Temperature-Regulated Bleaching and Lysis of the Coral *Pocillopora damicornis* by the Novel Pathogen *Vibrio coralliilyticus*

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Coral bleaching is the disruption of symbioses between coral animals and their photosynthetic microalgal endosymbionts (zooxanthellae). It has been suggested that large-scale bleaching episodes are linked to global warming. The data presented here demonstrate that *Vibrio coralliilyticus* is an etiological agent of bleaching of the coral *Pocillopora damicornis*. This bacterium was present at high levels in bleached *P. damicornis* but absent from healthy corals. The bacterium was isolated in pure culture, characterized microbiologically, and shown to cause bleaching when it was inoculated onto healthy corals at 25°C. The pathogen was reisolated from the diseased tissues of the infected corals. The zooxanthella concentration in the bacterium-bleached corals was less than 12% of the zooxanthella concentration in healthy corals. When *P. damicornis* was infected with *V. coralliilyticus* at higher temperatures (27 and 29°C), the corals lysed within 2 weeks, indicating that the seawater temperature is a critical environmental parameter in determining the outcome of infection. A large increase in the level of the extracellular protease activity of *V. coralliilyticus* occurred at the same temperature range (24 to 28°C) as the transition from bleaching to lysis of the corals. We suggest that bleaching of *P. damicornis* results from an attack on the algae, whereas bacterium-induced lysis and death are promoted by bacterial extracellular proteases. The data presented here support the bacterial hypothesis of coral bleaching.

Coral diseases have increased in frequency, intensity, and geographic extent over the last few decades (34, 35). On a global scale, the most serious destruction of corals is due to bleaching (20). Entire coral reef systems have been damaged following bleaching events (30). Coral bleaching is the disruption of symbioses between coral animals and their photosynthetic microalgal endosymbionts, the zooxanthellae (19). Loss of the zooxanthellae and/or their photosynthetic pigments results in fading or bleaching of the coral due to the low concentration of algal pigment and increased visibility of the white calcareous skeleton. If the process is not reversed within a few weeks or months, depending upon the specific coral species and conditions, the coral dies, since a major portion of a coral's nutrition comes from the photosynthetic products of the algae (14). In addition to eventually killing corals, bleaching affects coral populations by greatly reducing the reproductive capacity, reducing the growth rate, and increasing the susceptibility to secondary diseases (9, 38). Although most coral biologists have not considered bleaching a disease (18, 20, 33, 34), it fits precisely the definition of a disease—a process resulting in tissue damage or alteration of function, producing visible physiological or microscopic symptoms. Evidence showing that the coral bleaching disease can be considered an infectious disease is the subject of this report.

In general, coral bleaching occurs during the hottest period of the year (15, 18, 28) and is most severe at times of warmer-than-normal conditions (20). Also, a number of other environmental factors, such as decreased seawater temperature (31), increased irradiation (13), and reduced salinity (40), have been suggested to cause coral bleaching. In principle, the correlation between increased seawater temperature and infectious disease could be the result of increased sensitivity of the host to the pathogen, increased virulence of the pathogen, higher frequency of transmission, or a combination of these three things. In the case of bleaching of the coral *Oculina patagonica* by *Vibrio shiloi* in the Mediterranean Sea, the major effect of increased temperature is induction of bacterial virulence factors, including an adhesin that binds to a β-galactose-containing receptor on the coral surface (39), superoxide dismutase (1), and toxins that inhibit photosynthesis and bleach and lyse the zooxanthellae (3, 4).

How general is bacterial bleaching of corals? The consensus among coral biologists is that bleaching is the result of direct environmental stress, primarily temperature and/or light stress, of the coral, resulting in expulsion of the algae (20). Determining whether coral bleaching is the result of an infection or coral stress is crucial, because this information affects the design and interpretation of experiments and is fundamental to the development of technology to prevent or cure the disease. For example, if bleaching is the result of infection, then one of the target strategies for preventing the disease is to interfere with its transmission. Recently, it has been found that in the case of bleaching of *O. patagonica*, the winter reservoir of *V. shiloi* and a potential vector of the disease is the fireworm *Hermodice carunculata* (37). The data presented here show that *Vibrio coralliilyticus* is the etiological agent of bleaching of *Pocillopora damicornis* on coral reefs and thus provide support for the bacterial hypothesis of coral bleaching. Seawater temperature is the critical environmental parameter that determines the outcome of the infection.

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MATERIALS AND METHODS

Microorganisms, media, and growth conditions. *V. coralliilyticus* YB1 (= ATCC BAA-450) was isolated from *P. damicornis* as previously described (5, 6). *V. coralliilyticus* YB2, YB3, and YB4 were isolated from different colonies of bleached

or partially lysed *P. damicornis* on a reef near Eilat in the Gulf of Aquaba, Red Sea, during August 2001, as described previously (6). Bacterial concentrations were estimated by plating triplicate samples of appropriate dilutions. *V. coralliilyticus* YB2, YB3, and YB4 were each obtained in pure culture by picking a colony from a thiosulfate-citrate-bile-sucrose (TCBS) agar plate after 48 h of growth and restreaking it three times in order to purify the strain.

