

Virulence mechanisms of the coral bleaching pathogen *Vibrio shiloi*

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Abstract *Vibrio shiloi* is the causative agent of bleaching of the coral *Oculina patagonica* in the eastern Mediterranean Sea. Infection and subsequent bleaching occur only when water temperatures approach their maximum of 29–30°C. Virulence mechanisms of *V. shiloi* include: (i) chemotaxis to the mucus of *O. patagonica*, (ii) adhesion to a β -galactoside-containing receptor on the coral surface, (iii) penetration into coral epidermal cells, (iv) differentiation into a viable-but-not-culturable (VBNC) state, (v) intracellular multiplication, and (vi) production of toxins that inhibit photosynthesis, and bleach and lyse zooxanthellae. The toxin that inhibits photosynthesis is a proline-rich, 12 amino acid peptide. The adhesion, multiplication and toxin production steps occur only at high temperature, providing a biochemical explanation for the effect of temperature on the bleaching of *O. patagonica*. The generality of the bacterial bleaching hypothesis is discussed in terms of existing indirect evidence and how the hypothesis can be tested using information from the *V. shiloi*/*O. patagonica* model system.

Keywords Coral bleaching, *Vibrio*, VBNC, *Oculina*, virulence

Introduction

Previously, we have demonstrated that the causative agent of bleaching of the coral *Oculina patagonica* in the Mediterranean Sea is *Vibrio shiloi* (Kushmaro et al. 1996, 1997). In addition to applying Koch's postulates, it was shown that antibiotics block the infection and subsequent bleaching in controlled aquaria experiments. Based on DNA:DNA hybridization, 16S rDNA sequence data, and classical microbiology, *V. shiloi* has been

classified as a new species of the genus *Vibrio* (Kushmaro et al. 2001).

The effect of seawater temperature on bacterial bleaching of *Oculina patagonica* has been studied in the field and in aquaria experiments (Kushmaro et al. 1998). In the western Mediterranean Sea, *O. patagonica* undergoes massive bleaching (more than 70% of the colonies show extensive bleaching) each summer when the seawater temperature reaches a maximum of 29–30°C. In the winter when the seawater temperature drops to 16°C, the corals recover (less than 5% of the colonies show bleaching). This cyclic behavior has occurred every year since bleaching was first observed in 1993 (Fine and Loya 1995). In general, there is a strong correlation between high seawater temperature and coral bleaching (e.g., Brown 1997; Glynn 1991; Hoegh-Guldberg 1999; Jokiel and Coles 1991). In the case of bleaching *O. patagonica* by *Vibrio shiloi*, as few as 120 bacteria caused bleaching at 29°C, whereas no bleaching occurred at 29°C when no *V. shiloi* were present, nor at 16°C when the corals were inoculated with 10^7 *V. shiloi* (Banin et al. 2000a). Thus, bleaching of *O. patagonica* requires both the causative agent, *V. shiloi*, and the permissive environmental condition, high water temperature.

The previously published data summarized above lead to three key questions:

- (1) How does infection with *Vibrio shiloi* result in coral bleaching, i.e., what are the virulence mechanisms?
- (2) How does temperature affect the process? At the biochemical level, what is the effect of temperature on virulence gene expression?

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- (3) How general is bacterial bleaching of corals? Is it restricted only to *Oculina patagonica*, or is it the major mechanism by which corals bleach globally?

In this paper we will present experiments which have provided some fundamental answers to the first two questions, concentrating on how the information obtained by studying the *V. shiloi*/*O. patagonica* model system might be useful in testing the general bacterial hypothesis of coral bleaching.

Methods

Microorganisms and corals

Vibrio shiloi (ATCC BAA-91) was isolated from bleached coral and maintained as described previously (Kushmaro et al. 1996, 1997). Intact colonies of *Oculina patagonica* were collected from depths of 1 to 3 meters along the Mediterranean coast of Israel. 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 lighting regime. Coral fragments were allowed to recover and regenerate for 15 days. The corals were then shifted gradually to the experimental temperature and allowed to equilibrate for at least a week prior to infection.

Chemotaxis, adhesion and penetration of *Vibrio shiloi* onto and into *Oculina patagonica*.

