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Effect of temperature on bleaching of the coral *Oculina patagonica* by *Vibrio* AK-1

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ABSTRACT: Laboratory aquaria experiments demonstrated that *Vibrio* AK-1 caused rapid and extensive bleaching of *Oculina patagonica* at 29°C, slower and less complete bleaching at 25°C and 20°C, and no bleaching at 16°C. The effects of temperature on the bacteria-induced bleaching experiments in aquaria were consistent with the natural bleaching of *O. patagonica* in the Mediterranean Sea. *In situ* bleaching increased rapidly from late May to September, following the rise of surface seawater temperature, which reaches 29°C in August. During the winter, when the seawater temperature drops to 16°C, most of the bleached coral colonies recovered. *Vibrio* AK-1 was isolated from all bleached corals examined in the summer, but could not be isolated from healthy (unbleached) corals in the summer or from bleached and unbleached corals in the winter. The mechanism by which increased temperature causes the coral bleaching by *Vibrio* AK-1 is at present not clear. The bacteria grow in the laboratory relatively rapidly at 16°C (doubling time 2 h), indicating that bacterial growth is probably not the critical factor. We suggest that temperature-regulated factors affecting bacterial virulence may play a role in the bleaching process.

KEY WORDS: Coral · Bleaching · Bacterial infection · *Vibrio* · *Oculina* · Mediterranean · Temperature

INTRODUCTION

The symbiotic association between coral hosts and their photosynthetic microalgal endosymbionts, the zooxanthellae, is the key to scleractinian coral success in the coral reef ecosystem. Coral bleaching results from the disruption of this symbiosis, which leads to loss of the pigmentation, either from a reduction in zooxanthellae density and/or from decreased concentration of the photosynthetic pigments in the algal cells (Gates et al. 1992, Buddemeier & Fautin 1993, Brown et al. 1995, Brown 1997). Coral bleaching events can result in coral mortality, since a major portion of the coral's nutrition comes from the photosynthetic products of the algae (Muscatine 1990, Brown et al. 1995). Such events, of unprecedented frequency and global extent, were reported in the 1980s and 1990s (Glynn 1991a, Goreau 1994, Hoegh-Guldberg & Salvat 1995). It is not yet possible to determine conclusively that bleaching episodes and the consequent damage to reefs is due to global climate change, but there is firm evidence that the coral reef ecosystem is sensitive to increases in seawater temperature (Jokiel & Coles 1990, D'Elia et al. 1991, Glynn 1991b, Brown et al. 1996, Brown 1997). Although such increases are one of the most likely causes of most coral reef bleaching episodes, many bleaching events occurred with no evidence of temperatures higher than normal (Fisk & Done 1985, Glynn 1991a, Atwood et al. 1992). A number of other causes have been suggested for bleaching, including increased irradiance alone or combined with increased temperature (Glynn et al. 1992, Gleason & Wellington 1993, Glynn 1993, Shick et al. 1995, Brown 1997) and bacterial infection (Kushmaro et al. 1996, 1997). Despite its ecological impact, the biological mechanisms responsible for coral bleaching remain unknown.

Recently, we reported that bleaching of the coral *Oculina patagonica* from the Mediterranean Sea is the

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result of a bacterial infection (Kushmaro et al. 1996, 1997). The causative agent, *Vibrio* AK-1, was obtained in pure culture and shown to cause bleaching in controlled aquaria experiments. Furthermore, we have shown that bacteria-induced bleaching by *Vibrio* AK-1 can be inhibited by antibiotics.

The purpose of the present study was to determine and compare the effect of seawater temperature on (1) bleaching of the coral *Oculina patagonica* in the Mediterranean Sea and (2) bleaching of the coral in experimental aquaria induced by *Vibrio* AK-1. In this study we suggest an additional hypothesis for coral bleaching: namely, that elevated temperatures can cause the coral-bleaching pathogens to become more virulent.

