Role of endosymbiotic zooxanthellae and coral mucus in the adhesion of the coral-bleaching pathogen Vibrio shiloi to its host

Ehud Banin a, Tomer Israely a, Maoz Fine b, Yossi Loya b, Eugene Rosenberg a,*

* Department of Molecular Microbiology and Biotechnology, Tel Aviv University, Ramat Aviv 69978 Tel Aviv, Israel
b Department of Zoology, Tel Aviv University, Ramat Aviv 69978 Tel Aviv, Israel

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Abstract

Vibrio shiloi, the causative agent of bleaching the coral Oculina patagonica in the Mediterranean Sea, adheres to its coral host by a β-D-galactopyranoside-containing receptor on the coral surface. The receptor is present in the coral mucus, since V. shiloi adhered avidly to mucus-coated ELISA plates. Adhesion was inhibited by methyl-β-D-galactopyranoside. Removal of the mucus from O. patagonica resulted in a delay in adhesion of V. shiloi to the coral, corresponding to regeneration of the mucus. DCMU inhibited the recovery of adhesion of the bacteria to the mucus-depleted corals, indicating that active photosynthesis by the endosymbiotic zooxanthellae was necessary for the synthesis or secretion of the receptor. Further evidence of the role of the zooxanthellae in producing the receptor came from a study of adhesion of V. shiloi to different species of corals. The bacteria failed to adhere to bleached corals and white (azooxanthellate) O. patagonica cave corals, both of which lacked the algae. In addition, V. shiloi adhered to two Mediterranean corals (Madracis and Cladocora) that contained zooxanthellae and did not adhere to two azooxanthellate Mediterranean corals (Phyllangia and Polycyathus). V. shiloi demonstrated positive chemotaxis towards the mucus of O. patagonica. The data demonstrate that endosymbiotic zooxanthellae contribute to the production of coral mucus and that V. shiloi infects only mucus-containing, zooxanthellate corals. ß 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Coral bleaching is a widespread phenomenon that occurs in the world’s three major oceans and involves more than 50 countries [1]. Coral bleaching results from the disruption of the symbiotic association between the coral hosts and their photosynthetic microalgal endosymbionts (zooxanthellae). The loss of the pigmented zooxanthellae causes the coral to turn white. If the process is not reversed, the coral will die because a major portion of its nutrition comes from photosynthetic products of the algae [2]. Coral bleaching events of unprecedented frequency and global extent have been reported in the last 20 years [1]. It has been suggested that coral bleaching is triggered by environmental factors, especially increased seawater temperature [3–6] that impose stress on the coral. Thus, it is possible that global warming could result in alterations to, or destruction of, coral reefs, the consequences of which could be devastating to tourist and fishing industries, to coastlines that are protected by coral reefs and, most importantly, to the health of the sea. Thus, it is essential to understand the mechanism(s) of coral bleaching.

The coral Oculina patagonica exists as colonies throughout the Mediterranean Sea in shallow reefs and tidal pools to depths of greater than 10 m. For at least the last 6 years there has been massive bleaching of O. patagonica every summer in the eastern Mediterranean, when seawater temperatures approach a maximum of 30°C. When the temperature drops in the fall and winter, the corals recover. We have demonstrated that the causative agent of bleaching of O. patagonica is a new species of Vibrio, Vibrio shiloi [7–9]. The infectious process requires at least five distinct and sequential steps: (i) Adhesion of the bacte-
rium to the coral surface [10], (ii) penetration into the coral epidermal layer [11], (iii) differentiation into a viable but not culturable (VBN) state [12], (iv) intracellular multiplication, reaching over $10^8$ bacteria per cm$^3$ coral tissue [11,12] and (v) production of extracellular toxins that inhibit photosynthesis, bleach and lyse zooxanthellae [13,14]. The adhesion, intracellular multiplication and toxin production steps are all temperature dependent, i.e., the necessary virulence factors are produced only when the bacteria are at high (summer) temperatures [10,15,16]. Thus, the data from the *V. shiloi*/*O. patagonica* model system of coral bleaching indicate that increased seawater temperature triggers coral bleaching, not by acting directly on the coral, but rather by causing *V. shiloi* to become virulent.