Strains were routinely cultivated in liquid MBT medium (1.8% marine broth, 0.9% NaCl, 0.5% Bacto Tryptone [Difco]) at 30°C. Liquid cultures were prepared in 125-ml flasks containing 10 ml of MBT medium inoculated with one colony, and they were incubated at 30°C with shaking at 160 rpm for 24 to 48 h. The strains were maintained on MB agar (1.8% marine broth, 0.9% NaCl, 1.8% Bacto Agar[Difco]). Stock cultures were maintained in 15% glycerol at −70°C.

Phenotypic and genotypic characterization of *V. coralliilyticus* strains. Carbon compound utilization tests were performed with Biolog GN2 microplates (Biolog Inc., Hayward, Calif.) as previously described (6). Biochemical tests were performed by using the API 20NE system (Biomerieux, Marcy l'Etoile, France). The standard protocol was used, except that the NaCl concentration in the media was adjusted to 3%. Protease activity was measured by the standard azocasein method (12). One unit of protease activity was defined as the concentration that yielded an A_{450} of 1.0 after 5 min of incubation at 37°C. The results were expressed in units of specific activity per unit of culture turbidity (A_{600}).

Genomic DNA was isolated from 2-ml overnight bacterial cultures by using a Wizard genomic DNA purification kit (Promega, Madison, Wis.). 16S ribosomal DNA (rDNA) was amplified by PCR, and the reaction products were purified and sequenced as described previously (6).

Microscopy. Scanning electron microscopy was performed with an overnight culture of *V. coralliilyticus* YB1 after negative staining with 1% uranyl acetate, and preparations were examined with a JEOL 840A scanning electron microscope. To examine coral tissue, fragments were fixed in 2.5% glutaraldehyde in sterile seawater (SSW) for 24 h, washed, postfixed in 1% OsO₄, and washed again. These samples were decalcified in a mixture containing equal volumes of formic acid (50%) and sodium citrate (15%) for 15 h. They were then dehydrated in a graded ethyl alcohol series and embedded in glycid ether 100 (Epon; Serva Feinbiochemica & Co., Heidelberg, Germany). Ultrathin sections stained with uranyl acetate and lead citrate were viewed with a JEOL (Peabody, Mass.) 1200 EX electron microscope.

Collection and maintenance of corals. Corals for laboratory infection experiments were collected by SCUBA divers off Eilat in the Gulf of Aquaba, Red Sea, from depths of 2 to 6 m. For infection experiments conducted at temperatures lower than 26°C, the corals were collected in November 2001, when the seawater temperature was 24°C. The corals were broken into 1- to 2-cm² pieces and then allowed to recover and regenerate in 3- to 10-liter, glass-covered, aerated aquaria at the same seawater temperature (21 to 26°C) at which they were collected. After tissue recovery the corals were slowly acclimated to the experimental temperature by increasing the temperature no more than 0.5°C every other day. Then the corals were maintained at the experimental temperature for a further 7 to 14 days before the experiment was begun. If any fragment failed to heal (completely cover the broken edges with new tissue and exhibit dark pigmentation), it was not used in the experiments. The corals were maintained in freshly prepared artificial seawater (Instant Ocean) adjusted to a salinity of 35 to 37 ppt. The water was replaced every 3 to 6 days. The aquaria were aerated and illuminated with fluorescent lamps (Sylvania Aquastar 10,000K) by using a regimen consisting of 12 h of light and 12 h of darkness.

Infection experiments. After *V. coralliilyticus* YB1 was grown in MBT medium for 30 to 48 h at 30°C with aeration (160 rpm), 10-ml cultures were centrifuged at 7,000 rpm (Sorval SS-34) for 5 min at the ambient temperature, washed in 10 ml of SSW, and resuspended in 0.5 to 1 ml (final volume) of SSW. Viable counting on MB agar and TCBS agar was performed in order to estimate the precise size of the inoculum in each experiment. Infection experiments were all performed under controlled illumination and temperature conditions in 2- to 3-liter aerated and covered aquaria. Each aquarium contained two or four coral fragments. Infection experiments were conducted by moving the coral fragments from the water into an empty sterile petri dish and immediately inoculating the fragments directly with 10 to 30 μl of *V. coralliilyticus* (containing 10⁷ bacteria) without disturbing the coral tissue. The standard size of the inoculum used for the infection experiments was chosen following preliminary experiments performed with inocula of different sizes (5). After 1 min the coral fgraments were carefully returned to the aquaria. The control preparations were treated in the same manner except that inoculation was with 10 to 30 μl of SSW. Observations on the corals were recorded every day. The level of tissue lysis (expressed as a percentage) was determined visually by estimating the size of the diseased area compared to the total size of the tissue. A coral was considered lysed when at least 50% of the tissue was degraded, leaving only a bare skeleton. A coral was considered bleached when the coral tissue appeared to be totally transparent but otherwise looked intact. A coral was considered 100% bleached when the entire coral was white, 90% bleached when it was white except for pale tan in the polyps, and 70% bleached when all of the tissue was pale tan, compared to the dark brown color of the controls.

