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Penetration of the Coral-Bleaching Bacterium *Vibrio shiloi* into *Oculina patagonica*

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Inoculation of the coral-bleaching bacterium *Vibrio shiloi* into seawater containing its host *Oculina patagonica* led to adhesion of the bacteria to the coral surface via a β-D-galactose receptor, followed by penetration of the bacteria into the coral tissue. The internalized *V. shiloi* cells were observed inside the exodermal layer of the coral by electron microscopy and fluorescence microscopy using specific anti-*V. shiloi* antibodies to stain the intracellular bacteria. At 29°C, 80% of the bacteria bound to the coral within 8 h. Penetration, measured by the viable count (gentamicin invasion assay) inside the coral tissue, was 5.6, 20.9, and 21.7% of the initial inoculum at 8, 12, and 24 h, respectively. The viable count in the coral tissue decreased to 5.3% at 48 h, and none could be detected at 72 h. Determination of *V. shiloi* total counts (using the anti-*V. shiloi* antibodies) in the coral tissue showed results similar to viable counts for the first 12 h of infection. After 12 h, however, the total count more than doubled from 12 to 24 h and continued to rise, reaching a value 6 times that of the initial inoculum at 72 h. Thus, the intracellular *V. shiloi* organisms were transformed into a form that could multiply inside the coral tissue but did not form colonies on agar medium. Internalization of the bacteria was accompanied by the production of high concentrations of *V. shiloi* toxin P activity in the coral tissue. Internalization and multiplication of *V. shiloi* are discussed in terms of the mechanism of bacterial bleaching of corals.

During the past two decades there have been an increasing number of reports of a disease of corals referred to as coral bleaching (2, 8, 10). Coral bleaching is the disruption of the symbiotic association between the coral hosts and their photosynthetic microalgal endosymbionts (zooxanthellae). Bleaching or paling of corals results from a reduction in the density of zooxanthellae in the coral's gastrodermal tissues and/or from decreased concentrations of photosynthetic pigments in the algal cells (6). The loss of zooxanthellae greatly affects the coral host, because these photosynthetic symbionts supply as much as 63% of the coral's nutrients (5). Algae remaining in bleached corals suffer severe damage to photosystem II (24).

Coral bleaching is a widespread disease that occurs in the world's three major oceans and involves more than 50 countries (25). 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 (2, 5, 9, 12, 15). 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, to 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.

Recently, it was reported that bleaching of the coral *Oculina patagonica* from the Mediterranean Sea is the result of a bacterial infection (13–15, 20). The causative agent, *Vibrio shiloi* (1, 20), was obtained in pure culture and shown to cause bleaching in controlled aquarium experiments. Furthermore, it was shown that bacterium-induced bleaching by *V. shiloi* could be inhibited by antibiotics. The infection and resulting coral bleaching were temperature dependent, occurring only at elevated seawater temperatures (15).

By using the *V. shiloi-O. patagonica* model system to study coral bleaching, it was demonstrated that the first step in the infectious process is the adhesion of *V. shiloi* to a β-galactoside-containing receptor on the coral surface (23). The temperature of bacterial growth is critical for the adhesion of *V. shiloi* to the coral. When the bacteria were grown at a low temperature, there was no adhesion to the coral, regardless of the temperature at which the coral had been maintained. However, bacteria grown at the elevated seawater temperature adhered avidly to corals maintained at either low or high temperatures. The important ecological aspect of these findings was that the environmental stress condition was causing the coral-bleaching pathogen to express its virulence determinants.

In an attempt to understand how *V. shiloi* causes the destruction or loss of the algae, it was discovered that *V. shiloi* cells produce both a heat-stable extracellular toxin that inhibits the photosynthesis of zooxanthellae and also heat-sensitive toxins that bleach and lyse algal cells isolated from corals (1, 20). In this report, we demonstrate that *V. shiloi* penetrates into the coral epidermis following adhesion to the coral surface. Shortly after penetrating the epidermis, the bacteria multiply in the tissue and enter a state in which they fail to form colonies on media that normally support the growth of *V. shiloi*.

