1 were to an endoglucanase and firefly luciferase. Over 20 beta 1, 4 endoglucanses (cellulases) have been isolated from animals including nematodes, termites and molluscs (Wang et al. 2003; Watanabe and Tokuda 2001). Numerous examples exist of cellulases functioning in the onset of symbiosis in plant-microbe associations (Caldelari et al. 2003; Mateos et al. 2001; Mateos et al. 1992). Recently, a member of another glycosyl hydrolase family, a chitinase, was isolated from the cnidarian *Hydractinia echinata*. The expression pattern of this gene suggests a double role for that enzyme, pattern formation and host defense against fungal and nematode pathogens (Mali et al. 2004). Interestingly, the symbiont in the present study, a dinoflagellate, has a cell wall composed of cellulose (Taylor 1987). In

this association it is possible that a host cellulase could be utilized as a defense mechanism, for feeding, or for ridding the cell of incompatible or nonviable symbionts.

Firefly luciferase, the other top threading hit for EST 1, belongs to the Acyl-CoA synthetase family. While there is no known symbiosis function for luciferase itself, other members of the family do engage in related functions. The acyl-CoA synthetases include, along with the firefly (beetle) luciferases, long-chain acyl-CoA synthetases, acetate CoA synthetases, 4-coumarate:CoA ligases (4CL), and aromatic acid synthetases. Beetle luciferases are most closely related to the 4CLs which are involved in the biosynthesis of plant phenylpropanoids; important pathogen defense agents. Gene expression of 4Cl has been shown to be sharply upregulated (and localized around the necrotic spot) in parsely leaves under attack by fungal pathogens (Schmelzer et al. 1989).

The search hit with the lowest E-value for EST 66 was tartrate dehydratase. This enzyme belongs to the Fumarase Class I superfamily. Fumarases are tricarboxylic acid cycle enzymes that catalyze the interconversion of fumarate to L-malate. In cnidarian-dinoflagellate symbioses, fumarate is among the organic acids translocated from the symbiont to the host (Muscatine 1980; Trench 1979). And in *E.coli*, superoxide radicals have been shown to cause an increase in the expression of certain fumarase genes (Park and Gunsalus 1995).

Superoxide radicals also tend to induce expression of GST, a hit which appeared repeatedly during searches of crystal structure databases with EST 66 frame 1. GST constitutes an ancient superfamily found in prokaryotes and eukaryotes with limited overall sequence identity between members, yet a generally similar folded structure (Sheehan et al. 2001). GSTs are an important part of the cellular detoxification system which protects cells against reactive oxygen metabolites by catalyzing the conjugation

of activated xenobiotics to an endogenous water-soluble substrate, such as reduced glutathione. The superfamily is widespread and complex and consequently is subdivided into several classes. Two of the eukaryotic classes have been linked to herbicide resistance, pathogenic attack, and the onset of symbiosis in plants. In the *Eucalyptus globules – Pisolithus microcarpus* ectomycorrhiza, it has been shown that an auxin-induced *Eucalyptus* GST is upregulated during the onset of the symbiosis (Tagu et al. 2003). A separate study using proteome analysis of *Medicago trunculata* proteins during the onset of symbiosis with the arbuscular mycorrhizal fungus *Glomu mosseae,* showed an increase of GST in this association as well (Bestel-Corre et al. 2002).

As with EST 1, the EST 89 search resulted in a hit to an Acyl-CoA synthetase. This sequence generated the lowest E-value in the EST 89 results. Also intriguing were the several hits (in both reading frames) to lectins. Lectins are a class of structurally diverse proteins, found in a wide range of organisms, that bind carbohydrates and agglutinate cells. They serve primarily as recognition determinants and some of their functions include immune response against antigens, mediation of targeted cell death, and phagocytosis (Sharon and Lis 1989). Lectins have long been implicated in mediating the attachment of host and symbiont (Smith 1979) and experimental studies on the freshwater cnidarian *Hydra* supported this hypothesis in that plant-animal symbiosis (Meints and Pardy 1980). Many studies in leguminous plants have provided a wealth of evidence as well (Hirsch 1999; Kalsi and Etzler 2000; Sharma et al. 1993). Recently, a D-galactose binding lectin from the cnidarian *Sinularia lochmodes* (an octocoral) was isolated, characterized, and localized to the animal's nematocysts as well as to the surface of its symbiotic dinoflagellate, *Symbiodinium* sp., suggesting the presence of a lectin-mediated interaction between the two (Jimbo et al. 2000).