Measurement of maximal fluorescence of the corals. A portable underwater Mini pulse amplitude modulation fluorometer (Walz, Effeltrich, Germany) was used to measure the maximum fluorescence of zooxanthellae within the corals. This instrument permitted direct noninvasive measurement of the effective quantum yield of photosystem II under ambient light conditions and measurement of the maximal fluorescence of the photosynthetic pigments following a saturating light pulse (25, 36). In the experimental procedure used here, an intact coral was rinsed twice in SSW and transferred into a 50-ml beaker containing SSW, and the maximal fluorescence was measured. The optical fiber was 5 mm from the coral surface, the corals were light adapted prior to measurement, and the temperature was kept constant at 25°C. The fluorescence of a coral was measured 10 times by using different parts of each fragment, and there were 1-min intervals between measurements. The data reported below are the averages of these measurements.

Measurement of zooxanthella concentration in the coral tissue. In order to obtain the zooxanthellae, the coral fragments were removed from the aquaria and rinsed twice in SSW. After a coral fragment was weighed, the tissue was disrupted by water picking with ca. 100 ml of SSW. The resulting suspension was centrifuged at 20°C for 30 min at 4,000 rpm (Sorval SS-34). The pellet, resuspended in 1 ml of SSW, was then centrifuged in an Eppendorf 5402 centrifuge for 4 min at 10,000 rpm. The pellet was resuspended in 1 ml of SSW and then centrifuged for 21 min at 1,200 rpm. The final algal pellets from the bleached and healthy corals were resuspended in 0.5 and 1.5 ml of SSW, respectively. The zooxanthella concentration was determined microscopically with a hemacytometer. At least 10 1-mm² fields were counted. At the end of the analysis, the algal preparations were stored at −70°C for pigment extraction. The zooxanthella concentration was expressed as the number of intact pigmented algae per gram (wet weight) of tissue. The wet weight of tissue for each coral fragment was determined from the difference between the weight before water picking and the weight after water picking.

Measurement of chlorophyll *a* concentration in the coral tissue. The zooxanthella preparations from bleached and healthy coral tissues described above were defrosted on ice and centrifuged for 5 min at 5,000 rpm (Eppendorf 5402) at 4°C. After the supernatant fluid was discarded, chlorophyll was extracted by resuspending the zooxanthellae in 0.5 ml of 90% acetone. The samples were mixed well and then sonicated for 1 to 2 min (on ice) in order to break the remaining cells by using the method of Jeffrey et al. (22). During the whole procedure the samples were kept on ice in the dark. After the cells were disrupted, 90% acetone was added to each of the samples so that the total volume was 3.5 to 6.5 ml. The samples were then incubated at 4°C for 24 h in the dark. The acetone supernatants containing the pigments were collected after centrifugation at 4°C (10 min at 8,000 rpm with a Sorval SS-34), and their spectra were determined with an Ultrospec 2000 spectrophotometer (Pharmacia Biotech). The equation of Jeffrey and Humphrey (23) was used to calculate the chlorophyll *a* concentrations.

Purification and characterization of the extracellular protease of *V. coralliilyticus*. The extracellular protease of *V. coralliilyticus* was purified from a 1.2-liter culture after incubation for 24 h at 30°C with shaking in MBT medium. After removal of the cells by centrifugation for 30 min at $9,000 \times g$ and 4°C, the cell-free supernatant fluid was brought to 70% ammonium sulfate saturation and allowed to stand for 18 h at 4°C. The precipitate was collected by centrifugation and dissolved in 10 ml of TBS buffer (20 mM Tris-HCl [pH 7.4] in 0.9% NaCl). After dialysis against TBS buffer, the materials were loaded on a fast protein liquid chromatography column (HiLoad 16/60 Superdex 75; Pharmacia). Elution was carried out with 50 mM Tris buffer (pH 8.0) in 0.1 M NaCl at a flow rate of 1 ml/min. Fractions (2.5 ml) were collected and assayed for protease activity as described above. The purity of the active fractions was checked by dissolving an aliquot in a solution containing 2% sodium dodecyl sulfate, 4% β-mercaptoethanol, 8% glycerol, 50 mM Tris-HCl (pH 6.8), and 0.02% bromphenyl blue. After heating for 10 min at 100°C, a sample was applied to a 10% polyacrylamide gel and electrophoresed with a running buffer containing 0.1% sodium dodecyl sulfate, 192 mM glycine, and 25 mM Tris-HCl (pH 8.3). Prestained broad-range sodium dodecyl sulfate-polyacrylamide gel electrophoresis standards (Bio-Rad Labortories, Hercules, Calif.) were used as molecular markers. Gels were stained with Coomassie brilliant blue. Edman degradation was carried out at the University of California at Davis after the resolved protein was transferred to polyuranylidene difluoride paper.