MATERIALS AND METHODS

Microorganism, media and growth conditions. *Vibrio* AK-1 was isolated from bleached coral samples as described previously (Kushmaro et al. 1996, 1997). Forty-two bleached and 34 unbleached corals were sampled during the winter (January–March, see Table 1) and summer (June–September, see Table 1), diluted into sterile seawater, streaked onto TCBS Agar (Difco Laboratories, Detroit, MI, USA) and incubated at 30°C for 3 d. The strain was routinely cultivated in MB medium (0.9% NaCl, plus 1.8% Marine Broth, Difco Laboratories) or on MB agar (MB medium solidified with 1.8% Difco Bacto Agar). Unless stated otherwise, growth was carried out in 125 ml flasks containing 10 ml MB medium, with shaking (150 strokes min^{-1}), at 30°C. Growth rate constants were calculated from growth curves at 15, 20, 25, 30 and 35°C. Bacterial growth was initiated by introduction of a 1% inoculum obtained from a starter culture that was grown in 10 ml of MB medium in a 125 ml flask overnight at the corresponding temperature. Bacterial growth was followed by determination of turbidity in a Klett-Summerson photometer and by determination of viable counts by plating an appropriate dilution on MB agar of 5 replicates. Growth rates were also determined in 5 replicates in 1.5 ml Eppendorf tubes containing 0.5 ml of MB, coral mucus and coral tissue media. Mucus medium consisted of mucus removed from fresh corals, and tissue medium was produced by ground corals. In both cases, the medium was sterilized by filtration through a 0.2 µm filter.

Analysis of bleached corals in the sea. Line transects (Loya 1972) were used to assess the percentage of bleaching of the coral *Oculina patagonica* at 5 chosen sites along the Israeli Mediterranean coastline (Ashkelon, Bat Yam, Tel-Aviv Yafo, Hadera and Haifa). At each site percent bleaching within the coral population was estimated by counting the number of bleached and non-bleached coral colonies underlying the line (10 transects of 10 m each, at depths of 1 to 6 m). Seawater temperatures along the Israeli coastline for the years 1994 to 1996 were obtained from the Israeli Meteorological Center.

Collection and maintenance of corals. Intact colonies of *Oculina patagonica* were collected from depths of 1 to 3 m along the Mediterranean coast of Israel during June 1996. Seawater temperature at the time of collection was 25 to 26°C. Within 1 to 2 h after collection, each colony was split into several fragments and placed into 2 l aerated aquaria containing filtered seawater (0.45 µm) maintained at 25°C. The aquaria were illuminated with a fluorescent lamp under a 12 h light:12 h dark artificial lighting regime at an intensity of approximately 90 µEinst m^{-2} s^{-1}. Coral fragments were allowed to recover and regenerate for 15 d before the start of each experiment. If any piece failed to heal (complete cover of damaged skeleton by new tissue), it was discarded and not used in any experiment. For experiments at temperatures other than 25°C, the recovered corals were transferred to aquaria at the desired temperature and maintained for an additional 10 d for acclimation.

Laboratory aquaria bleaching experiments. In the first set of experiments, *Vibrio* AK-1 was inoculated directly into the water of 2 l aerated aquaria containing corals that had been acclimated for an additional 10 d to different temperatures, without removal of the corals. For each temperature (16, 20, 25 and 29°C), 3 aquaria, each containing 5 or 6 colonies of *Oculina patagonica* corals, were inoculated with *Vibrio* AK-1 to a final bacterial cell density of 10^6 cells ml^{-1}. Four control aquaria, 1 per each temperature, were inoculated with sterile medium in place of bacteria.

In the second set of experiments, 10 µl containing 1.2×10^4 cells of *Vibrio* AK-1 was placed directly onto each of 18 healthy corals, which were then replaced in 3 separate 2 l aerated aquaria (6 corals per aquarium) maintained at 16, 23 and 29°C. For controls, 18 additional corals were inoculated with 10 µl of sterile medium, and placed in 3 separate aquaria under the same temperature regimes.

