Unusual Mode of Symbiont Repopulation after Bleaching in *Anthosigmella varians*: Acquisition of Different Zooxanthellae Strains

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Abstract

Several Hadromerid sponge species harbor intracellular zooxanthellae, and these associations appear to be more stable than coral-zooxanthellae symbioses. In a series of transplant experiments, we observed bleaching and symbiont recovery in the sponge *Anthosigmella varians*. Six individuals of *A. varians* were transplanted from 20 m reefs in the Florida Keys to 1 m depth in Florida Bay. After 12 d, three sponges lost all pigmentation (the other half were obviously paler). Zooxanthellae were absent from sponge tissue in totally bleached individuals based on zooxanthella cell counts. Approximately 40 d after bleaching, we observed small localized circles of pigmentation. The circles continued to increase in diameter until the entire sponge surface had regained normal pigmentation. After recovery, algal densities were close to values recorded from healthy sponges. Recovered sponges harbored a zooxanthellae strain distinct from typical algal populations as evidenced by different RFLP patterns from amplified lsRNA gene sequences. It appears that repopulation did not occur from remnant algae, instead sequences from the novel symbiont matched sequences from a type of zooxanthellae commonly found

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in association with an anemone. *Anthosigmella varians* may provide a model system to test ecological and evolutionary hypotheses concerning the specificity and stability of algal-invertebrate associations.

1. Introduction

Many marine invertebrates, including reef-building corals, harbor uni-cellular dinoflagellate symbionts (zooxanthellae), which provide photo-synthetically derived, energy rich compounds utilized for host metabolism and growth (Davies, 1993; Trench, 1993). Ecological and physiological aspects of these associations have been studied extensively (Trench, 1971; Jokiel and Coles, 1990; Weis, 1991; Fitt et al., 1993; Gleason and Wellington, 1993; Yellowlees et al., 1993), but scientists have only recently been able to address questions concerning the evolution of these symbioses. Specifically, application of molecular techniques has improved the ability to distinguish among morphologically similar, but genetically distinct, dinoflagellate symbionts (Rowan and Powers, 1991a, b; McNally et al., 1993; Wilcox, 1997). It is now apparent that algal symbiont diversity is much greater than originally believed (Rowan and Powers, 1991a, 1992; Wilcox, 1997). High algal symbiont diversity, and the consequent increase in the potential number of interactions among host and symbiont species, highlights the need to understand the evolutionary processes that influence the composition and stability of algal invertebrate symbioses.

Although reef-building corals are the most obvious invertebrates that form symbioses with zooxanthellae, associations between zooxanthellar symbionts and several Hadromerid sponge species (especially individuals in the Clionid family) are quite common. Algal symbionts appear to strongly influence the physiology and ecology of their host sponges (Hill, 1996). Sponge-zooxanthellae associations, however, appear to be more stable than coral-algal symbioses since environmental factors responsible for loss of algal pigmentation in corals (i.e., bleaching), such as high temperature, increased UV light exposure, or changes in salinity (Jokiel and Coles, 1990; Glynn, 1991; Fitt et al., 1993; Gleason and Wellington, 1993), do not appear to affect sponges. For example, Caribbean sponges maintained healthy algal symbiont populations during a widespread coral bleaching event in 1987 (Vicente, 1990). It is unclear why sponges (or their algal symbionts) are more resistant to environmental fluctuations than are corals, however, relatively little is known

of the general biology of poriferan-zooxanthellae symbioses (Sara and Liaci, 1964; Vacelet, 1982; Wilkinson, 1987, 1992; Rosell and Uriz, 1992; Rosell, 1993).

Most investigators view bleaching as a pathological condition (e.g., Glynn, 1991). It has been proposed, however, that bleaching may be an adaptive response on the part of the host to changing environmental conditions (Buddemier and Fautin, 1993; Ware et al., 1996). According to these authors, corals bleach as the environment changes in order to acquire a new algal complement which is better adapted to current conditions. However, few studies have directly examined the 'adaptive bleaching' hypothesis, due in part to the lack of experimental systems in which the assumptions and predictions of the hypothesis can be tested.

Here we report on a recently observed bleaching event and an unusual form of symbiont repopulation in the sponge *Anthosigmella varians* forma *incrustans*. When a typical coral recovers from a bleaching episode, pigmentation appears to increase more or less uniformly. In contrast, reinfection in our experimental sponges was first noticeable as small localized circular patches of dark pigmentation, which increased in diameter until the entire sponge had regained pigmentation. More important, however, was our discovery that *A. varians* acquired a type of algae during recovery that was distinct from the symbiont with which it is normally associated. These findings are discussed as they relate to the evolutionary biology of algal-invertebrate symbioses.