MATERIALS AND METHODS

Microorganisms and corals. *V. shiloi* AK-1 was isolated from a bleached coral as described previously (13, 14). The strain was maintained on MB agar (1.8% marine broth plus 0.9% NaCl solidified with 1.8% agar [both products of Difco Laboratories, Detroit, Mich.]). 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. The coral *Oculina patagonica* was collected and maintained as described previously (13, 14, 23).

Adhesion and penetration of *V. shiloi* onto and into *O. patagonica*. An overnight culture of *V. shiloi*, grown at 30°C in MB broth with aeration, was centrifuged at 5,000 × *g* for 10 min, and the cell pellet was washed twice and then resuspended to ca. 10^9 cells per ml in sterile seawater. The bacteria were inoc-

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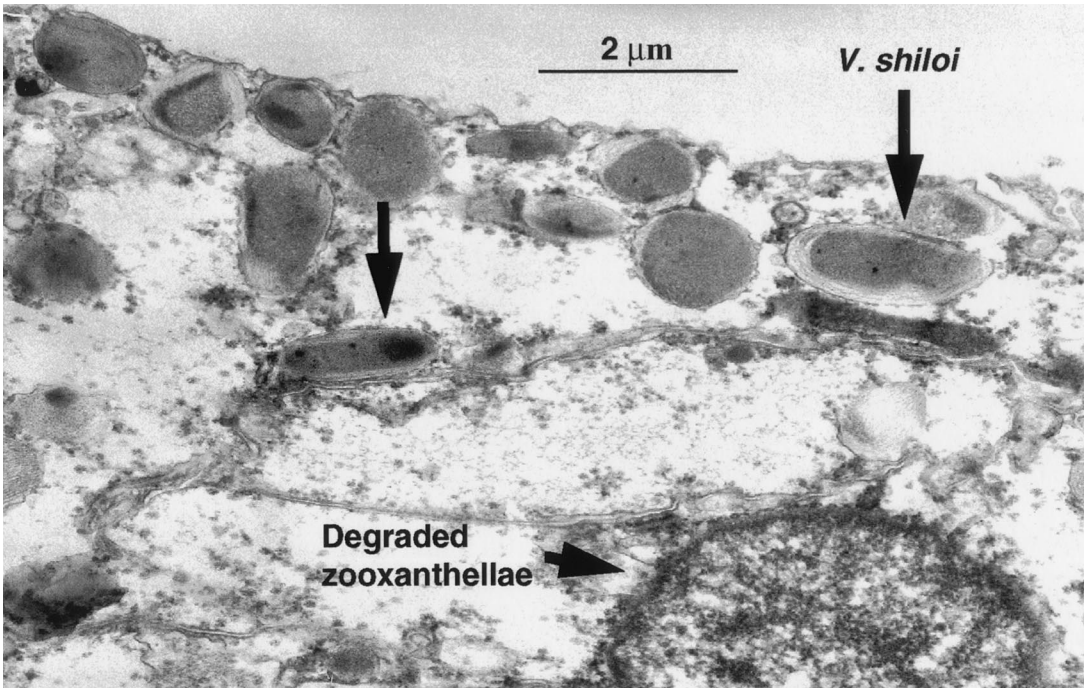

FIG. 1. Electron micrograph of a section of *O. patagonica* after infection with *V. shiloi*.

ulated into a 125-ml flask containing fragments of *O. patagonica* (~1 cm^3) in 25 ml of sterile seawater to the desired initial bacterial concentration. The flasks were incubated at 29 ± 1°C with gentle shaking on a "Belly Dancer" (Stovall Life Sciences Inc., Greensboro, N.C.). During the incubation period, the coral fragments were illuminated with a fluorescent lamp on cycles alternating 12 h of light and 12 h of darkness. Adhesion was determined by removing water samples at timed intervals and plating on MB agar and/or thiosulfate-citrate-bile-sucrose (TCSB) agar (Difco) as described previously (23). In each experiment, a no-coral control was included and the percent bacteria adhering to these flasks was subtracted from the values obtained with the coral experiments to obtain net adhesion. All values reported refer to net adhesion.