The first step in the infection process is the adhesion of *V. shiloi* to a β-d-galactopyranoside-containing receptor on the coral surface [10]. This step is essential for infection and subsequent bleaching because non-adhering mutants are not virulent [15]. To better understand the mechanism and specificity of the adhesion, we have examined the roles of the coral mucus and the endosymbiotic zooxanthellae in this process. The data demonstrate that the β-d-galacto-side-containing receptor is in the coral mucus and that zooxanthellae must be present and photosynthetically active for the receptor to be produced and secreted.

2. Materials and methods

2.1. Microorganism and growth media

*V. shiloi* (ATCC BAA-91) was maintained on MB agar (1.8% Marine broth plus 0.9% NaCl solidified with 1.8% agar (both products of Difco Laboratories, Detroit, MI, USA)). After being streaked onto MB agar, the cultures were incubated at 30°C for 2 days and then allowed to stand at room temperature for 1 week. MBT medium is MB medium supplemented with 0.5% tryptone. *V. shiloi* grows more rapidly and achieves a higher cell density in MBT medium than MB medium.

2.2. Collection and maintenance of corals

Colony fragments of specified corals (ca. 1 cm$^3$) were collected from depths of 0.5-10-m sites along the Israeli coast of the Mediterranean Sea. Azooxanthellate *O. patagonica* cave corals (white) were collected at Rosh Hanikra, Israel. Within 1 h of collection, the coral fragments were placed in aerated aquaria containing filtered seawater at 25°C. The aquaria were illuminated with a fluorescent lamp at 12 h light: 12 h dark intervals (160 μmol quanta m$^{-2}$ s$^{-1}$). Cave corals were maintained in covered aquaria protected from light. Coral pieces were allowed to recover and regenerate for 15 days 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.

2.3. Chemotaxis

A modification of the quantitative capillary assay [17] was used to measure *V. shiloi* chemotaxis. Bacteria were grown at 30°C in MBT medium overnight, harvested by centrifugation at 6000 × g for 5 min and resuspended in an equal volume of sterile seawater. This procedure was repeated three times in order to remove residual medium. A suspension of washed bacteria in seawater (ca. $10^7$ cells ml$^{-1}$) was dispensed in 0.2-ml aliquots into 1.5-ml Eppendorf tubes. A 1-μl capillary tube, heat-sealed at one end and containing the substrate to be tested (seawater control or undiluted coral mucus), was inserted 1 mm below the culture surface. After incubating for 60 min at room temperature, the capillaries were removed, the outside rinsed with sterile seawater, and their contents expelled into 0.3 ml of sterile seawater. Viable counts were performed on MB agar. The chemotactic activity of the mucus was expressed as the ratio of the bacterial density inside the capillary containing the mucus compared to the density of bacteria in control seawater capillaries. Mucus for these experiments was collected by placing pieces of coral into a funnel and allowing the secreted mucus to drip into a tube. The mucus was then filtered through a 0.2-μm Millipore filter.

2.4. Adhesion to corals

An overnight culture of *V. shiloi*, grown at 30°C in MB medium with aeration, was centrifuged at 5000 × g for 10 min, the cells washed twice in sterile seawater and then resuspended to ca. $10^9$ cells per ml. The bacteria were inoculated into a 125-ml flask containing fragments of coral (~1 cm$^3$) in 25 ml sterile seawater to an initial bacterial concentration of $2 \times 10^7$. The flasks were incubated at 29 ± 1°C with gentle shaking on a ‘Belly Dancer’ (Stowell Life Sciences Inc., Greensboro, NC, USA). During the incubation period, the coral fragments were illuminated with a fluorescent lamp (160 μmol quanta m$^{-2}$ s$^{-1}$) on cycles alternating 12 h of light and 12 h of darkness. Adhesion was determined by removing liquid samples at timed intervals and plating on MB agar. In each experiment, a no-coral control was performed and the percent bacteria adhering to the flask alone was subtracted from the experimental value to obtain net adhesion. Each time point represents the average of three independent experiments with standard errors from 0 to 5%. Several adhesion experiments were carried out with mucus-depleted corals in which the mucus was allowed to drip off the coral and then extensively washed with seawater. 3-(3,4-dichloro-phenyl)-1,1-Dimethyl urea (DCMU), used to inhibit algal photosynthesis in some of the adhesion experiments, was a product of Sigma Co., St. Louis, MO, USA.
2.5. Adhesion to mucus