2. Materials and Methods

During the summer of 1994, six *Anthosigmella varians* forma *incrustans* individuals were transplanted from 20 m on the ocean side of the Florida Keys (Tennessee Reef – 24°45'N; 80°45'W) to 1 m depth in the Florida Bay (adjacent to the Key Largo Marine Research Laboratory – 25°06'N; 80°27'W). These sites differ in many ways including: nutrient levels, salinity, temperature, light levels, and wave energy (W. Fitt, pers. comm.). After 2 d of exposure to ambient illumination, sponges were transferred to a shaded position. We measured zooxanthella density on transplanted sponges at two times, immediately after bleaching and after full-recovery, using the procedure described in Hill (1996). We also measured zooxanthella densities on 8 healthy (i.e., unmanipulated) sponges that were collected from 20 m on Tennessee Reef.

One affected sponge was chimeric: one half of its tissue did not bleach while the other half bleached and recovered. We collected two tissue samples (1 cm^2) from the bleached/recovered portion of this sponge, and three samples from the region that had never lost its zooxanthellae. Tissue sections were ground with an ice-cold mortar and pestle in the presence of algal isolation buffer (IB: 0.4 M NaCl, 10 mM EDTA, 20 mM Tris-HCl, pH 7.6, 8 mM DTT,

0.05% (v/v) Triton-X100), and the resulting slurry was transfered to a 1.5 ml microfuge tube. Algal cells were separated from the animal tissue by repeated centrifugation and rinsing with IB, until a uniformly dark pellet was recovered. Total nucleic acids were extracted from algal cells following standard procedures (Coffroth et al., 1992). We amplified the 5' end (900 bp) of the large-subunit ribosomal RNA gene (lsRNA) of the algae using standard PCR techniques and the primers ls1.5 (5' CGCTGAAATTAAGCATATAAGTAAG 3') and ls1.3 (5' AACGATTTGCA CGTCAGTATC 3'), and used the restriction enzymes *Hae*III and *Cfo*I to digest the PCR products. The resulting restriction fragment length polymorphism (RFLP) generated by these two enzymes can distinguish between the major clades of *Symbiodinium*, as well as among algae within a clade. This same methodology (PCR amplification followed by restriction digests) was used on the algae *Symbiodinium microadriaticum* (cultured from *Cassiopiea xamanchana*) and *S. bermudense* (cultured from *Aiptasia pallida*).We also amplified and cut algal DNA from one *A. varians* forma *incrustans* collected at 20 m, and one *A. varians* forma *varians* collected off of Long Key, FL (1 m). These sponges served as unmanipulated 'controls' for the algae typically associated with *A. varians* throughout its habitat range.

Finally, we sequenced the 5' end of the PCR products to determine whether RFLP patterns represented the same sequence and to assess the evolutionary affinities of the sponge symbionts. Previously obtained sequence data from zooxanthellae collected from *Montastrea franksii, Agaricia fragilis,* as well as cultured *Symbiodinium bermudense, S. microadriaticum, S. pulchorum, S. pilosum* and two free-living *Gymnodinium* species were used to construct a phylogenetic tree using maximum likelihood analysis. Phylogenetic trees were estimated using PAUP* (v4.054d; Swofford, 1997) with Felsenstein's (1984) model of molecular evolution. Rate heterogeneity was incorporated using a discrete gamma model of among site rate variation with four rate classes (Swofford et al., 1996; Yang, 1996). All substitution and rate parameters were estimated simultaneously using the successive approximation approach outlined by Swofford et al. (1996).

3. Results

Approximately 12 d after transplantation, half of the sponges had lost all pigmentation while the other three were obviously paler, and 40 d after bleaching sponges began to show signs of increased pigmentation. Zooxanthellae appeared to be absent from sponge tissue in the totally bleached *Anthosigmella varians* individuals (Table 1). {Total darkness has also been observed to cause total loss of zooxanthellae in this species (MH pers.

Table 1.   Average zooxanthella densities measured from transplanted and unmanipulated
            control *Anthosigmella varians*. Two measurements were made on transplanted
            sponges: the first was made immediately after bleaching (after 12 d), the second
            was made 90 d post transplantation (after regaining pigmentation). There were
            no significant differences in algal density between control and recovered *A.
            varians* (students two-tailed t-test; P>0.25).

