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Growth, differentiation and death of *Vibrio shiloi* in coral tissue as a function of seawater temperature

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ABSTRACT: The coral pathogen *Vibrio shiloi* was present during the summer in the tissues of all of its host corals, *Oculina patagonica*, which were bleached. During the winter, when seawater temperatures dropped below 20°C, *V. shiloi* could not be detected in either healthy or bleached corals, even when the corals were placed in aquaria and the temperature was slowly increased to 28°C. Using specific anti-*V. shiloi* antibodies to enumerate the bacterium, it was shown that 99 to 99.99% of *V. shiloi* inside coral tissues were in a viable-but-not-culturable (VBNC) state. All attempts to culture VBNC *V. shiloi* in liquid or on solid media were unsuccessful. However, it was demonstrated that VBNC *V. shiloi* was infectious, i.e., it adhered to, penetrated into and multiplied inside corals. Laboratory aquaria experiments indicated that when corals were infected with *V. shiloi* at 28°C and then shifted slowly to their winter *in situ* temperature (16°C), the bacteria died and lysed. Since the bacteria remain viable under the same temperature shifts outside the coral, either in growth media or seawater, it follows that the coral must have a host-defense mechanism for killing intracellular bacteria. The rapid killing of intracellular *V. shiloi* at 16°C and their absence from corals during the winter suggest that bleaching of *O. patagonica* in the Mediterranean Sea requires a fresh infection each spring, rather than the activation of dormant intracellular bacteria. The lessons learned from the *V. shiloi*/*O. patagonica* model systems are discussed in terms of the general problem of the coral bleaching disease.

KEY WORDS: *Vibrio* · Coral bleaching · Temperature and infection · Temperature and coral bleaching

INTRODUCTION

During the last 2 decades, coral bleaching events of unprecedented frequency and global extent have been reported (Hoegh-Guldberg 1999). Coral bleaching is the disruption of symbioses between coral animals and their photosynthetic microalgal endosymbionts, the zooxanthellae (Iglesias-Prieto et al. 1992). As a result of the loss of the algae and/or their pigments, the coral turns white. The sudden loss of zooxanthellae greatly affects the coral host because these photosynthetic pigments supply up to 63% of the corals' nutrients (Glynn 1991a). The energy source derived from the algae facilitates calcification. Coral bleaching is a widespread disease that occurs in the world's 3 major oceans and involves over 50 countries (Wilkinson 1998). It has been suggested that coral bleaching is triggered by environmental factors that impose stress on the coral. The most frequently reported stress condition is increased seawater temperature (Jokiel & Coles 1990, Glynn 1991b, Brown 1997, Kushmaro et al. 1998). Thus, it is possible that global warming could result in alterations to or destruction of coral reef systems, the consequences of which could be devastating—to tourist and fishing industries, islands that are protected by coral reefs and, most importantly, to the health of the sea. Consequently, it is essential to understand the mechanism(s) of coral bleaching.

Bleaching of the coral *Oculina patagonica* from the Mediterranean Sea is the result of a bacterial infection (Kushmaro et al. 1996, 1997). The causative agent, *Vibrio shiloi* (Rosenberg et al. 1998, Kushmaro et al. 2001), was obtained in pure culture and shown to cause

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bleaching in controlled aquarium experiments. Furthermore, it was shown that bacterial-induced bleaching by *V. shiloi* could be inhibited by antibiotics. The infection and resulting coral bleaching was temperature-dependent, occurring only at seawater temperatures above 25°C (Kushmaro et al. 1998).

Using the *Vibrio shiloi*/*Oculina patagonica* model system to study coral bleaching, it was demonstrated that the first step in the infectious process was the adhesion of *V. shiloi* to a β-galactoside-containing receptor on the coral surface (Toren et al. 1998). The temperature of bacterial growth was critical for the adhesion of *V. shiloi* to the coral. When the bacteria were grown at the winter seawater temperature (16°C), there was no adhesion to the coral, regardless of what temperature the coral had been maintained. However, bacteria grown at summer seawater temperatures (25 to 30°C) adhered avidly to corals maintained at either low or high temperatures. The important ecological aspect of these findings was that the environmental stress condition, i.e., high temperature, was causing the coral bleaching pathogen to become virulent. In an attempt to understand how *V. shiloi* causes the destruction or loss of the algae, it was discovered that *V. shiloi* produces a heat-stable extracellular toxin that inhibits photosynthesis of zooxanthellae and heat-sensitive toxins that bleach and lyse the algae isolated from corals (Rosenberg et al. 1998, Ben-Haim et al. 1999, Banin et al. 2001).