The best hits for EST 158, a human and a mouse protein kinase, came at the amino acid sequence level. Kinases engage in reversible phosphorylation, a common strategy used to control the activity of proteins in eukaryotic cells. They mediate signal cascades by phosphorylating serines, threonines or tyrosines on target proteins. Complex and organized networks of signaling pathways help coordinate the cell's activities, drive the cell cycle, and relay signals into the cell from the cell's environment (Alberts et al. 1994). The kinases bearing similarity to EST 158 are involved in programmed cell death (apoptosis), a cellular mechanism used widely by organisms, including cnidarians, for a multitude of reasons including roles in development, symbiosis, and host defense (DosReis and Barcinski 2001; Dunn et al. 2002; Foster et al. 2000; Mali and Frank 2004; Seipp et al. 2001).

The lack of an open reading frame in any of the EST 165 translations suggests that this portion of the cDNA sequence may be part of the 3' or 5' untranslated region. The best search hit for EST 165 was a D-amino acid aminotransferase. This enzyme participates in the reversible conversion of the amino acid D-alanine to pyruvate. In cnidarian-algal symbioses, studies have suggested that the presence of host free amino acids stimulates the symbiotic dinoflagellate to release its photosynthetic products to the host (Gates et al. 1999; Gates et al. 1995). While there is controversy surrounding this hypothesis (Cook and Davy 2001; Wang and Douglas 1997; Withers et al. 1998), one study has shown that increasing alanine concentration does cause a significant increase in the release of fixed carbon from the symbiont (Cook and Davy 2001).

EST 165 searches also generated several hits to cytokines. Cytokines are small, secreted, soluble glycoproteins found in all animals that function in intercellular signaling and regulate a broad spectrum of biological activities like growth, differentiation, and defense. Two of these hits were to IL6 and one, oncostatin m, is a part of the IL6 subfamily. IL6 is a multi-functional cytokine which is the central regulator of the host defensive mechanisms; its role is to stimulate a wide range of

target cells against hostile agents (Silvennoinen and Ihle 1996). IL6 production is increased in response to stress reactions like bacterial infections, whereupon it coordinates the immune response, acute phase reaction, and is a main regulator of the systemic inflammatory response. Numerous studies have found an increase in IL6 production and activity in animals upon infection with alveolate intracellular parasites such as *Plasmodium* and *Toxoplasma* (Nagineni et al. 2000; Seixas et al. 2001). Symbiotic dinoflagellates also belong to this group of evolutionarily related protists (Bhattacharya et al. 2003). IL19, another of the cytokine threading hits, is part of the IL10 subfamily; this family functions in regulation of the immune and inflammatory responses as well.

While none of the six sequences investigated showed homology with any protein family currently known, search algorithms based on primary or secondary structure yielded interesting possibilities in most cases. The paucity of lower invertebrate, and in particular cnidarian, sequence data makes it necessary to rely on the more highly conserved secondary structure of proteins for similarity comparisons. And while the database of protein crystal structures is growing, it is still much smaller than the database of amino acid sequences. With the acquisition of entire ORFs for each of these ESTs and rapidly expanding sequence databases, the identity of these symbiosis-upregulated *F. scutaria* genes will become increasingly clear.

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CHAPTER 5

DISCUSSION

The onset of symbiosis as described in many other mutualistic and pathogenic intracellular associations results in a multitude of biochemical and molecular responses both from the host and symbiont. These responses are often evident in the first few days of the association. However, in cnidarian – dinoflagellate associations nothing is known at these levels about host or symbiont response to the onset of symbiosis. In the association between the scleractinian coral *Fungia scutaria* and the dinoflagellate *Symbiodinium* C1f, relatively little host response appears to be occurring by 2 or 3 days into the symbiosis.