The optimum pH was determined by using sodium citrate buffer (pH 3.7 to 7.0) and Tris buffer (pH 7.0 to 12.2). Four class-specific protease inhibitors were
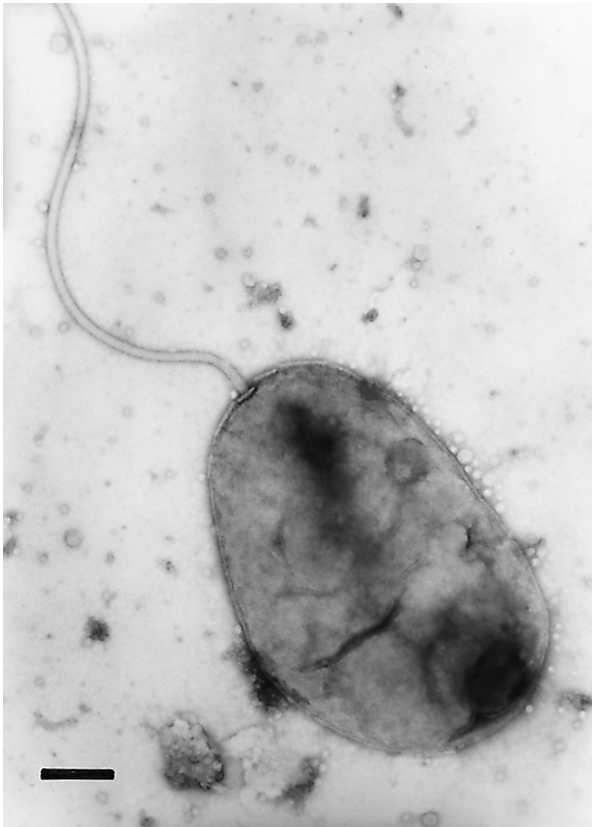
FIG. 1. Electron micrograph of negatively stained *V. coralliilyticus* YB1. Bar = 0.2 μm.

tested: phenylmethanesulfonyl fluoride for serine proteases, iodoacetic acid for cysteine proteinases, pepstatin for aspartic proteinases, and EDTA for metalloproteinases. In order to test for metal requirements, the purified enzyme was incubated for 18 h in the presence of 1 mM EDTA at 37°C. Addition of 10 mM $ZnCl_2$ and 10 mM $CaCl_2$ to the apoenzyme was used to determine the recovery of proteolytic activity.

RESULTS

Isolation of *V. coralliilyticus* strains from diseased corals. The first strain of *V. coralliilyticus*, isolated recently from bleached *P. damicornis* on the reef off Mawi Island near Zanzibar in the Indian Ocean, was demonstrated to be a new species of the genus *Vibrio* based on its morphology, biochemical characteristics, and 16S rDNA sequence (5). This isolate, *V. coralliilyticus* YB1, is a gram-negative, rod-shaped, motile bacterium that is 1.3 by 0.9 μm and has a single polar sheathed flagellum (Fig. 1).

During the summer of 2001 the temperature of the seawater surrounding the Eilat reef reached 26 to 27°C, which is higher than the normal maximum temperature. During that time bleaching and partial tissue lysis of *P. damicornis* were observed. To determine if these effects were associated with the presence of a specific bacterium, coral fragments were removed, rinsed with SSW, and crushed, and serial dilutions were spread on TCBS agar (Table 1). *V. coralliilyticus* was present in all four bleached corals and five partially lysed corals but was absent from the five healthy corals examined (limit of detection, 100 CFU/cm^3 of coral tissue). *V. coralliilyticus* was

recognized by its characteristic colony morphology (6). In addition, several colonies from diseased corals were isolated and shown to be *V. coralliilyticus* by genotypic and phenotypic characterization (6). It should be pointed out that the values in Table 1 are minimum values, because a significant number of *Vibrio* cells may be in the viable but nonculturable state, as has been shown for *V. shiloi* (21).