Recently it was reported that *Vibrio shiloi* penetrates into epithelial cells shortly after adhering to the coral surface (Banin et al. 2000). The intracellular *V. shiloi* were observed by electron microscopy and fluorescence microscopy using specific anti-*V. shiloi* antibodies to stain the bacteria. Once inside their host, *V. shiloi* multiplied intracellularly, reaching over 10^8 cells cm^{-3} of coral tissue. Furthermore, intracellular *V. shiloi* were transformed into a viable-but-not-culturable (VBNC) state, which has been defined as 'a cell that can be demonstrated to be metabolically active, while being incapable of undergoing the sustained cellular division required for growth in or on a medium normally supporting growth of that cell' (Oliver 1993). In this study, we measured the numbers of intracellular *V. shiloi* in corals taken from the sea at different times of the year, the occurrence of the VBNC state in bleached corals, the infectivity of *V. shiloi* in the VBNC state and the effect of temperature shifts on the viability of *V. shiloi* in seawater, growth media and in coral tissues.

MATERIALS AND METHODS

Microorganism and growth media. *Vibrio shiloi*, a new species of *Vibrio* (Kushmaro et al. 2001), was isolated from a bleached coral as described previously (Kushmaro et al. 1996). The strain was maintained on MB agar (1.8% marine broth plus 0.9% NaCl solidified with 1.8% agar, both products of Difco Lab). After being streaked onto MB agar, the cultures were incubated at 30°C for 2 d and then allowed to stand at room temperature for 1 wk. TCBS agar (Difco MA2216), a selective medium for *Vibrio*, was used periodically to confirm the purity of the strain. MBTG medium is MB medium supplemented with 0.5% Tryptone and 0.1% glycerol.

Collection and maintenance of *Oculina patagonica*. Colony fragments of *O. patagonica* (ca 1 cm^3) were collected at different times during 1999–2000 from depths of 0.5 to 1.5 m at 2 sites along the Mediterranean Sea coastline (Bat-Yam and Jaffo). The extent of bleaching was estimated visually and the seawater temperature at the time of collection was recorded. Within 1 h of collection, the coral fragments were placed in aerated aquaria containing filtered seawater at the seawater ambient temperature. The aquaria were illuminated with a fluorescent lamp at 12 h light:12 h dark intervals. Coral pieces were allowed to recover and regenerate for 15 d before the start of each experiment (unless indicated differently). If any piece failed to heal (complete cover of damaged skeleton by new tissue), it was discarded and not used in any experiment.

Enumeration of *Vibrio shiloi*. The number of *V. shiloi* inside coral tissues was determined by a modification of the gentamicin invasion assay (Isberg & Falkow 1985). The antibiotic gentamicin kills only bacteria outside the coral because it does not penetrate into coral tissues (Banin et al. 2000).

Corals were rinsed in sterile seawater and then transferred to a 50 ml tube with 5 ml of sterile seawater containing 0.01% of methyl-β-galactopyranoside and 200 μg ml^{-1} of gentamicin (both products of Sigma, St. Louis, MO, USA) in order to desorb and kill non-internalized bacteria, respectively. After incubation for 3 h at 29°C, the coral was removed, rinsed in sterile seawater, then crushed in 5 ml of sterile seawater using a mortar and pestle, and finally vortexed in a 50 ml tube for 1 min. Samples were taken after 2 min from the upper part of the liquid, after allowing the heavy materials to settle to the bottom of the tube. Two methods were used in order to quantitate the internal *Vibrio shiloi*. The first method (colony forming units, cfu) involved estimating the number of internal bacteria by plating triplicate samples of appropriate dilutions in sterile seawater on MB agar and TCBS agar. *V. shiloi* has a characteristic colony morphology on TCBS agar. Confirmation that the colonies were *V. shiloi* was obtained by checking the cells with specific anti-*V. shiloi* antibodies. The standard errors for all determinations of cfu were less than 10%.