Penetration of *V. shiloi* into the coral tissue was determined by modifications of the gentamicin invasion assay (11). The infected coral was removed from the flask at the appropriate time, rinsed with sterile seawater, and then transferred to a 50-ml tube with 5 ml of sterile seawater containing 200 μg of gentamicin per ml and 0.01% methyl-β-D-galactopyranoside in order to desorb and kill noninternalized bacteria. After incubation for 3 h at 29°C, the coral was removed, rinsed in sterile seawater, then crushed in 5 ml of seawater with a mortar and pestle, and finally vortexed in a tube for 1 min. The first method (viable count) involved estimating the number of internal bacteria by plating appropriate dilutions on MB agar and TCSB agar. *V. shiloi* has a characteristic colony morphology on TCSB agar. Confirmation that the CFU were due to *V. shiloi* was obtained by checking the cells with anti-*V. shiloi* antibodies. The second method (total count) involved determining the number of *V. shiloi* cells in the tissue 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 to 3 h. The fixed samples were then washed three times in Tris-buffered saline (TBS) (10 mM Tris-HCl [pH 7.5]–150 mM NaCl) and attached to microscope slides covered with poly-L-lysine (50 μg/ml). After incubation for 1 h, the slides were washed once in TBS and incubated for 12 h at 4°C with polyclonal antibodies to *V. shiloi* (1:500 dilution in TBS). The antibodies were affinity purified by using fixed *Escherichia coli* and *Vibrio mediterranei* cells. The slides were then washed three times in TBS and incubated with 5 μg of Amca-conjugated anti-rabbit immunoglobulin G (IgG) (Jackson ImmunoResearch, West Grove, Pa.)/ml. After the incubation, the slides were washed three times in TBS and mounted with a solution of 90% glycerol containing 1 mg of *p*-phenylenediamine (Sigma, St. Louis, Mo.)/ml. Coverslips were sealed, and 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 Amca.

The viability of intracellular *V. shiloi* was examined with the Live/Dead Baclight Bacterial Viability Kit (Molecular Probes, Eugene, Oreg.). 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.

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 (25%) and sodium citrate (10%) for 15 to 25 h (19). After decalcification, the tissue was rinsed in fresh water and transferred into 70% ethanol. The tissue was embedded in paraffin by use of a Citadel Embedding apparatus. Sections (4 to 6 μm thick) of polyps were attached to microscope slides covered with poly-L-lysine. The paraffin was removed by successive washing with *O*-xylene and decreasing concentrations of ethanol. The slides were then washed in TBS and stained with the antibodies as described above.

For electron microscopy, coral fragments were fixed in 2.5% glutaraldehyde in filtered (pore size, 0.2 mm) 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 *V. shiloi* toxin P activity. A portable underwater Mini Pulse-Amplitude-Modulation (PAM) fluorometer (Walz GmbH, Effeltrich, Germany) was used to measure the quantum yield of zooxanthellae. This instrument allows the direct, noninvasive measurement of the effective quantum yield (Y) of photosystem II under ambient light. Good correlations between measurements of quantum yield and photosynthetic rates (determined by O_2 evolution and CO_2 uptake) have been reported for plants (7) and cyanobacterial symbionts of lichens (22).

In the experimental procedure used here, the quantum yields of 0.05-ml samples containing zooxanthellae in seawater (5×10^6 algae ml^{-1}) were measured in enzyme-linked immunosorbent assay (ELISA) plates at room temperature with the Mini-PAM (Y_0). Then 0.05 ml of the experimental sample was added to the algae, and the quantum yield was measured after 5 min (Y_t). The percent quantum yield at each time was $Y_t/Y_0 \times 100$. One unit of toxin P activity is defined as the amount that causes a 10% net inhibition of the quantum yield in the presence of 10 mM NH_4Cl. NH_4Cl is required for toxin P activity (1). Toxin P was obtained from the infected corals by extraction with ethyl acetate, removal of the solvent with a stream of nitrogen gas, and dissolving of the dried material in sterile seawater.