	Average zooxanthella densities ($\times 10^6$ cells cm^{-2}; $\pm$ SE)
Bleached sponge, N = 4	0
Recovered sponge, N = 3	1.05 ($\pm$ 0.66)
Control sponge, N = 8	0.86 ($\pm$ 0.05)

obs.).} The manner of re-infection was unusual for this sponge in that it was
localized to small circles (Fig. 1) which increased in diameter throughout the
summer until the entire surface of the sponge had regained normal
pigmentation. Zooxanthellae cell counts of these recovered individuals
indicated that symbiont densities were not significantly different from values
recorded from healthy sponges (Table 1).

The symbiont RFLP pattern from the 'normal' sections of the chimeric sponge
matched the symbiont RFLP pattern from unbleached *A. varians* collected from
the reef and bay (Fig. 2; Lanes 4–8). 'Recovered' sections of the chimeric sponge,
however, harbored zooxanthellae with a RFLP pattern identical to that from
zooxanthellae usually associated with the anemone *Aiptasia pallida* (Fig. 2;
Lanes 2, 3, and 10). Interestingly, there was an *A. pallida* individual growing
immediately adjacent to the recovering sponge (see lower left portion of sponge
– Fig. 1). Phylogenetic analysis of algal sequences revealed that the recovered
sponge was infected by a strain of zooxanthellae distantly related to the algae
normally associated with the sponge, but very similar to cultured symbionts
isolated from *Aiptasia* anemones (Fig. 3).

4. Discussion

This is the first report of environmentally-induced bleaching and recovery in
a tropical sponge. More significant, however, was our observation of apparent
replacement of the typical algal symbiont with a different species of algal
symbiont under natural conditions (Fig. 2). Although this has been
hypothesized to occur (Rowan and Powers, 1991b; Buddemeier and Fautin, 1993;

Figure 1. Recovering *Anthosigmella varians* approximately two months after bleaching. The sponge is approximately 12 cm in length along the fishing line. Note "freckle-like" round brown patches on the surface of the sponge (Z). These are areas of reinfection. Notice *Aiptasia pallida* at lower right edge of sponge (arrow).
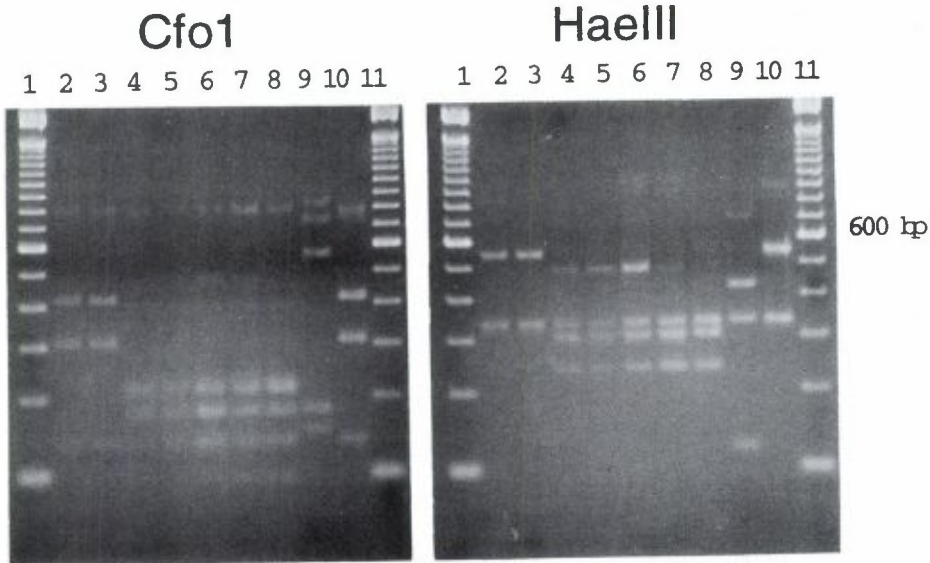
Figure 2.   Restriction enzyme digests of nuclear large ribosomal subunit PCR products amplified from isolated algal symbionts. Lanes 1 and 11: 100 bp ladder. Lanes 2 and 3: algae from recovered *Anthosigmella varians*. Lanes 4–6: algae from unbleached portion of same *A. varians* individual. Lane 7: algae from *A. varians* forma *incrustans* collected at 20 m. Lane 8: algae from *A. varians* forma *varians* collected in Florida Bay. Lane 9: cultured *Symbiodinium microadriaticum*. Lane 10: cultured *S. bermudense*, originally collected from *Aiptasia pallida*.
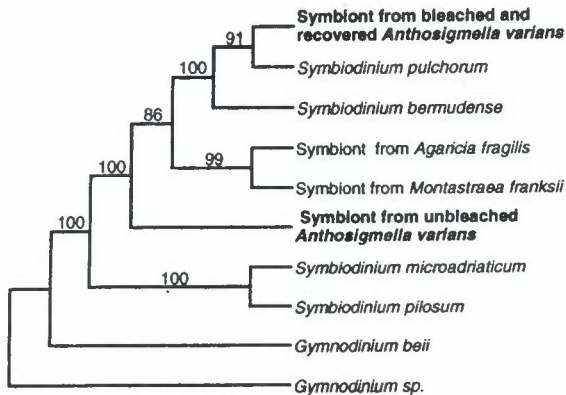