The second method (total count) involved determining the number of *Vibrio shiloi* cells in the coral tissue microscopically after staining with specific polyclonal anti-*V. shiloi* antiserum. The crushed coral samples (0.5 ml) were fixed at room temperature, with freshly prepared 4% paraformaldehyde in 0.45 M NaCl for 1 to 3 h. The fixed samples were then washed 3 times in TBS (10 mM Tris-HCl, pH 7.5, in 150 mM NaCl), and after 24 h at 4°C the cells were attached to microscope multitest slides (ICN, Mesa, CA, USA) covered with poly-L-lysine (50 µg ml^{-1}). After incubation for 1 h, the liquid was removed and the slides were dried for 5 min in 60°C. Polyclonal antibodies raised against *V. shiloi* (Banin et al. 2000) were added to the fixed cells (30 µl of 1:500 dilution in TBS) for 12 h at 4°C. (The antibodies were affinity purified by using fixed *Escherichia coli* and *Vibrio mediterranei* cells.) The slides were then washed 3 times in TBS and incubated in the dark with 5 µg ml^{-1} of Amca-conjugated anti-rabbit immunoglobulin G (IgG) (Jackson Immuno Research, West Grove, PA , USA). After the incubation, the slides were washed 3 times in TBS and mounted with a solution of 90% glycerol containing 1 mg ml^{-1} of *p*-phenylenediamine. Cover slips were used to seal the walls containing the fixed, stained and mounted bacteria. The sample was stored at –20°C until examination. Examination was carried out using a Leica fluorescence microscope (model DMR) with filter A (UV). For each determination, at least 3 separate fields were counted. The viability of intracellular *V. shiloi* was examined with a Live/Dead BacLight Bacterial Viability Kit (Molecular Probes, Eugene, OR, USA). The bacteria were stained according to the manufacturer's protocol and then examined by fluorescence microscopy with a Leica B/G/R filter. Live bacteria fluoresce green and dead bacteria fluoresce red.

Electron microscopy. For electron microscopy, 0.5 ml samples of exponentially growing *Vibrio shiloi* in MB medium and crushed coral fragments were fixed in 2.5% glutaraldehyde in filtered (pore size, 0.2 µm) seawater. After staining with 2% uranyl acetate, the bacteria were viewed with a JEOL 1200 EX electron microscope.

Laboratory infection experiments. An overnight culture of *Vibrio shiloi*, grown at 30°C in MB broth with aeration, was centrifuged at 5000 × *g* for 10 min, and the cell pellet was washed twice and then resuspended in sterile seawater to ca 10^9 cells ml^{-1}. The bacteria were then inoculated into aerated aquaria containing colonies of *Oculina patagonica* in 2 l filtered seawater (0.45 µm) at 28°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}. After standing for 3 d at 28°C to allow for the adhesion of the bacteria onto the coral, penetration into the coral tissues and intracellular multiplication (Banin et al. 2000), the corals were either maintained at 28°C or transferred to different aquaria at varying temperatures. For each set of experiments, no bacterial inoculum controls were performed under the same conditions. At timed intervals, duplicate or triplicate corals were removed from the aquaria for determination of internal *V. shiloi* by fluorescence microscopy using anti-*V. shiloi* antibodies (total count) and cfu, as described above.

RESULTS

Seasonal variation in bleaching and internal *Vibrio shiloi*

The percentage of coral colonies that showed distinct bleaching areas reached over 80% each summer from 1996 to 2000 when water temperatures exceeded 28°C (Fig. 1). During the winter (minimum temperature of 16°C) most of the bleached colonies recovered. The number of bleached colonies increased each spring when the water temperature exceeded 20°C and reached a peak in August/September, approximately a month after the maximum water temperature.

Since *Vibrio shiloi* is the causative agent of bleaching *Oculina patagonica*, it was of interest to examine the concentration of *V. shiloi* inside the bleached coral tissue monthly (Table 1). From December through March less than 10^5 (limit of detection) bacteria were found per cm^3 coral fragment. Starting in April, the number of internal *V. shiloi* increased, reaching a maximum of 8.4×10^8 cm^{-3} in June. The numbers then

Table 1. Seasonal variation (1999 to 2000) of total counts of *Vibrio shiloi* inside bleached *Oculina patagonica*. The number of *V. shiloi* in 1 cm^3 coral fragments taken directly from the sea were determined by fluorescence microscopy using specific anti-*V. shiloi* antibodies after removing external bacteria as described in 'Materials and methods'

Month	Temperature (°C)	Corals examined	Total internal *V. shiloi* × 10^6
Jan	18	2	<0.1
Feb	16	1	<0.1
Mar	16	3	<0.1
Apr	19	4	15.0
May	22	3	26.5
Jun	26	4	843
Jul	28	4	124
Aug	30	4	35
Sep	28	2	4.1
Oct	27	3	0.1
Nov	24	4	1.8
Dec	20	2	<0.1

decreased through the remainder of the summer and fall. Thus, the maximum concentration of bacteria in the coral tissue preceded the maximum percent bleaching by 1 to 2 mo.