Three *V. coralliilyticus* strains isolated from separate coral colonies (bleached and partially lysed) of *P. damicornis* on the Eilat reef were characterized further and compared to the original strain, strain YB1 from Zanzibar. The 16S rDNA of the Eilat isolates, designated YB2, YB3, and YB4, exhibited more than 99% sequence identity to the 16S rDNA of strain YB1 (6). For comparison, the 16S rDNA of the most closely related other species of *Vibrio*, *V. tubiashii*, *V. nereis*, and *V. shiloi*, exhibited 97.2, 96.8, and 96.6% identity, respectively, to the 16S rDNA of strain YB1. Biochemical tests (performed with the API 20NE system) and carbon compound utilization analyses (performed with Biolog GN2 microplates) indicated that all three Eilat strains gave the same qualitative results as the Zanzibar strain for at least 112 of the 117 tests employed. Thus, the four *V. coralliilyticus* strains form a tight genomic group within the genus *Vibrio*. Interestingly, YB1 and YB4 gave identical results in all 117 tests, although YB1 was isolated from the Indian Ocean and YB4 was isolated from the Red Sea. As determined by the methods described below, all four strains were pathogenic for *P. damicornis*.

Laboratory aquarium infection experiments. Inoculation of corals at temperatures between 24.5 and 29.0°C with *V. coralliilyticus* caused either bleaching or lysis of the coral tissue (Table 2). Figure 2 shows healthy, bacterium-bleached, and bacterium-lysed *P. damicornis* fragments. At 24.5 and 25°C, all 13 corals remained healthy and pigmented for 10 days after infection. Bleaching began at day 12, and after 15 to 20 days most of the corals were bleached. By day 25, 12 of the 13 infected corals were bleached. The corals remained bleached, showing no signs of lysis, for an additional 2 weeks. However, infection at 27 or 29°C resulted in different symptoms than infection at the lower temperatures; by day 10, most of the corals were lysed, and by day 15, all 20 infected corals were lysed and had died. There was no sign of bleaching at these temperatures. Tissue lysis typically started in small spots, usually on the verrucae of the branches, which slowly united into white patches, and progressed until the entire tissue was degraded, leaving only the bare skeleton (5). Control corals that were inoculated with SSW at all four temperatures remained healthy and pigmented for at least 2 months (results for only

TABLE 1. Enumeration of *V. coralliilyticus* in healthy and diseased *P. damicornis*[a]

Condition of coral	n	*V. coralliilyticus* concn (CFU/cm^3)	
		Mean ± SEM	Range
Healthy	5	$<10^2$	$<10^2$
Bleached	4	$1.8 \times 10^5 \pm 1.2 \times 10^5$	0.11×10^5–11×10^5
Partially lysed	5	$10.5 \times 10^5 \pm 5.1 \times 10^5$	1.1×10^5–24×10^5

[a] Coral fragments (ca. 2 cm^3) were obtained from depths of 2 to 6 m on the Eilat reef during the summer of 2001. The numbers of CFU were determined by plating crushed fragments on TCBS agar.

TABLE 2. Bleaching and lysis of *P. damicornis* by *V. coralliilyticus* as a function of temperature[a]

Temp (°C)	No. of fragments/total no. of fragments after:										
	10 days		15 days			20 days			25 days		
	Healthy fragments	Lysed fragments	Healthy fragments	Bleached fragments	Lysed fragments	Healthy fragments	Bleached fragments	Lysed fragments	Healthy fragments	Bleached fragments	Lysed fragments
20.0	3/3		3/3			3/3			3/3		
22.0	4/4		4/4			4/4			4/4		
24.5	9/9		3/9	6/9		1/9	8/9		1/9	8/9	
25.0	4/4		4/4			2/4	2/4		4/4		
27.0	3/9	6/9			9/9			9/9			9/9
29.0	1/11	10/11			11/11			11/11			11/11
Control	30/30		30/30			30/30			30/30		

[a] *P. damicornis* fragments (two to four coral fragments per 2.5 liter aquarium) were infected with a 30-h culture of *V. coralliilyticus* (final concentration 10^7 cells per coral) as described in Materials and Methods. The fractions of healthy, bleached, and lysed corals are indicated. Healthy corals were dark-brown-pigmented corals; bleached corals were corals in which the tissue was transparent but otherwise intact; and lysed corals were dark-brown-pigmented corals exhibiting lysis of 50% or more of the tissue. The control consisted of 30 uninoculated corals maintained at 29°C.

the 29°C control are shown in Table 2). Corals infected at 20 or 22°C showed neither bleaching nor lysis for at least 6 weeks. At the end of the experiment, healthy and bacterium-bleached corals were crushed, diluted in SSW, and plated on TCBS agar. No *V. coralliilyticus* was detected in the healthy corals, while all the bleached corals contained high concentrations of the bacterium. Furthermore, electron micrographs of thin sections of the bleached corals showed that there were bacteria inside the coral tissue (Fig. 3). No bacteria were found in the tissue of the uninfected control corals.

In addition to visual determinations of bleaching, quantitative measurements of bacterium-induced bleaching were obtained (Table 3). The four control corals contained 66×10^3 zooxanthellae per g (wet weight) of coral tissue (mean value); the numbers of zooxanthellae in the two corals that appeared visually to be 70% bleached were 10.9 and 11.8% of the numbers of zooxanthellae in the controls, whereas the numbers of zooxanthellae in the corals that appeared visually to be 90% bleached were 8.8% of the numbers of zooxanthellae in the controls. The concentrations of photosynthetic pigments in the bleached corals were also reduced compared to the concentrations in the controls, but to a lesser degree than the algal concentrations. For example, the coral that was 90% bleached contained 16% of the chlorophyll *a* found in the unbleached controls and exhibited 23% of the maximum fluorescence of the unbleached controls.