Determination of bleaching. Percentage of bleaching in the coral population was determined qualitatively by visual observation of corals in the laboratory and in the field. For this study, a coral was considered 'bleached' when at least 10% of its surface appeared white. Number of algae were determined directly from histological sections of 42 bleached and 34 unbleached coral tissue samples. Coral samples were fixed in 4% formaldehyde solution in seawater for 24 h, rinsed in fresh water and transferred to 70% ethanol. Decalcification was carried out using a solution of 1:1 (v:v)

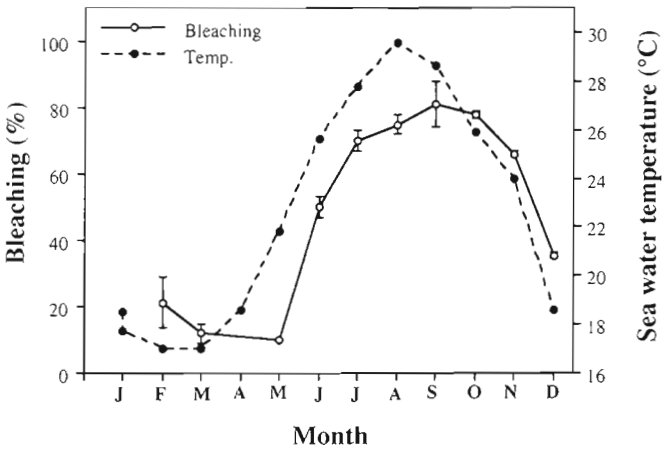

Fig. 1. *Oculina patagonica*. Mean percent ± SD of bleaching of the coral population in the Mediterranean Sea from 1994 to 1996 in relation to seawater temperature

formic acid (initial concentration 42.5%) and sodium citrate (initial concentration 20%) for 24 h. The tissue was then rinsed in water and transferred into 70% ethanol. The dehydrated tissue was embedded in paraffin. Sequential cross-sections of the tissue were mounted on glass slides, stained with hematoxylin and eosin, and examined under a Nikon light microscope at magnification of 1000× using a calibrated eyepiece micrometer.

RESULTS

***In situ* bleaching as a function of seawater temperature.** Most of the bleached colonies were found at depths of 1 to 3 m. The number of colonies showing distinct bleaching areas increased rapidly from late May to September following rising seawater temperature (Fig. 1). From July to October, 70 to 80% of the colonies showed bleaching. A maximum seawater temperature of 29°C was recorded in August. During the winter (with minimum temperature of 16°C), most of the bleached colonies recovered (Fig. 1). During the rise in seawater temperature from 20 to 29°C (April to August), the increase in percent bleaching in the coral population lagged 1 mo behind compared with the monthly temperature. Furthermore, a correlation of $r^2 = 0.8$ ($p < 0.001$) was found between seawater temperature and bleaching during the 3 yr study period (1994 to 1996), and a correlation of $r^2 = 0.9$ ($p < 0.001$) was obtained during the bleaching event from May to September (1994–1996). A typical example showing a progressive bleaching of *Oculina patagonica* colony collected from Bat Yam and maintained in an aquarium at 25°C is shown in Fig. 2. Histological sections of healthy corals contained $1.3 \pm 0.1 \times 10^6$ zooxanthellae cells cm^{-2}, whereas bleached corals contained $0.5 \pm 0.2 \times 10^6$ cells cm^{-2}.

Isolation of *Vibrio* AK-1 from *Oculina patagonica* during the winter and summer

The occurrence of *Vibrio* AK-1 in bleached and unbleached coral samples is presented in Table 1. In the summer, all 28 bleached coral samples examined yielded *Vibrio* AK-1, whereas *Vibrio* AK-1 could not be cultured from 24 healthy (unbleached) coral samples. *Vibrio* AK-1 could not be isolated from any of the 24 coral samples taken in the winter, regardless of the extent of bleaching.