An overnight culture of *V. shiloi*, grown at 30°C in Marine Broth (Difco) with aeration, was centrifuged, the pellet washed and then resuspended in sterile seawater. Chemotaxis was determined by a modification (Banin et al. 2001b) of the standard capillary method (O'Toole et al. 1999). The bacteria were inoculated ($1.6 \times 10^5 \text{ ml}^{-1}$ or a total of 4.0×10^6) into 125 ml flasks containing fragments of *Oculina patagonica* in 25 ml sterile seawater. Adhesion was determined by removing water samples at timed intervals and plating on MB Agar as described previously (Toren et al. 1998). Penetration of *V. shiloi* into the coral tissue was determined by a modification (Banin et al. 2000b) of the gentamycin invasion assay (Isberg and Falkow 1985). The infected coral was removed from the flask or aquarium, rinsed with sterile seawater and transferred to 5 ml seawater containing 1 mg gentamycin and 0.5 mg methyl- β -D-galactopyranoside, in order to desorb and kill non-internalized bacteria. After 3 h at 29°C, the coral was removed, rinsed and crushed in 5 ml of seawater with a mortar and pestle. Colony forming units were determined by plating an appropriate dilution on MB agar. Total counts were determined microscopically after staining with a specific polyclonal anti-*V. shiloi* antiserum as described below.

Microscopy

Crushed coral samples (0.5 ml) were fixed with freshly prepared 4% paraformaldehyde in seawater for 1-3 h. The fixed samples were then washed 3 times in TBS (10 mM Tris-HCl, pH 7.5, and 150 mM NaCl) and attached to microscope slides covered with poly-L-lysine (50-100 $\mu\text{g/ml}$). After incubation for 1 h, the slides were washed once in TBS and incubated for 12 h at 4°C with *V. shiloi*-specific polyclonal antibodies (1:500 dilution in TBS). Preparation of the antibody has been described previously (Banin et al. 2000b). The slides were then washed 3 times in TBS and incubated with 5 $\mu\text{g/ml}$ anti-rabbit IgG-Amca conjugated (Jackson Immuno Research, West Grove, Penn. USA). After the incubation, the slides were washed 3 times in TBS and mounted with a solution of 90% glycerol containing 1 $\mu\text{g/ml}$ p-phenylenediamine (Sigma, St. Louis). Cover slips were sealed and the sample stored at -20°C until examination. Examination was carried out using a Leica fluorescence microscope (model DMR) with filter A (UV) for Amca.

For examining coral sections, samples at different stages of infection were fixed in 4% formaldehyde in seawater for 24 h, rinsed in fresh water and transferred to 70% ethanol for preservation. Decalcification was carried out using a solution of formic acid and sodium citrate for 15-25 h. After decalcification, the tissue was rinsed in fresh water and transferred into 70% ethanol. Embedding of the tissue in paraffin was done using a Citadel Embedding apparatus. Sections (4-6 μm thick) of polyps were attached to microscope slides covered with poly-L-lysine and then stained with the antibodies as described above.

For electron microscopy, coral fragments were fixed in 2.5% glutaraldehyde in 0.2 μm filtered seawater and decalcified in a mixture of equal volumes of formic acid (50%) and sodium citrate (15%) for 15 h. They were then dehydrated in graded series of ethyl alcohol and embedded in Epon. Sections stained with uranyl acetate and lead citrate were viewed with a Jeol 1200 EX electron microscope.

Measurement of photosynthesis inhibition

A portable underwater Mini Pulse-Amplitude-Modulation (PAM) Fluorometer (Walz GmbH, Germany) was used to measure the quantum yield of zooxanthellae. This instrument allows for the direct non-invasive measurement of effective quantum yield (Y) of photosystem II under ambient light (Genty et al. 1990). In the experimental procedure used here, the quantum yield of 0.05 ml freshly isolated zooxanthellae in seawater (5×10^6 algae ml^{-1}) was measured in an Elisa plate at room temperature with the Mini-PAM (Y_0). Then, 0.05 ml of the experimental sample was added to the algae, and quantum yield was measured after 5 min (Y_t). The percent quantum yield at the different times was $Y_t/Y_0 \times 100$.