In addition to the bleached corals, healthy corals were also examined monthly for the present of internal *Vibrio shiloi*. During the summer, from June through November, none of the 15 coral fragments examined showed any internal *V. shiloi* (>10^5 cm^{-3}). This is consistent with the fact that *V. shiloi* is the causative agent of bleaching; if *V. shiloi* were present in these corals during this period of warm water, then one would expect them to bleach. From December through February, none of the 7 healthy corals examined showed *V. shiloi*. Thus, no *V. shiloi* could be found inside 7 healthy or 5 bleached corals in the winter. The only time that *V. shiloi* was found in healthy (unbleached) corals was in the spring. From March through May, 4 of the 10 corals examined showed significant numbers (0.2 to 1.8 × 10^6 cm^{-3}) of *V. shiloi*. Presumably, these corals had been infected recently and would eventually bleach.

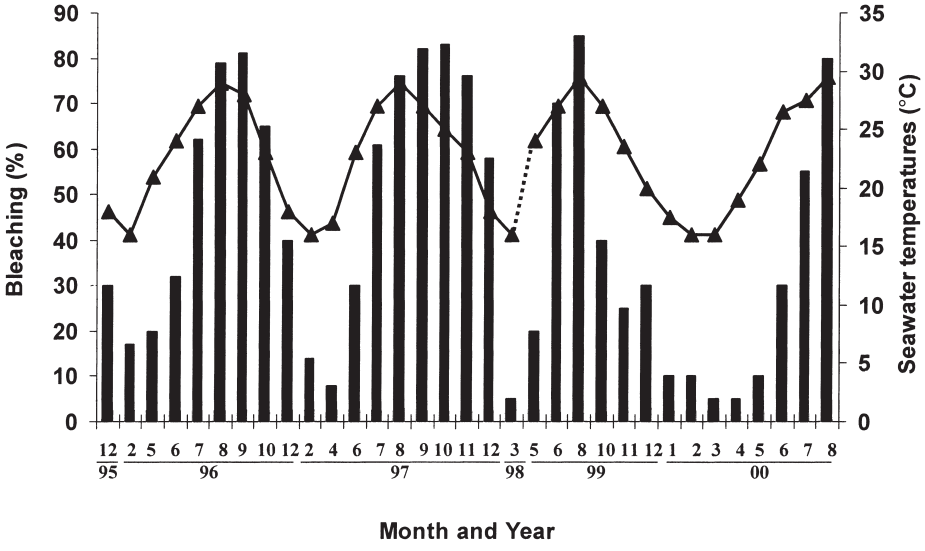

Fig. 1. Bleaching of *Oculina patagonica* in the Mediterranean Sea from 1995 to 2000 in relation to seawater temperature. Seasonal variations in seawater temperature (▲) and percent of bleached coral colonies (bars) are plotted

Temperature-shift experiments

In order to better understand how water temperature affects survival of *Vibrio shiloi* inside coral tissues, laboratory temperature-shift experiments were performed. After infecting the corals at 28°C and allowing sufficient time (3 d) for the bacteria to adhere to the corals and penetrate into the tissue, half of the corals were transferred to a second aquarium, where the temperature was decreased 2°C d^{-1} (Fig. 2). In the corals that were maintained at 28°C, the bacteria multiplied reaching almost 10^{10} cm^{-3} and then slowly decreased to ca 5 × 10^8 cm^{-3}. This concentration was maintained for 25 d at which time the experiment was discontinued. In the infected corals that were shifted to lower temperatures, there was a 67% decrease in bacteria 2 d after the temperature shifted down (24°C) and an 83% decrease after 3 d (22°C). After 13 d (5 d at 16°C), no detectable (>10^5) bacteria were observed.