Figure 3.   Maximum likelihood phylogenetic analysis of the 5' end of the large ribosomal subunit recovered a single well supported tree. It is clear that bleached specimens of *Anthosigmella varians* became colonized by an alga quite distinct from the symbiont normally found in this sponge species. Numerals above the branches represent the number of 100 bootstrap replicates that contained each clade.

Ware et al., 1996), there have been no studies of symbiont replacement under field conditions.

Replacement of the normal strain of zooxanthellae may have occurred from 1) remnant algal cells that were present in the sponge before bleaching and/or 2) free-living algal cells that were present in the surrounding environment. The following evidence lends support to the second explanation. During typical bleaching events involving corals, algal densities remain relatively high (e.g., Fitt et al., 1993; Gleason and Wellington, 1993), and recovery most likely stems from growth of this residual population. In this study, however, there were no detectable algal cells after bleaching in *A. varians* (Table 1). In addition, different sized patches of newly colonized sponge tissue (i.e., circles of different diameters – Fig. 1) may have represented zooxanthella populations initiated from independent colonization events that had been growing for different periods of time. Finally, there was no evidence of sequence heterogeneity in the RFLP patterns or sequencing reactions obtained from any of the unbleached sponge samples, suggesting the presence of only a single algal lsRNA genotype in the host prior to bleaching. In cases where the restriction fragments sum to a molecular size greater than the undigested amplified product, it appears that the PCR products were only partially digested (perhaps due to a restriction site that was relatively inaccessible). However, all RFLP patterns have been consistently reproduced, and sub-cloned PCR products always recovered a single sequence (data not shown). For example, RFLP patterns produced from cultured *Symbiodinium microadriaticum* (Lane 9; HaeIII digest in Fig. 2) sum to more that the undigested product. However, this pattern (from cultured algae) should contain only a single type of zooxanthellae, and therefore suggests that one of the restriction sites is difficult to cut.

For algal repopulation to proceed from external sources of algae, environmental pools of symbiotic zooxanthellae must be available to colonize bleached adult hosts. Although aposymbiotic larvae clearly acquire symbionts from the environment, it is not clear that bleached adults also have this capability. In addition, little is known of the natural density of symbiotic zooxanthellae in plankton communities, however, several reports indicate that these types of algal cells are continuously released into the environment (e.g., Steele, 1975; Stimson and Kinzie, 1991). Recently, McCloskey et al. (1996) found that *Anthopluera elegantissima* expelled a sizeable fraction of its zooxanthellae, and Matuyama and Heslinga (1997) found that *Tridacna derasa* released approximately 6.7% of its newly produced algal population daily. Of particular importance, Hoegh-Guldberg et al. (1987) demonstrated that several cnidarian species expel zooxanthellae in the field. Thus, symbiotic zooxanthellae may be common in the plankton, and expelled algae may be able to repopulate recently bleached adult hosts in the field.

Buddemeier and Fautin (1993) suggested that when environmental conditions become stressful, host invertebrates will expel and replace their resident algae with a symbiont more adapted to current environmental conditions. It is unlikely, however, that the replacement of the resident algal strain in *A. varians* represents a case of 'adaptive bleaching'. *Anthosigmella varians* occurs as two different morphotypes, an encrusting morphology (forma *incrustans*) found in open reef environments and a branching morphology (forma *varians*) found in shallow, lagoonal habitats. These environments differ significantly in terms of salinity, water clarity, nutrient concentrations, sedimentation and wave action. However, *A. varians* collected in either environment contain indistinguishable algal symbionts (on the basis of lsRNA sequences). Yet, when *A. varians* forma incrustans was transplanted from the open reef environment to Florida Bay, bleaching and recovery resulted in the replacement of the resident algal strain with another type of zooxanthellae. The reinfecting species may have come from another, unidentified, host species. In any case, our observation does not support the adaptive bleaching hypothesis (Buddemeier and Fautin, 1993) since reinfection should have involved the type of zooxanthellae normally found in *A. varians* forma *varians* (which according to the adaptive bleaching hypothesis, should represent the best combination of symbiont and host in that environment). Nonetheless, *A. varians* provides a model system to test hypotheses concerning ecological and evolutionary aspects of algal-invertebrate associations.

Acknowledgments

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