Laboratory aquaria bleaching experiments

In the first set of experiments, we tested the effect of inoculating the water in the aquaria at different temperatures (Fig. 3). The corals in the aquaria maintained at 29°C showed an average of 44% bleaching 8 d after inoculation and reached 92% bleaching by 22 d. At 25°C and 20°C there was considerable bleaching, but at a slower rate and extent than at 29°C. At 16°C, the corals remained healthy with no sign of bleaching for 45 d, at which time the experiment was terminated. In control aquaria, where corals were held at each of the temperatures from 16°C to 29°C without inoculation of *Vibrio* AK-1, no bleaching was observed.

The second set of experiments tested the effect of direct inoculation of *Vibrio* AK-1 on healthy corals at different temperatures (Fig. 4). Using a constant inoculum of 1.2×10^4 bacteria, bleaching was first observed after 6 d at 29°C. On Day 11 and Day 22 at 29°C, bleaching was observed in 67% and 100% of the corals, respectively. At 23°C, bleaching was first ob-

Table 1 *Oculina patagonica*. Occurrence of *Vibrio* AK-1 in the coral during the winter and summer

Season	Coral condition[a]	Fraction of corals yielding *Vibrio* AK-1
Winter	Normal	0/10
	Bleached	0/14
Summer	Normal	0/24
	Bleached	28/28

[a]Pieces from different coral colonies were vortexed in sterile seawater and spread on TCBS agar. Bacterial colonies morphologically similar to *Vibrio* AK-1 were further examined by biochemical tests and antibiotic sensitivity patterns