Results

The following sequential steps are involved in the infection of *Oculina patagonica* by *Vibrio shiloi*: chemotaxis to the coral, adhesion to the coral surface, penetration of *V. shiloi* into coral epithelial cells, differentiation of *V. shiloi* into a viable-but-not-culturable (VBNC) state, intracellular multiplication and toxin production.

Chemotaxis and adhesion

Using the capillary tube assay (O'Toole et al. 1999), *Vibrio shiloi* showed positive chemotaxis towards mucus obtained from *Oculina patagonica*. In a typical experiment, the number of *V. shiloi* attracted to the mucus was 26 ± 3 fold higher than seawater control. Once *V. shiloi* makes contact with *O. patagonica* it adheres avidly to a β -galactoside-containing receptor on the coral surface (Toren et al. 1998). Adhesion occurs during the first eight hours after inoculation (Fig. 1) and is a prerequisite for successful infection because non-adhering mutants of *V. shiloi* fail to bleach the corals (Rosenberg et al. 1998). The β -galactose-containing receptor is present in the mucus of *O. patagonica*, since *V. shiloi* adheres to slides coated with the mucus, and the adhesion is inhibited by methyl- β -D-galactoside. Interestingly, the bacteria do not adhere to bleached corals (Banin et al. 2001b).

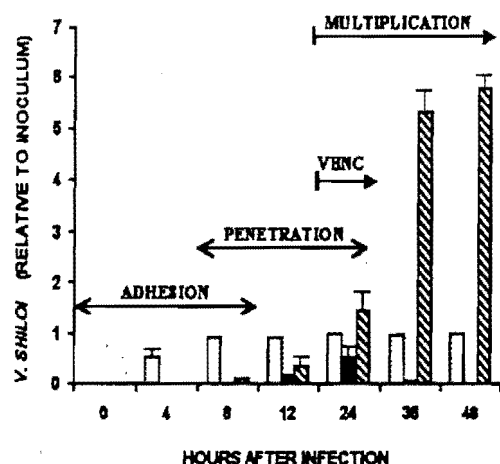


Fig. 1 *Vibrio shiloi* infection of *Oculina patagonica* at 29°C. Healthy pieces of coral were inoculated with a total of 4.0×10^6 *V. shiloi* and incubated with gentle shaking. Adhesion (□), internal total (▨) and internal colony forming units (■) were determined as described in the Methods. The value of *V. shiloi* cells is presented relative to the number inoculated.

Adhesion of *Vibrio shiloi* is temperature dependent. When the bacteria were grown at 16°C, they failed to adhere to corals maintained either at 16°C or 28°C, whereas bacteria grown at 28°C adhered to the corals contained at either temperature (Toren et al. 1998). The fact that adhesion is required for infection and that the bacteria do not express their adhesion genes at the winter seawater temperature (16°C), provided the first biochemical mechanism to explain the effect of temperature on bleaching *Oculina patagonica* by *V. shiloi*.

Penetration and multiplication

Electron micrographs of thin sections of *Oculina patagonica* following infection with *Vibrio shiloi* demonstrated large numbers of bacteria in the epidermal layer of the coral (Banin et al. 2000b). Using antibodies specific to *V. shiloi*, it was shown that the observed intracellular bacteria were, in fact, *V. shiloi* (Banin et al. 2000b). The gentamycin invasion assay was used to measure the kinetics of *V. shiloi* penetration into the epidermal cells (Fig. 1). After adhesion was complete (ca. 8 h), the bacteria began to penetrate inside the coral as determined by both total counts and colony forming units. Based on total counts, *V. shiloi* multiplies intracellularly from 24 h to ca. 1 week (Fig. 1, Table 1) reaching ca. 10^9 bacteria cm^{-3} .

The effect of temperature on the intracellular growth and death of *Vibrio shiloi* is summarized in Table 1. Corals that were infected with *V. shiloi* at 28°C contained 7×10^3 cm^{-3} *V. shiloi* after 3 d. When they were maintained at 28°C, the concentration of bacteria increased further for a week and remained high for 17 d. However, when the temperature was decreased 2°C/d to 16°C, the bacteria stopped growing and died off, so that by 17 d, they were no longer detectable. Controls of *V. shiloi* in seawater or MB medium (data not shown) showed less than a 2-fold decrease in counts when exposed to the same temperature decrease. Thus, the coral must have a mechanism for killing and lysing intracellular bacteria.