RESULTS

Qualitative microscopic observations of corals infected with *V. shiloi*. Thin sections of *O. patagonica* that were infected with *V. shiloi* showed numerous bacteria in the coral tissue visual-

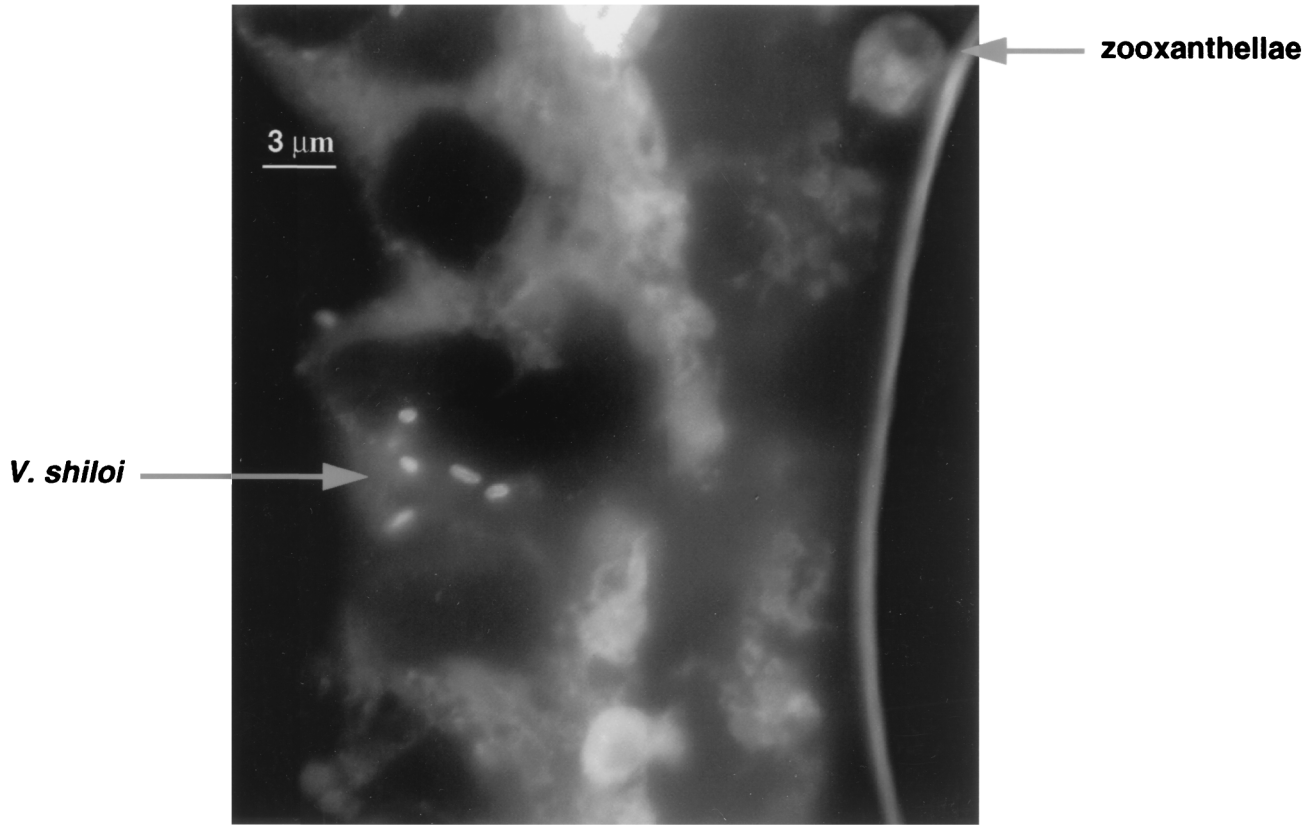

FIG. 2. Section of *O. patagonica* 24 h after infection with *V. shiloi* stained with anti-*V. shiloi* antibodies.