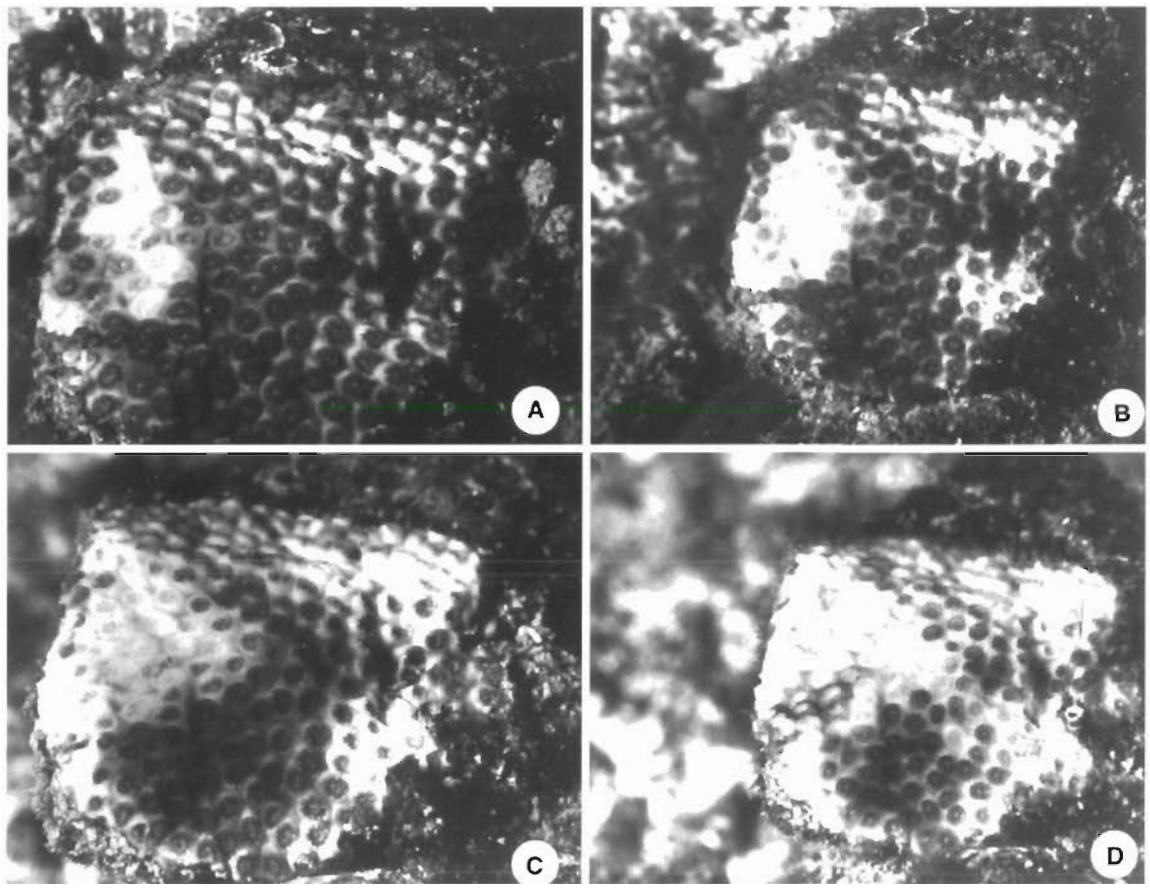

Fig. 2. *Oculina patagonica*. Series of photographs showing the progressive bleaching of the coral in an aquarium. (A) Initial bleaching, (B) bleaching after 1 wk, (C) bleaching after 2 wk, and (D) bleaching after 4 wk

served on Day 11 and reached 67% after 22 d. At 16°C, no bleaching was observed throughout the experiment. Controls, containing no added bacteria, showed no bleaching at any of the temperatures for 22 d.

Growth rate of *Vibrio* AK-1 as a function of temperature

Growth rate constants during the late exponential growth phase, from 10^6 to 10^8 cells ml^{-1}, are presented in Fig. 5. The growth rate constants, calculated from growth curves at 15, 20, 25, 30 and 35°C, were 0.24, 0.66, 0.92, 1.44 and 1.32 generations h^{-1}, respectively (in 10 ml MB medium) In order to measure the growth rates of *Vibrio* AK-1 in a medium which contained coral mucus as the sole nutrient, the experiment was scaled down to 0.5 ml total volume. Under these conditions the growth rate constants of *Vibrio* AK-1 were 1.8, 1.2 and 1.5 generations h^{-1} at 29°C in MB, mucus and tissue media, respectively. At 16°C, the growth rate constants were 0.53, 0.47 and 0.50 generations h^{-1} in MB, mucus and tissue media, respectively.

DISCUSSION

There have been a large number of studies indicating a correlation between increased seawater temperature and coral bleaching (e.g. Jokiel & Coles 1990, D'Elia et al. 1991, Glynn 1991b, Brown et al. 1996, Brown 1997). Furthermore, it has been shown that raising the water temperature experimentally in aquaria can cause such bleaching (Jokiel & Coles 1990, Glynn et al. 1992). This has led to the speculation that increased seawater temperature, resulting from global warming or El Niño events, is the direct cause of coral bleaching (Glynn 1991a). Direct cause implies that the increased temperature directly affects the zooxanthellae and/or the host coral in a manner that disrupts the symbiosis. However, coral bleaching is not necessarily the direct response to elevated seawater temperature. For example, Oliver (1985) showed that extensive