Differentiation of *Vibrio shiloi* into the viable-but-not-culturable (VBNC) state

A comparison of the colony forming units and total counts (Fig. 1) indicate that most of the *Vibrio shiloi* that were enumerated by fluorescence microscopy after 24 h of infection were unable to form colonies on MB agar.

These bacteria are clearly viable because they are actually multiplying intracellularly. Recently, we demonstrated that *V. shiloi* in the VBNC state is infectious, i.e., they can adhere, penetrate and multiply inside *O. patagonica* (Israely et al. 2001).

Toxin Production

Vibrio shiloi produces extracellular toxins that block photosynthesis, and bleach and lyse zooxanthellae (Ben-Haim et al, 1999). The

Table 1 Effect of temperature on *Vibrio shiloi* inside coral tissue^a (Banin et al)

	Incubation (days)	Temperature (°C)	Intracellular <i>V. shiloi</i> ^b	
			(cells/cm ²)	(Relative to 3 d)
I	3	28	7 × 10 ⁸	1.0
	4	28	2 × 10 ⁹	2.9
	5	28	4 × 10 ⁹	5.7
	7	28	6 × 10 ⁹	8.6
	17	28	5 × 10 ⁸	0.7
II	3	28	7 × 10 ⁸	1.0
	4	26	4 × 10 ⁸	0.6
	5	24	2 × 10 ⁸	0.3
	7	20	1 × 10 ⁸	0.1
	17	16	< 10 ⁵	< 0.001

^a The fragments were maintained at 28°C (I) and half-healthy fragments of *Oculina patagonica* were infected with 5 × 10⁶ *V. shiloi* ml⁻¹ at 28°C. After 3 d, half of them were transferred to a separate aquarium in which the temperature was reduced at a rate of 2°C/d (II).

^b Intracellular *V. shiloi* concentration was determined by the gentamycin invasion assay, using fluorescence microscopy to enumerate total counts.

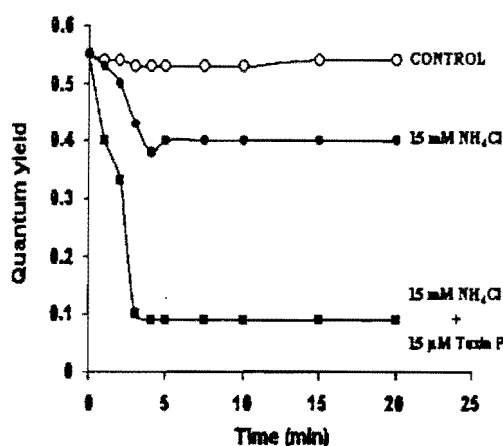


Fig. 2 The inhibition of photosynthetic quantum yield of zooxanthellae by Toxin P. The algae were incubated in 15 mM NH₄Cl (●), 15 mM NH₄Cl plus 15 μM Toxin P (PYPVYAPPPVVP) (■) or seawater (○). Quantum yield was measured fluorometrically as described in the Methods.

toxin responsible for inhibition of photo-synthesis is the following proline-rich peptide: PYPVYAPPPVVP (Banin et al. 2001a). In the presence of NH₄Cl, the toxin causes a rapid decrease in the photosynthetic quantum yield of zooxanthellae (Fig. 2). Evidence has been presented that the toxin binds to algal membranes, forming a channel that allows NH₃ to rapidly pass, and thereby destroying the pH gradient across the membrane and blocking photosynthesis (Banin et al. 2001a). This mode of action can help explain the mechanism of coral bleaching by *V. shiloi*.

Vibrio shiloi also produces heat-sensitive, high molecular weight toxins that bleach and lyse isolated zooxanthellae. These toxins have not yet been isolated and characterized. The heat-stable peptide toxin and the heat-sensitive toxin(s) are produced at much higher levels at 28°C than at 16°C (Rosenberg et al. 1998).