Mucus removed from a healthy zooxanthellate coral was fixed to a 96-well ELISA plate using a 0.1-M sodium carbonate fixation buffer, pH 9.0. After fixation, the plate was washed three times with TBS (10 mM Tris, pH 7.4, 150 mM NaCl) buffer and then blocked with 1% skim milk for 12 h in the cold. The plate was then washed an additional three times with TBS buffer. An overnight culture of *V. shiloi* was centrifuged and the cells washed two times in sterile seawater. Then the washed cells (0.2 ml containing $4 \times 10^5$ bacteria) were added to each well and incubated with slow agitation at 29°C. Bacteria remaining in the seawater (unbound) were determined by viable counts at timed intervals. Net adhesion was calculated by comparison to a sample in a control well containing no mucus. Each time point is the average of three determinations, with a standard error of 0–3%. Where indicated 0.01% methyl-β-D-galactopyranoside was added to the cell suspension to check inhibition of adhesion.

3. Results

3.1. Chemotaxis of *V. shiloi* to *O. patagonica* mucus

Using the capillary tube assay, *V. shiloi* showed positive chemotaxis towards mucus obtained from *O. patagonica*. When the bacteria were pregrown at 25°C, the number of *V. shiloi* in the capillary (attributed to the mucus) was $9.0 \times 10^4$, compared to $0.3 \times 10^4$ for the seawater control, or a 30-fold enhancement. When the bacteria were pregrown at 16°C, the concentration of *V. shiloi* in the capillary was $4.6 \times 10^4$, compared to $0.3 \times 10^4$ for the seawater control, or a 15-fold enhancement. The same data were obtained with undiluted mucus obtained from corals maintained at 16 and 25°C.

3.2. Adhesion of *V. shiloi* to healthy, bleached and azooxanthellate *O. patagonica*

*O. patagonica* colonies can be found in the Mediterranean Sea in three forms – normal healthy colonies, bleached colonies and cave colonies. The cave colonies are apparently healthy corals that are white because they do not have any zooxanthellae. Nevertheless, they can maintain themselves under the dark conditions of the cave. Since we could never isolate *V. shiloi* from cave corals, it was decided to check the ability of *V. shiloi* to adhere to these corals. The results, summarized in Fig. 1, demonstrate that *V. shiloi* can not adhere to cave *O. patagonica*, nor to bleached corals. Thus, the presence of zooxanthellae inside the coral is required for the bacteria to adhere to the coral surface.

3.3. Adhesion of *V. shiloi* to *O. patagonica* mucus

One of the obvious differences between bleached and cave colonies compared to healthy pigmented colonies of *O. patagonica* is the amount of mucus secreted by the corals. Healthy pigmented corals secrete large amounts of mucus compared to cave and bleached colonies. This led us to check the role of the mucus in adhesion of *V. shiloi* to *O. patagonica*. Mucus was removed from a healthy coral and fixed to wells in an ELISA plate. When suspensions of *V. shiloi* in seawater were placed in the wells, the bacteria adhered rapidly to the coral mucus (Fig. 2). Methyl-β-D-galactopyranoside blocked the adhesion, indicating that the β-D-galactoside-containing receptor is found in the coral mucus. In order to better understand the role of the coral mucus and the zooxanthellae in the adhesion process, several pieces of corals were treated to remove the mucus and then incubated with 50 μM
DCMU to inhibit photosynthesis of zooxanthellae during the adhesion experiment (Fig. 3). The kinetics of adhesion to mucus-depleted corals was much slower than to corals containing the normal amount of mucus. The fact that mucus-depleted corals treated with DCMU did not allow any significant adhesion suggests the key role of photosynthetically active zooxanthellae in the production of the L-D-galactoside-containing receptor in the mucus.

3.4. Adhesion of V. shiloi to other coral species from the Mediterranean Sea

The adhesion of V. shiloi to five different coral species is summarized in Table 1. Three of the corals from the Mediterranean Sea (Madracis, Cladocora and O. patagonica) possess zooxanthellae, while the other two (Phyllangia and Polycyathus) do not contain the algae. V. shiloi adhered only to the Mediterranean corals that contained zooxanthellae.