Protease activity of *V. coralliilyticus* YB1. During studies of the classification of *V. coralliilyticus* YB1 it was observed that the strain produced a potent extracellular protease (5, 6). Since in several cases the virulence of *Vibrio* pathogens is associated with protease activity (16, 17, 41), we investigated the production and properties of the *V. coralliilyticus* protease. Extracellular protease activity was a function of the growth temperature and the phase of growth (Fig. 4). When a 0.5% inoculum was used, the maximum culture turbidity was achieved within 24 h over the entire temperature range studied (16 to 30°C), and the maximum values differed by only 20% at temperatures from 20 to 30°C. However, production of the extracellular protease was greatly influenced by the growth temperature, with the greatest change in specific activity occurring between 24°C (0.8 U/A_{600}) and 28°C (5.9 U/A_{600}). At all temperatures, the specific activity was three- to fivefold higher during the stationary phase than during the exponential phase.

The *V. coralliilyticus* protease was purified to homogeneity as described in Materials and Methods. The molecular mass, as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, was 36 kDa. The first 17 amino acids from the N terminus of the protease were sequenced. Comparative analysis of the protein sequence (Fig. 5) showed that this protein exhibited high levels of homology to a range of proteases found in members of the family *Vibrionaceae*, including the well-studied hemagglutinin-protease of *Vibrio cholerae* (10). The *V. coralliilyticus* protease exhibited maximum activity at pH 8 to 11 and 30 to 40°C. The enzyme was inactivated by 1 mM EDTA, and the apoenzyme was reactivated by addition of 10 mM Ca^{2+} or Zn^{2+}, indicating that it is a metalloproteinase.
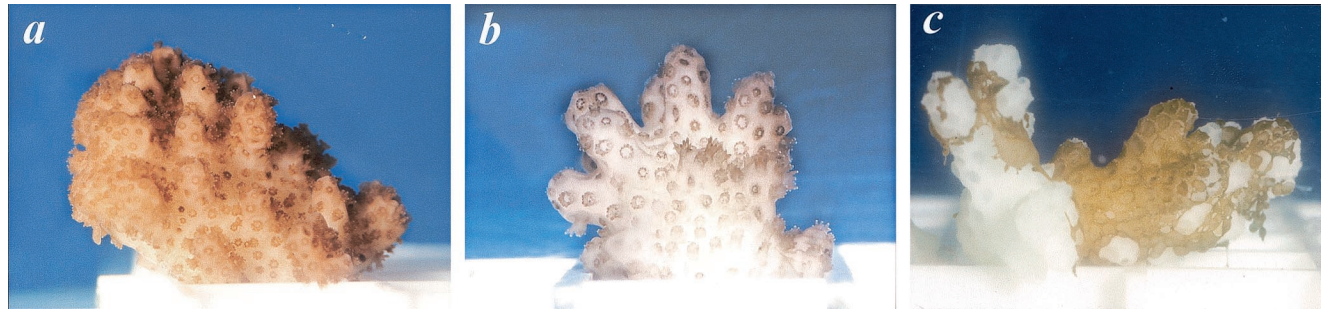