Discussion

Although considerable data have been obtained on the virulence mechanisms of the *Vibrio shiloi*/*Oculina patagonica* model system and how temperature affects these processes, very little information is available on the generality of the bacterial hypothesis of coral bleaching. The following indirect evidence suggests coral bleaching is an infectious disease and not the direct effect of environmental stress on the coral:

1. **Patchy spatial distribution.** It has been argued that the random mosaic patterns of bleaching are difficult to attribute to the effect of temperature stress, since neighboring regions of the colony must be exposed to the same extrinsic conditions (Hayes and Bush, 1990). There is no evidence that neighboring corals of the same species, where one is bleached and the other not, are genetically different.

2. **Spreading.** Several authors have reported on the spreading nature of coral bleaching (e.g., Fisk and Done, 1985; Jokiel and Coles, 1990; Lang et al. 1992). Spreading is highly symptomatic of bacterial and viral infectious diseases.

3. **Analogy with bleaching of *Oculina patagonica*.** Bleaching of *O. patagonica* appears similar to the bleaching of other corals – loss of zooxanthellae, induction by high seawater temperature and reversibility when the temperature decreases. In pathology, diseases that exhibit similar symptoms most often are caused by similar (although not identical) pathogens.

4. **The effect of ultraviolet radiation.** Investigators have observed that corals in shallow reefs and tidal pools show lower bleaching than the same coral species in deeper water (e.g., Loya et al. 2001). Also, Brown et al (2000) showed that the shallow water coral *Goniastrea aspera* exposed to high solar radiation prior to maximum seawater temperature was protected from bleaching. In the case of *Oculina patagonica*, shallow water corals show very little bleaching, even at high temperature (Fine et al. unpublished). In this latter case, it was shown that the high ultraviolet light in the shallow water killed over 99.9% of the intracellular *Vibrio shiloi*. In general, bacteria are much more sensitive to ultraviolet radiation than corals or zooxanthellae.

5. **The effect of temperature.** Although there is a general correlation between high seawater temperature and coral bleaching, the correlation is

not absolute. For example, Oliver (1985) and Fisk and Done (1985) showed that extensive bleaching in the Great Barrier Reef during 1982 was not associated with any major sea surface temperature increase. There are also numerous examples of corals that did not bleach when exposed to high temperature. One interpretation of these data is that environmental stress is insufficient; the infectious agent must also be present for bleaching to occur. Of the numerous diseases that have documented links with climatic factors, most are water-borne infections and toxin-related illnesses (Colwell 1996). Black band disease in corals, caused by a microbial consortium dominated by the cyanobacterium *Phormidium corallyticum*, is prevalent in the summer when seawater temperatures are above 28°C (Edmunds 1991; Kuta and Richardson 1996).

The above arguments are clearly not decisive and direct experimental proof is necessary to support the generality of the bacterial bleaching hypothesis. Several conclusions obtained from the *Oculina patagonica*/*Vibrio shiloi* model system should be useful in designing these experiments. From the studies on the specificity of adhesion, one would expect that different strains of bacteria are responsible for bleaching different genera, and possibly different species, of corals. If a bacterium can not adhere to a specific coral, it can not infect it and cause bleaching. Second, for the potential pathogen to attack the zooxanthellae, it must penetrate into the coral tissue. Thus, we suggest that potential pathogens be isolated from the interior of corals undergoing bleaching. The coral mucus contains a vast array of nonpathogenic bacteria (Ducklow and Mitchell 1979; Ritchie et al. 1994) that could interfere with the isolation of a pathogen. Third, the VBNC state of intracellular bacteria could complicate the isolation of a potential pathogen. One way to overcome this problem would be to use crushed bleached corals to isolate particles the size of bacteria in order to infect healthy corals of the same species. Finally, the presence of anti-algal toxins following infection should be indicative of a potential pathogen.

Hopefully, during the next few years the bacterial hypothesis of coral bleaching will be tested. The answer to this question is not trivial because it impinges directly on the design and interpretation of experiments and possible methods for prevention and cure of coral bleaching. For example, if bacteria are responsible for coral bleaching, then the most likely method to prevent the disease is interfering with its mode of transmission from one coral to another.

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