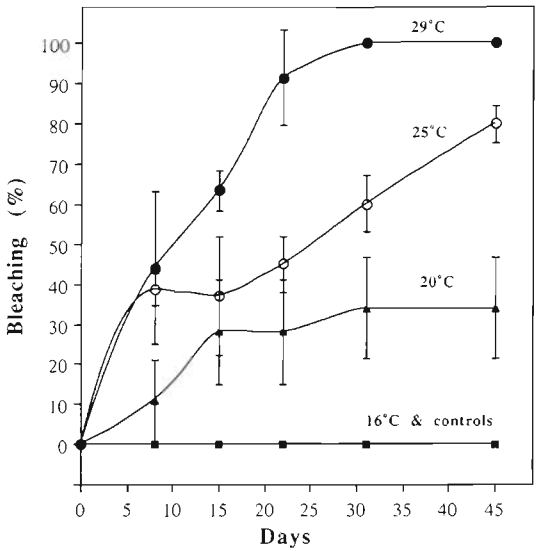

Fig. 3. *Oculina patagonica*. Bacterial bleaching of the coral in experimental aquaria at different temperatures. *Vibrio* AK-1 was inoculated into the water of 2 l aerated aquaria containing 5 or 6 corals. The final bacterial cell density was 10^6 cells ml^{-1}. For each temperature, 3 separate aquaria were used. Values for average percent bleaching (± SD) are plotted as a function of time. Control aquaria at each of the 4 temperatures were treated in exactly the same manner but with sterile medium in place of bacteria

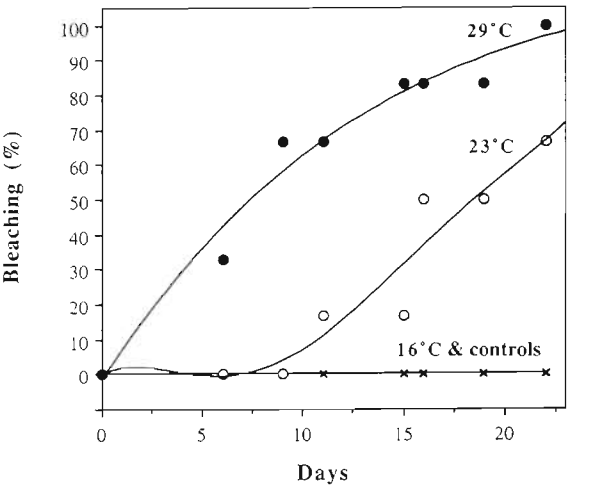

Fig. 4. *Oculina patagonica*. Bacterial bleaching of the coral in experimental aquaria at different temperatures by direct inoculation of *Vibrio* AK-1 onto the coral. Ten µl containing 1.2×10^4 of *Vibrio* AK-1 was placed on each of 18 healthy corals and the corals were then replaced in 3 separate aquaria (6 corals per aquarium) maintained at the indicated temperatures. Percent bleaching denotes the proportion of corals bleached out of the 6 corals observed in each aquarium at each temperature

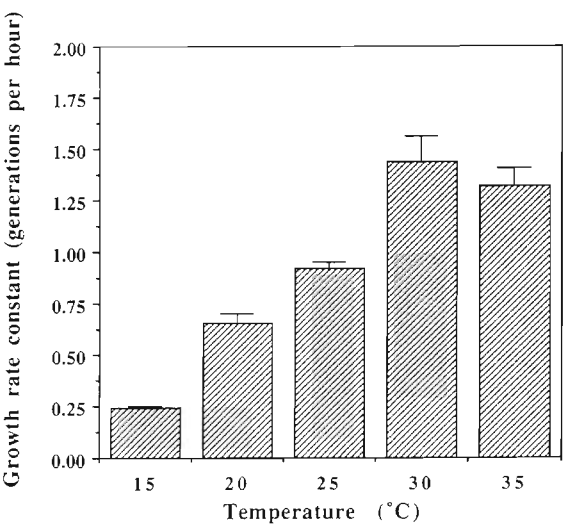

Fig. 5 *Vibrio* AK-1. Growth rate constants calculated from growth curves at 15, 20, 25, 30 and 35°C. Growth of *Vibrio* AK-1 was carried out in 125 ml flasks containing 10 ml MB, medium with shaking (150 strokes min^{-1}), and followed by determination of turbidity. Results are average ± SD of 5 replicates for each temperature