ized in the electron microscope (Fig. 1). The intracellular bacteria were short rods, approximately 2.5 by 0.8 μm. The fact that these intracellular bacteria were *V. shiloi* was demonstrated using specific anti-*V. shiloi* antibodies and fluorescence microscopy (Fig. 2). Control experiments demonstrated that the antibodies did not react with other bacteria, including the closest known relative of *V. shiloi*, *V. mediterranei* (14). Eight hours after inoculation of *V. shiloi* into seawater containing *O. patagonica*, most of the bacteria were seen adhering to the coral surface. After 24 h, many *V. shiloi* bacteria had penetrated into the coral tissue.

TABLE 1. Adhesion and penetration of *V. shiloi* onto and into *O. patagonica*[a]

Time after infection (h)	% Adhesion[b] ± SE	% Penetration[c] ±SE
4	36 ± 10	0.9 ± 0.5
8	83 ± 0	5.6 ± 2.0
12	77 ± 16	20.9 ± 8.5
24	86 ± 7	21.7 ± 7.6
48	100 ± 0	5.3 ± 4.3
72	100 ± 0	0.0 ± 0

[a] An overnight culture of *V. shiloi* was inoculated into 125-ml flasks containing healthy pieces of coral in 25 ml of sterile seawater. The flasks were incubated at 29°C with gentle shaking (80 rpm) with cycles alternating 12 h of light and 12 h of darkness. Data are averages from three experiments, each performed in duplicate with initial bacterial concentrations of ca. 4×10^6 per ml.

[b] Calculated from CFU lost from the seawater.

[c] Calculated from the CFU in the crushed coral (compared to the inoculated bacteria) following treatment of the coral with methyl-β-D-galactopyranoside and gentamicin.

Adhesion and penetration of *V. shiloi* onto and into *O. patagonica* as determined by viable counts. As summarized in Table 1, approximately 80% of the bacteria inoculated into seawater adhered to the coral within 8 h. At that time only 6% of the input bacteria were recovered as CFU in the crushed tissue after desorbing and killing of external bacteria with methyl-β-D-galactopyranoside and gentamicin, respectively.

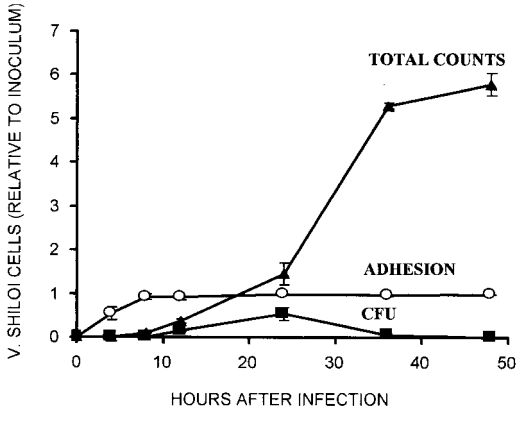

FIG. 3. Multiplication of *V. shiloi* bacteria in coral tissue. Healthy pieces of coral were inoculated with 1.6×10^5 bacteria per ml and incubated with shaking as described in Table 1, footnote *a*. At timed intervals, bacterial adhesion (○) to the coral, culturable internal bacteria (■), and total internal bacteria (▲) were measured. Total internal *V. shiloi* bacteria were quantitated by using specific anti-*V. shiloi* antibodies. The ordinate indicates the number of cells relative to the inoculum. Error bars, standard errors.