Since the limit of detection for internal *Vibrio shiloi* was 10^5 cm^{-3}, temperature shift-up experiments were performed on corals taken from the sea in the winter. The rationale for this experiment was that even if a few viable *V. shiloi* were present in the coral, then by slowly raising the temperature, they would multiply and become detectable. The temperature of 10 coral fragments, taken from the sea from December to March, was slowly increased from their *in situ* temperature (16 to 20°C) to 28°C. After being maintained at 28°C for 2 to 3 wk, the corals were crushed and examined for total counts and cfu. No bacteria were observed in any of the 10 coral fragments by either method.

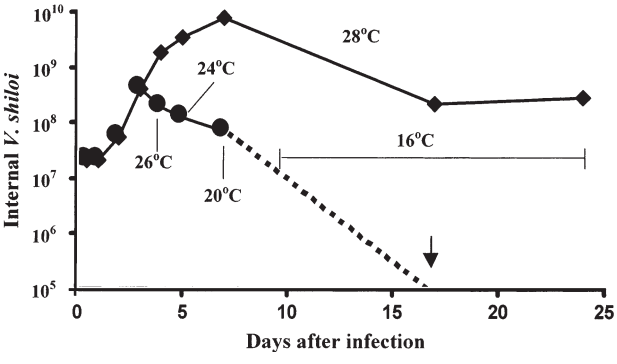

Fig. 2. Effect of temperature shift-down on *Vibrio shiloi* inside coral tissue. Healthy pieces of coral were infected with *V. shiloi* at 28°C. After 72 h, half of the corals were transferred to a second aquarium at 28°C, and then each day the temperature was lowered 2°C, until the temperature reached 16°C. At timed intervals, 2 corals were taken from each aquarium for the determination of total *V. shiloi* by fluorescence microscopy

Temperature shift-up experiments were also performed on laboratory-infected corals that had been previously shifted to 16°C, as described in Fig. 2. Four coral fragments were infected at 28°C, slowly adjusted to 16°C, maintained at 16°C for 1 wk and then slowly brought back to 28°C. After 1 wk at 28°C, the corals were crushed and examined for internal *Vibrio shiloi*. No bacteria were detected by total counts or cfu, further indicating that at the winter temperature the bacteria are completely killed inside the coral tissue.

The death of *Vibrio shiloi* in coral tissue when shifted to temperatures below 25°C is due to the intracellular coral environment and not the temperature shift alone (Table 2). Bacteria grown for 3 d at 28°C and then suspended in seawater or rich culture medium showed only a small decrease in cfu when the temperature was lowered. When the bacteria were inside the coral tissue, no bacteria could be detected by the antibody method for total counts after the same temperature shift-down. Although colony counts for *V. shiloi* inside coral tissue are not reliable, because of the transition into the VBNC form (see next section), no *V. shiloi* could be detected after the intracellular bacteria were at 16°C for 1 wk.

Table 2. Killing of *Vibrio shiloi* when shifted slowly from 28 to 16°C in coral tissue compared to shake flask cultures. For determination of total counts in coral tissue, healthy corals, maintained at 28°C, were infected with *V. shiloi*. After 3 d, the temperature of the aquarium was decreased 2°C d^{-1} until it reached 16°C. For shake flask culture, *V. shiloi* was inoculated into MBTG medium and allowed to grow for 3 d at 28°C. The bacteria were then harvested and suspended in fresh MBTG medium or sterile seawater. At timed intervals, colony forming units (cfu) were determined on MB agar. The temperature shifts were the same as those for the bacteria in the corals

Incubation (d)	Temperature (°C)	Counts of *V. shiloi* in: Coral tissue (total count)	MBTG (cfu ml^{-1})	Seawater (cfu ml^{-1})
3	28	4.0×10^8	4.5×10^9	4.5×10^9
5	24	1.3×10^8	4.9×10^9	3.2×10^9
6	22	7.0×10^7	2.9×10^9	3.4×10^9
16	16	$<10^5$	3.8×10^9	2.8×10^9
28	16	$<10^5$	8.7×10^8	9.5×10^8