FIG. 2. Healthy (a), bacterium-bleached (b), and bacterium-lysed (c) *P. damicornis*. The bleached coral (b) was estimated to be 90% bleached compared to the uninoculated control (a); the lysed coral (c) was estimated to be 50% lysed.
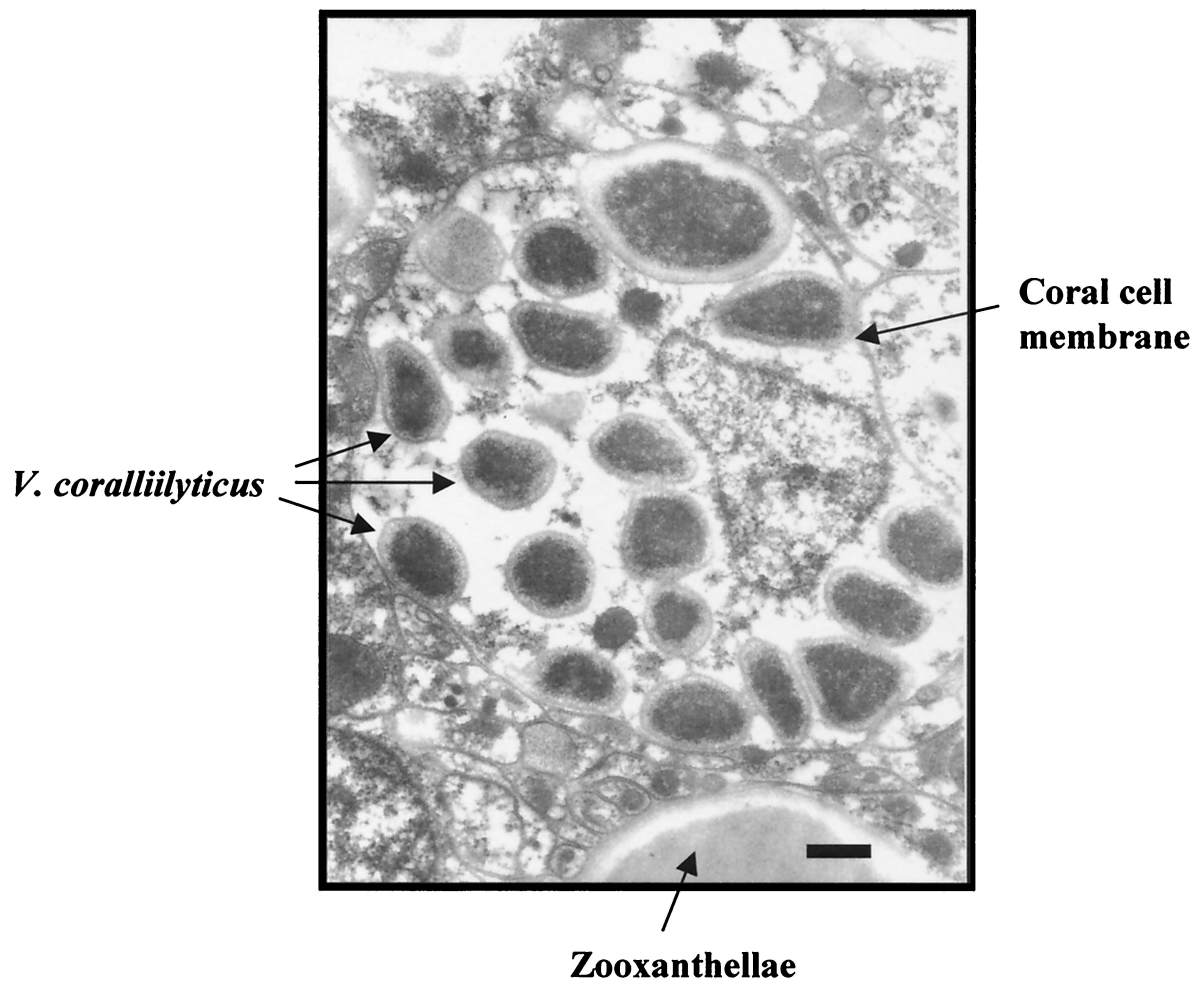
FIG. 3. Electron micrograph of a thin section of a bleached *P. damicornis* coral, 13 days after infection with *V. coralliilyticus* at 24.5°C. Bar = 0.5 μm.

Enzyme activity was not affected by phenylmethanesulfonyl fluoride, iodoacetic acid, or pepstatin.

Preliminary experiments indicated that addition of the purified protease to coral fragments caused tissue damage within 18 h at 27°C, as measured by release of protein and pigments (maximum absorbance at 320 nm) from the coral. The effect was proportional to the enzyme concentration; in the presence of 2, 4.5, and 9 U of the purified enzyme per ml at 28°C, the A_{320} in aquaria containing the corals were 0.5, 2.1, and 4.8, respectively, after 18 h. EDTA inhibited the release of pigment. There were no significant changes in the pH of the seawater during the treatments.

DISCUSSION

The two most significant findings described in this report are (i) that *V. coralliilyticus* is an etiological agent of the bleaching disease of *P. damicornis* and (ii) that seawater temperature is the critical environmental parameter in determining the outcome of the infection. Koch's postulates were satisfied: (i) *V. coralliilyticus* was present in diseased corals and absent from healthy tissue; (ii) the strain was obtained in pure culture; (iii) infection of healthy corals in controlled aquarium experiments

caused the disease; and (iv) the pathogen was reisolated from the infected host. This is the second demonstration of bacterial bleaching of corals; the first was the bleaching of the temperate coral *O. patagonica* by *V. shiloi* (26, 27). In each case, a specific different *Vibrio* species was the responsible pathogen. These

TABLE 3. Zooxanthella and chlorophyll *a* contents of healthy and bacterium bleached coral tissue[a]

Coral	Concn of algae (10³ cells g [wet wt] of tissue⁻¹)	Concn of chloro- phyll *a* (μg [wet wt] of tissue⁻¹)	Maximum fluorescence
Control 1	43.7	120	3,540
Control 2	45.5	90	3,460
Control 3	111.7	219	3,630
Control 4	62.6	183	3,860
Avg ± SEM	65.9 ± 15.9	153 ± 29	3,630 ± 90
70% bleached	7.2	55	1,040
70% bleached	7.8	70	1,430
90% bleached	5.8	24	820