Very few symbiosis-induced translational changes were found to be occurring in *F. scutaria* larvae. Only one symbiosis-specific change in the larval profile of standing-stock proteins could be detected at 2 and 3 days post-infection. All attempts to identify this protein spot failed due to an inability to recover sufficient amounts of the protein from polyacrylamide 2-dimensional gels. Additionally, it seems unlikely that the larvae have begun facilitating inorganic carbon transfer to the symbiont at this early stage. No expression of the CA isoform found in an adult sea anemone to be functioning in inorganic carbon supply to the symbiont could be detected in the coral larvae, although expression of a phylogenetically related isoform was found expressed in coral adults. The CA isoform being expressed by the larvae did not vary in expression level as a function of the early symbiotic state and is more similar to membrane-bound forms of the enzyme which have not been implicated in symbiosis.

There does seem to be symbiosis-induced gene expression occurring in 5 day-old larvae (2 days post-infection) collected in 1998, as indicated from the primary screen

of the subtracted library. The secondary screen of the six ESTs chosen for examination, and performed on the same larval sample used to construct the library, showed two of the sequences (EST 1 & 135) to be expressed only in the symbiotic larvae, one of the sequences (EST 89) to be differential, though the small "n" value resulting from sample shortage prevents establishment of significance, and another of the sequences (EST 158) to be significantly differential in expression level at that point in time. Of the remaining two sequences examined: one (EST 66) appears equally expressed in both symbiotic and aposymbiotic larvae and the other (EST 165) was un-testable at that point in time due to a shortage of 5 day-old 1998 larval sample. However, all of the EST fragments were found to be expressed significantly more in symbiotic larvae at some point in time during the early days of the symbiosis, though for most the significance occurred in samples other than the one used to create the subtracted library.

The evidence for symbiosis-induced gene expression in the host, while there, is not overwhelming. The remainder of the data obtained from real-time quantitative PCR suggests that the differences in gene expression between symbiotic and aposymbiotic larvae, though significant at times, are miniscule and highly variable; often there is only a 100-fold (1 PCR cycle) difference in expression between the two symbiotic states. Additionally, the level of expression for each EST examined, in both aposymbiotic and symbiotic larvae, was very low; amplification of all but one of the sequences (EST 158) occurred towards the end of the PCR reaction (cycles 35-39). These issues present challenging obstacles when attempting to confirm differential expression using real-time quantitative PCR, as well as in identifying the differential sequences. Low expression levels decrease the success rate of RACE by PCR, a technique often used to obtain complete open reading frames. Without the complete open reading frame, particularly when dealing with lower invertebrate sequences, homology is more difficult to ascertain.

Perhaps the difficulties encountered lie not in the lack of a host response but in the size and location of the response. Each individual larva has an average of only 25 symbionts; a maximum of 25 larval cells directly engaged in symbiosis. The translational and transcriptional changes occurring in those larval cells could easily be obscured by the lack of changes occurring in the remaining thousands of larval cells not exposed to a symbiont. Use of a population further muddies the water. The range of natural variation in transcriptional or translational processes occurring in a population of these larvae is unknown and may be very great, further obscuring any symbiosis-specific changes. The symbiotic populations used, furthermore, were not wholly symbiotic. In all cases, at least 10% of the "symbiotic" population remained aposymbiotic. One possible way to circumvent these problems, at least when looking at the symbiosis-induced transcriptional changes revealed by the subtracted library, would be to do real-time quantitative PCR experiments on individual symbiotic and aposymbiotic larvae, as opposed to a whole population of larvae. Perhaps eliminating the variability introduced by using a population of larvae that are not 100% infected, combined with the sensitivity of PCR, would be enough to overcome the background noise produced by the uninfected cells in an individual larva. For translational questions, looking at newly-synthesized proteins in wholly symbiotic populations of larvae may be enough to discern any changes.

Another simple explanation for the apparent lack of response is that the host, at 2 or 3 days post-infection, is still oblivious to the presence of the symbiont. Though the host has responded by this point in time at a cellular level, phagocytosis of each dinoflagellate and production of the surrounding symbiosome membrane, these responses are attributable to feeding behavior. The acceptance or rejection of each individual alga, and the commencement of the mutualism, may occur later than the time points chosen for examination; at 2 and 3 days post-infection, the host may not yet be aware of the permanence of the association. As is often the case, the data presented in this thesis provide more questions than answers.

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