The VBNC state of intracellular *Vibrio shiloi*

Previously, we demonstrated that *Vibrio shiloi* enters the VBNC state shortly after infecting corals in laboratory aquarium experiments (Banin et al. 2000). To test if *in situ* bleached corals also contained *V. shiloi* in the VBNC state, coral fragments taken from the sea were crushed and examined for total counts and plate counts. The data are presented as the ratios of total counts per colony forming unit (Table 3). This ratio was 1 to 3 for the control bacteria grown in MBTG medium and suspended in either seawater or rich medium. All 28 bleached corals taken from the sea showed a much higher value for total counts than plate counts, indicating the presence of the VBNC state in naturally infected corals. All of the intracellular bacteria that stained with the antibodies also scored as viable by the Live/Dead BacLight Viability Kit. In corals taken in the spring and fall, an average of 0.014 and 0.63%, respectively, of the observed *V. shiloi* gave rise to colonies on standard agar plates. In corals examined in the summer, at the height of the bleaching, only 0.00013% of the *V. shiloi* were platable. An even smaller percentage of the bacteria gave rise to colonies in controlled laboratory infection experiments, 4 to 7 d after inoculation. It appears that during the most active part of the infection cycle, when bacteria are multiplying intracellularly and reaching high cell densities, they exist almost exclusively in the VBNC state.

Intracellular *Vibrio shiloi* has a distinctly different morphology than the same bacterium grown in culture medium (Fig. 3). Exponentially growing *V. shiloi* is a Gram-negative, motile, rod-shaped bacterium (2.4 × 1.6 µm) that has a single polar sheathed flagellum. When growing intracellularly in coral tissue, the bacteria lack the flagellum and are smaller (2.0 × 1.0 µm).

Vibrio shiloi in the VBNC state is infectious

From both theoretical and practical considerations it is important to know whether or not *Vibrio shiloi* in the

Table 3. The viable-but-not-culturable (VBNC) state of *Vibrio shiloi* in corals. The total and the cfu of *V. shiloi* were determined in laboratory-infected corals and bleached corals taken directly from the sea. As a control, *V. shiloi* was grown in MBTG medium for 3 d, the cell harvested and resuspended in either sterile seawater of MBTG medium. The results are presented as the ratio of total count per cfu. n: number of corals examined

Samples examined (n)	*Vibrio shiloi* (total count/cfu) Average	Range
Bleached corals from the sea		
April, May (7)	7×10^2	$3–9 \times 10^2$
June–August (12)	8×10^4	$3–13 \times 10^4$
September–November (9)	1.6×10^2	$0.4–3 \times 10^2$
Laboratory-infected corals		
After 1–3 d (6)	24	3–50
After 4–7 d (6)	3×10^5	$10^5–10^6$
Bacteria grown in MBTG medium		
1–17 d in seawater (5)	2	1–3
1–17 d in MBTG (5)	2	1–3

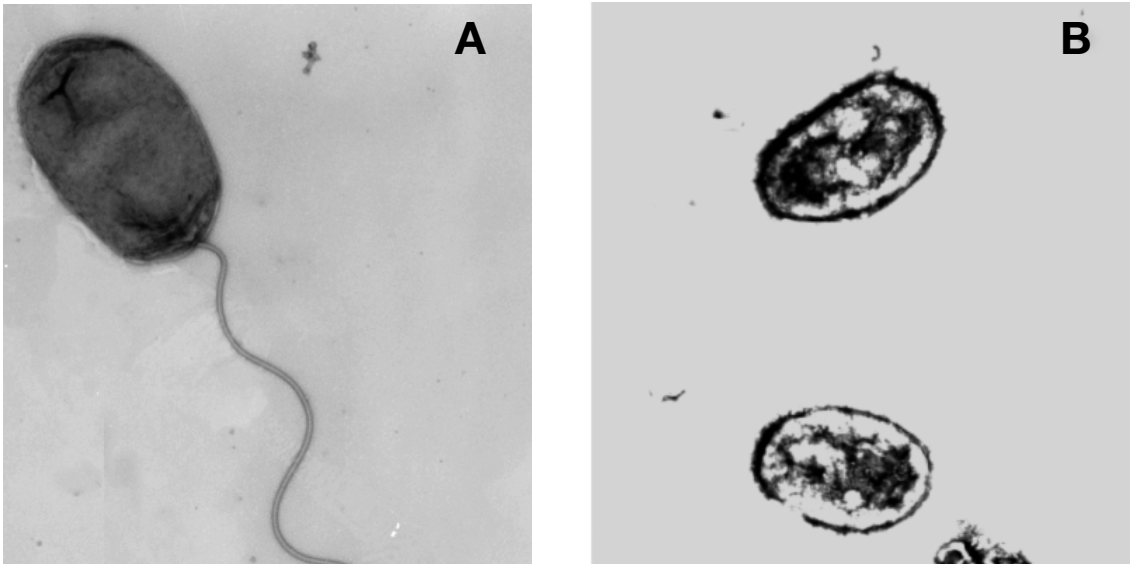

Fig. 3. Electron micrographs of negatively stained *Vibrio shiloi*. (A) Exponentially growing cells; (B) intracellularly growing cells