bleaching in the Great Barrier Reef (Australia) during the summer of 1982 was not associated with any major sea surface temperature anomalies. He further hypothesized that a synergistic effect between high temperature and high light or a water-borne pathogen could have been involved. Fisk & Done (1985) were unable to correlate bleaching in the Eastern Pacific with regional temperature anomalies. The patterns of coral mortality with depth and within colonies are in some respects consistent with solar radiation (Fisk & Done 1985). Several authors have reported on the patchy spatial distribution and the spreading nature of coral bleaching (Fisk & Done 1985, Oliver 1985, Jokiel & Coles 1990, Lang et al. 1992). Hayes & Bush (1990) suggested that the random mosaic pattern of bleaching within a coral colony is difficult to attribute solely to environmental variables such as seawater temperature. They showed that segments of colonies, rather than the entire colonies, responded to stress by expulsion of zooxanthellae. There was no consistency to the size of the bleached zones or to their location within the coral colony. Observations of progressive recovery in the coral colony have suggested that bleaching may have begun within a small zone and spread from there into adjacent areas of the colony (Hayes & Bush 1990). More recently, Rowan et al. (1997) have attributed mosaic bleaching patterns to irradiance influences.

In the present study, temperature dependence of bacteria-induced bleaching of *Oculina patagonica* was clearly demonstrated. The controls (no inoculum) showed no bleaching even at 29°C, whereas bleaching occurred even at 20°C in the presence of the pathogen

(Fig. 3). There are several possible explanations for the effect of temperature on the bleaching of *O. patagonica* by *Vibrio* AK-1. Temperature has often been shown to be one of the major factors correlated with the seasonal variation of bacterial abundance and specific growth rates in coastal ecosystems (Shiah & Ducklow 1994). For example, the growth of bacteria in the Chesapeake Bay (USA) is primarily limited by temperature during the cooler seasons when temperatures fall below 20°C (Shiah & Ducklow 1994). In the case of *Vibrio* AK-1, the simple effect of temperature on growth rate does not seem to be the critical factor. In spite of its potential for growth at 16°C (ca 0.5 generations h^{-1}), we were unable to isolate *Vibrio* AK-1 from corals in the sea during the winter, even from colonies that were partially bleached. It may be that during the winter, when seawater temperatures were low (with a minimum temperature of 16°C) *Vibrio* AK-1 enters into a viable but not-culturable state, as has been shown for other marine *Vibrios* (Oliver et al. 1995). Recovery from this state in natural environments may result from a rise in seawater temperature (Oliver et al. 1995).

Toren et al. (1998) demonstrated that the coral-bleaching pathogen *Vibrio* AK-1 adheres to its host coral, *Oculina patagonica*, in a specific manner. The adhesion is blocked by D-galactose and by very low concentrations of methyl-β–D-galactopyranoside. Adhesion did not occur when *Vibrio* AK-1 was grown at 16°C, regardless of whether the corals were maintained at 16°C or 25°C, whereas bacteria grown at 25°C adhered to corals maintained at 16°C or 25°C. Thus, the *Vibrio* AK-1 adhesin recognizes β-D-galactopyranoside residues on the coral surface, and production of the bacterial adhesin is temperature regulated. The inability of *Vibrio* AK-1 to induce bleaching in *O. patagonica* at low temperatures may be due to repressed synthesis of virulence factors, including adhesins that are strictly temperature regulated.

In general, climate-related increases in sea surface temperature can lead to higher incidence of waterborne infections and toxin-related illnesses, such as cholera caused by *Vibrio cholera* (Colwell 1996, Patz et al. 1996). A strong seasonality was demonstrated in the black-band disease in corals. This disease, caused by the cyanobacterium *Phormidium corallyticum*, is most prevalent in the summer and early fall when seawater temperatures are above 28°C (Edmunds 1991, Kuta & Richardson 1996, Bruckner & Bruckner 1997). Recently Richardson et al. (1998) have reported that coral tissue damage in the Florida Keys (USA) is caused by a bacterium of the genus *Sphingomonas*. In the present paper we further suggest that bacterial-induced bleaching in *Oculina patagonica* is temperature regulated. The fact that bacteria are the causative agents of at least certain coral diseases does not diminish the critical importance of environmental factors and their effect on the disease process. Thus, it is important both to discover the causative agents for coral diseases and to study the effect of environmental factors on the pathogen/host interaction.

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