Table 1

<table>
<thead>
<tr>
<th>Coral</th>
<th>Source</th>
<th>Presence of zooxanthellae</th>
<th>Adhesion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>O. patagonica</td>
<td>Mediterranean Sea</td>
<td>+</td>
<td>90</td>
</tr>
<tr>
<td>Madrasis pharensis</td>
<td>Mediterranean Sea</td>
<td>+</td>
<td>79</td>
</tr>
<tr>
<td>Cladocora caespitosa</td>
<td>Mediterranean Sea</td>
<td>+</td>
<td>87</td>
</tr>
<tr>
<td>Phyllangia mouchezii</td>
<td>Mediterranean Sea</td>
<td>–</td>
<td>5</td>
</tr>
<tr>
<td>Polycyathus mulerae</td>
<td>Mediterranean Sea</td>
<td>–</td>
<td>10</td>
</tr>
</tbody>
</table>

Adhesion was measured by determining the number of V. shiloi cells remaining in the seawater compared to the initial inoculum, as described in Section 2.

4. Discussion

Previous studies have demonstrated that V. shiloi adheres to its coral host, O. patagonica, via a β-p-galactoside-containing receptor on the coral surface [10]. The data presented here demonstrate that the receptor is present in the coral mucus and that actively photosynthesizing zooxanthellae inside the coral are required for the receptor to be present in the mucus. Indirect evidence that the primary receptor is in the coral mucus came from examining the adhesion of V. shiloi to coral fragments that had most of their mucus removed. Bacterial adhesion to the mucus-depleted coral was greatly inhibited compared to normal mucus-containing corals during the first 8 h after inoculation, but gradually approached 100% adhesion as the coral produced and secreted fresh mucus. When the photosynthesis inhibitor DCMU was added to the mucus-depleted O. patagonica, the coral did not produce fresh mucus and no adhesion occurred. Direct evidence that the β-p-galactoside-containing receptor was in the mucus came from demonstrating that V. shiloi adhered avidly to mucus-coated plastic surfaces and that the adhesion was inhibited by methyl-β-p-galactopyranoside.

The discovery that V. shiloi adhered only to O. patagonica that contained photosynthetically active zooxanthellae is consistent with other data indicating that the bacteria are directed against the algae. V. shiloi produces extracellular toxins that inhibit photosynthesis, bleach and lyse the zooxanthellae [13,14]. There is no evidence that the bacteria damage the coral tissue. In fact, except for the loss of the endosymbiotic algae, the coral tissue generally appears healthy following V. shiloi infection. In the winter, when the seawater temperature drops, V. shiloi is no longer present in the coral [12] and the coral re-establishes its symbiosis with zooxanthellae.

It is at present not clear whether the intracellular algae synthesize the β-p-galactoside-containing receptor, or whether the algae induce the coral to produce the receptor. The observation that V. shiloi adheres to two other corals in the Mediterranean Sea that contain zooxanthellae suggests that the zooxanthellae may provide the specificity for receptor synthesis. It is known that the composition of mucus is coral species specific [18]. The finding that arabino, a sugar not commonly found in animal cells, was...
present in coral mucus [19] suggested that the zooxanthellae may contribute carbohydrates directly to the mucus.

One of the toxins that _V. shiloi_ produces is a proline-rich dodecapeptide that inhibits photosynthesis. The fact that inhibition of photosynthesis by DCMU as well as incubation in the dark (data not presented) inhibited the production of the β-D-galactoside-containing receptor, and that the dodecapeptide is produced in the coral tissue during infection [14], may suggest a mechanism in which intracellular _V. shiloi_ control expression of receptor production and thereby block adhesion of other bacteria to the coral once the infection process has begun.

Chemotaxis of other _Vibrio_ pathogens to their host mucus has been reported. _V. cholera_ exerts a chemotactic response towards the intestinal mucus layer which has been correlated with the pathogen’s competence in penetrating the mucus and reaching the deep intervillous space [20, 21]. _Vibrio anguillarum_, a fish pathogen, exhibits chemotaxis to different components in the fish mucus [17]. In the case of chemotaxis of _V. shiloi_ to the mucus of _O. patagonica_, we have not yet investigated the component(s) in the mucus responsible for the positive chemotaxis. Since proteases are induced when _V. shiloi_ comes into contact with the mucus (unpublished data), it may be that the chemotaxis is towards peptides and/or amino acids.

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### References