VBNC state is infectious, i.e., does it penetrate into coral tissue and multiply? To answer this question, a laboratory-infected coral, which contained *V. shiloi* primarily in the VBNC state, was crushed, diluted in sterile seawater and used to infect healthy corals (Table 4). Four corals were infected, each with 640 *V. shiloi* in the VBNC state and a calculated value of 0.002 cfu. After 48 h incubation, all 4 corals were infected, with average values of 2.0×10^3 cfu and 4.0×10^6 total counts. Since there was only a 1 in 500 chance that each coral would be infected if *V. shiloi* in the VBNC state was not infectious, the infection must have been the result of *V. shiloi* in the VBNC state. To reach 4.0×10^6 total cells from an inoculum of 640 would require 13 doublings. Assuming that all VBNC *V. shiloi* participated in the infection and multiplication, then the average doubling time during the first 48 h is 3.7 h. Since the previously determined doubling time of *V. shiloi* in coral tissue was 3.5 h (Banin et al. 2000), it follows that most of the VBNC bacteria must have penetrated into the tissue and multiplied.

Table 4. Infectivity of the VBNC state of *Vibrio shiloi*. Four corals, each in 50 ml seawater at 28°C, were inoculated with a million-fold dilution of a crushed coral that had been infected 96 h previously with *V. shiloi*. The number of *V. shiloi* was determined by fluorescence microscopy (total counts) and cfu

Samples examined (n)	*Vibrio shiloi*	
	cfu	Total count
Inoculum		
96 h crushed coral	2×10^3	6.4×10^8
Calculated inoculum per coral	0.002	640
Internal *V. shiloi* after:		
48 h	2.0×10^3	4.0×10^6
144 h	1.8×10^4	8.5×10^7
Non-inoculated control coral		
144 h	–	$<10^5$

DISCUSSION

The infectious cycle of *Vibrio shiloi*

The data presented here provide additional information on the infectious cycle of *Vibrio shiloi* in its coral host, *Oculina patagonica*. Previously, it has been demonstrated that (1) *V. shiloi* adheres to the coral via a β-galactoside-containing receptor on the coral surface (Toren et al. 1998), (2) penetrates into the epidermal layer of the coral (Banin et al. 2000), and (3) transforms into a VBNC state and multiplies intracellularly (Banin et al. 2000). During the summer months, when the seawater temperature is high, the bacteria remain at a high cell density, primarily in the VBNC state. In the winter, when the seawater temperature drops, the bacteria are no longer present in the coral tissue. At present, it is not known whether all of the bacteria are killed and lysed, or if some bacteria are released into the seawater. What is clear is that a fresh infection cycle must begin in the spring when the water temperature rises. Since *V. shiloi* can survive for long periods in seawater at 16°C, outside their host, it is possible that bacteria released by bleached corals provide the inoculum for new infections. Alternatively, fish and

other marine organisms that feed on corals (Miller & Hay 1998) could serve as a reservoir for *V. shiloi* during the winter months. In this regard, it is interesting that copepods are a reservoir of *Vibrio cholerae* (Lobitz et al. 2000). Studies are now being carried out on the mechanism of release of *V. shiloi* from infected corals and the mode of transmission of the coral disease.

The unique VBNC state of intracellular *Vibrio shiloi*

A large number of bacterial species have been shown to enter the VBNC state, including several *Vibrio* species: *Vibrio vulnificus* (Oliver et al. 1991), *Vibrio parahaemolyticus* (Chowdhury et al. 1990), *Vibrio cholerae* (Colwell et al. 1996), and *Vibrio fischeri* (Lee & Ruby 1995). All of these VBNC states were induced by stress conditions, such as starvation or low temperature. It has been suggested that the VBNC response may be a genetically programmed differentation which enhances survival during stress (Oliver 1993, McDougald et al. 1998). In the case of *V. shiloi*, the bacteria transform into the VBNC state shortly after they enter the epithelial cells of their coral host, in an environment which is ideal for their growth and multiplication. In fact, *V. shiloi* multiply intracellularly in the VBNC state, clearly demonstrating that they are viable. However, all attempts to obtain colonies on different agar media or growth in liquid media of VBNC *V. shiloi* have failed. It is possible that a signal present inside the coral is required for multiplication of VBNC *V. shiloi*.