[a] Coral fragments were taken from the experiment described in Table 2 performed at 24.5°C. Pigmented zooxanthella and chlorophyll *a* concentrations were determined as described in Materials and Methods. Maximum fluorescence was determined by using the intact coral fragments.
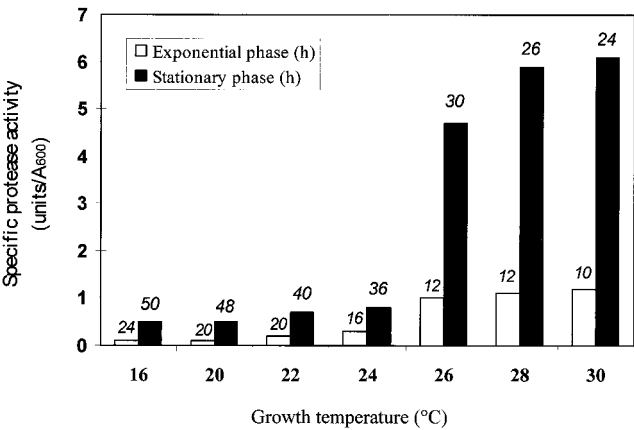
FIG. 4. Extracellular protease activity of *V. coralliilyticus* YB1 as a function of growth temperature. Strain YB1 was grown with aeration in MBT medium at different temperatures. Values for culture turbidity (A_{600}) and extracellular protease activity (units) were determined during the exponential and stationary phases. The numbers above the bars indicate the time (in hours) when the assay was performed at each growth temperature.

data provide support for the bacterial hypothesis of coral bleaching.

In addition to the two direct demonstrations that coral bleaching is an infectious disease, several indirect lines of evidence suggest that at least some other cases of coral bleaching resulted from infection. Several authors have described the patchy spatial distribution and spreading nature of coral bleaching (7, 11, 14, 24, 29, 32). It has been argued that the random mosaic patterns of bleaching observed in coral colonies are difficult to attribute solely to environmental stress, since neighboring regions of a colony must be exposed to the same extrinsic conditions (19). Even more significantly, the spreading nature of coral bleaching is highly symptomatic of an infectious disease. It is likely that coral bleaching is an array of diseases and that infection by bacteria is only one of the etiologies. We suggest that the patchy pattern of bleaching of *P. damicornis* observed in Eilat when the temperature reached ca. 24°C and shown here to be an infectious disease may be a model for patchy, distributed bleaching diseases seen on other coral reefs. Mass bleaching may have a different etiology.

The demonstration that bleaching and lysis of *P. damicornis* are the result of a bacterial infection does not diminish the

importance of temperature stress in coral diseases. It is not clear at this time, however, whether an increased temperature causes *P. damicornis* to become more susceptible to infection or makes the bacterium more virulent. For *P. damicornis* to either bleach or lyse, both the pathogen and the permissive temperature must be present. In the case of bleaching of *O. patagonica*, the temperature stress is primarily stress on the pathogen, causing expression of a variety of virulence genes (35).

Jokiel and Coles (24) reported that seawater temperatures slightly above the normal maximum temperature cause bleaching of *P. damicornis*, while temperatures a few degrees higher cause lysis and death of Hawaiian reef corals. These observations are similar to the data reported here for infected corals. Jokiel and Coles did not perform microbiological studies, so that it is not known if these corals were infected before the temperature was elevated.

Several marine *Vibrio* species are pathogenic to invertebrates (8, 16). The unusual feature of *Vibrio* bleaching of corals is that the target appears to be the intracellular zooxanthellae rather than the coral tissue. *V. shiloi* does not infect corals that lack algae (2), and the bacterium produces anti-algal toxins that inhibit photosynthesis and lyse the algae (3, 4). In the case of bleaching of *P. damicornis* by *V. coralliilyticus*, it also appears that the bacterium attacks the algae. The relatively higher level of photosynthetic pigments than of intact pigmented algae (Table 3) suggests that the intracellular algae are damaged, resulting in release of their pigments. An alternative explanation for the increased ratio of pigment to algae in the bleached tissue is an increase in the specific activity of pigment production due to the decreased self-shading resulting from the presence of fewer algal cells. Based on the existing data, we suggest that bacterial bleaching of *P. damicornis* results from an attack on the algae, whereas bacterium-induced coral lysis and death are caused by bacterial extracellular proteases. The temperature-regulated production of protease by *V. coralliilyticus* supports this suggestion. The great increase in enzyme production occurs at the same temperature range (24 to 28°C) as the transition from bleaching to lysis of the corals. We are currently attempting to obtain protease-negative mutants in order to test the hypothesis more vigorously.

FIG. 5. Comparison of the N-terminal amino acid sequences of *Vibrio* proteases. The boxes enclose amino acids that are identical in all five proteases.

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