It is particularly interesting that *Vibrio shiloi* in the VBNC state has the potential to infect healthy corals. Not only did the VBNC *V. shiloi* penetrate into and multiply in the corals (Table 4), but a significant proportion of them transformed back to a culturable form, i.e., cfu. We believe this is convincing evidence for resuscitation. Oliver & Bockian (1995) demonstrated that non-culturable *Vibrio vulnificus* was virulent in mice, and Colwell et al. (1996) reported that *Vibrio cholerae* resuscitated after passing through the digestive system of human volunteers. Also, *Legionella pneumophila* was transformed from the non-culturable state when passed through the amoeba *Acanthamoeba castellanii* (Steinert et al. 1997).

Effect of temperature on *Vibrio shiloi* and a novel coral defense system

There is a strong correlation between high seawater temperature and coral bleaching (Hoegh-Guldberg 1999). This is clearly the case with bleaching of *Oculina patagonica* in the Mediterranean Sea (Fig. 1). This correlation has led coral biologists to assume that the high temperature places a stress directly on the coral, causing the loss of the endosymbiotic zooxanthellae. However, the data that have been obtained from studying bleaching of *O. patagonica* by *Vibrio shiloi* indicate that high temperature exerts its effect on the pathogen rather than on the host. For example, adhesion of *V. shiloi* to *O. patagonica* requires an adhesin on the bacterial surface that recognizes a β-galactoside receptor on the coral surface. The adhesin is only produced at high temperatures, whereas the coral receptor is produced even at 16°C (Toren et al. 1998). In addition, the anti-algal toxins synthesized by the bacterium are produced at much higher levels at summer seawater temperatures than at winter temperatures (Ben-Haim et al. 1999). Furthermore, if not infected, *O. patagonica* remained unbleached and healthy for months at 30°C (several control experiments).

In the present study, it was shown that *Vibrio shiloi* inside coral tissues dies and lyzes when the water temperature is lowered in aquarium experiments or drops naturally in the sea. Since bacteria outside the coral do not die when the temperature is lowered, it follows that the coral must have a mechanism for killing intracellular bacteria. We postulate that one such mechanism is the production of superoxide free radicals (O_2^-) from the oxygen generated during photosynthesis. In support of this hypothesis, *V. shiloi* produces high levels of superoxide dismutase at 30°C and very low levels at 16°C (Banin et al. unpubl.). Furthermore, mutants of *V. shiloi* lacking superoxide dismutase adhere to corals, penetrate into the coral tissue and then rapidly die — even at 28°C.

Relevance of the *Vibrio shiloi/Oculina patagonica* model system to coral bleaching in general

Although there is no direct data for or against bacteria being the etiological agent of bleaching corals other than *Oculina patagonica*, the following circumstantial evidence suggests that bacterial bleaching of corals may be widespread. First, the pattern of *O. patagonica* bleaching closely resembles coral bleaching in other parts of the world: patchiness and spreading nature, loss of pigments and endosymbiotic zooxanthellae and reversibility when the temperature drops. By analogy with well-known animal and plant diseases, identical symptoms often suggest similar pathogens. Second, the frequently observed patchiness and spreading nature of coral bleaching (e.g., Oliver 1985, Lang et al. 1992) is more typical of an infectious agent rather than a stress phenomenon. One would expect adjacent corals in the sea to be exposed to the same conditions.

There are several phenomena discovered in the *Vibrio shiloi/Oculina patagonica* systsem that may be generally relevant to the study of coral bleaching. First, the

existence of the VBNC state may make the isolation of pathogens difficult; one way to overcome this problem may be to infect healthy corals with the internal tissues of freshly bleached corals of the same species. Second, since the first step in the infection cycle, adhesion of the bacteria to the coral, is host-specific, one would expect that different bacterial strains would be required to infect different species of corals. Third, a potential coral-bleaching pathogen should produce toxins directed at the zooxanthellae. The toxins produced by *V. shiloi* are not host-specific, since they kill zooxanthellae from different coral genera (Ben-Haim et al. 1999). Finally, we would like to stress that if the 'bacterial hypothesis of coral bleaching' is generally correct, then it must be considered in the design and interpretation of experiments and on the development of methods for preventing and curing the coral bleaching disease.

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