TABLE 2. Sensitivity of free and intracellular *V. shiloi* to treatment with gentamicin and methyl-β-D-galactopyranoside[a]

Source of *V. shiloi*	CFU	Microscopic count[b] (total)
Coral mucus		
Treated	$<10^2$	1.3×10^6
Untreated	1.1×10^6	1.0×10^6
Coral tissue (intracellular)		
Treated	6.6×10^4	1.0×10^8
Untreated	3.4×10^4	1.4×10^8

[a] The experiment was performed as described in the legend to Fig. 3 with an initial inoculum of 2.6×10^5 *V. shiloi* bacteria per ml. After incubation for 36 h, the corals were removed from the flasks and were shaken for 3 h in sterile seawater in either the presence (treated) or the absence (untreated) of gentamicin and methyl-β-D-galactopyranoside. Total microscopic counts and CFU were then determined on the treated and untreated samples of the free bacteria (from coral mucus) and the crushed coral tissue (intracellular bacteria).

[b] Microscopic counts were determined by using specific anti-*V. shiloi* antibodies.

The number of internalized CFU increased to 21% of the input bacteria after 12 h, remained constant until 24 h, and then decreased to 5% at 48 h. No CFU could be recovered inside the tissue after 72 h. Uninoculated controls showed no detectable internalized CFU throughout the experiment.

When different inoculum sizes of *V. shiloi* were compared, it appeared that both adhesion and penetration onto and into *O. patagonica* with the lower inoculum concentration (1.6×10^5 per ml) and the higher inoculum concentration (4×10^6 per ml) were similar with regard to both extent and kinetics. With both inocula, the number of internalized CFU decreased dramatically after 24 h.

Penetration and multiplication of *V. shiloi* in coral tissue. As shown in Fig. 2, antibodies specific for *V. shiloi* can be used to visualize the bacteria inside coral tissue. This technique was applied to quantitate the number of *V. shiloi* bacteria inside the coral as a function of time after infection, and these data were compared to the data obtained from viable counts (Fig. 3). During the first 12 h after infection, the number of *V. shiloi* bacteria that penetrated into the tissue was the same regardless of whether the bacterial concentration was determined by viable counts or total counts. From 24 h onward, however, the data obtained by the two methods were very different. Whereas the CFU count of *V. shiloi* inside the tissue decreased rapidly after 24 h, the total counts more than trebled from 24 to 36 h and continued to rise until 48 h, reaching a value of six times the number of bacteria that were introduced in the inoculum. Since the total number of *V. shiloi* bacteria initially inoculated into the 25 ml of seawater was 4×10^6, the number of *V. shiloi* cells in the 1-cm^3 piece of coral at 72 h was 2.4×10^7.

Further evidence that intracellular *V. shiloi* is viable but not culturable. One possible explanation for the failure of intracellular *V. shiloi* to form colonies was that the treatment of the corals with relatively high concentrations of gentamicin and methyl-β-D-galactopyranoside, prior to crushing, actually killed the intracellular bacteria. To test this possibility, a control experiment was performed (Table 2). Extracellular *V. shiloi* bacteria, obtained from the mucus on the outside of the coral, were efficiently killed when treated with a mixture of gentamicin and methyl-β-D-galactoside. However, when the coral fragments were treated with the antibiotic, essentially the same CFU count of intracellular bacteria was obtained after crushing the coral as with no antibiotic treatment. Thus, the fact that the CFU count of intracellular bacteria was more than a thousand times less than the microscopic count of specifically stained *V. shiloi* bacteria was not the result of the treatment killing the intracellular bacteria.

Another possible explanation for the unculturability of the intracellular *V. shiloi* is that the coral host responds to the infection by producing a compound that depresses plate counts. This hypothesis was eliminated by showing that adding *V. shiloi* to crushed coral did not change the CFU count.

Intracellular bacteria that bound the anti-*V. shiloi* antibodies were also scored as viable (green) by the Live/Dead BacLight Viability Kit (Fig. 4). Although it was not quantitated, it appeared that a high percentage of the intracellular *V. shiloi* bacteria were in close contact with the zooxanthellae in the crushed infected coral.

Production and degradation of toxin P activity in coral tissue following infection with *V. shiloi*. Flask cultures of *V. shiloi* produce a heat-stable extracellular photosynthesis inhibitor (toxin P) during stationary phase (1, 20). If toxin P plays a role in coral bleaching, then it must reach the intracellular zooxanthellae in the coral tissue. Because the bacteria penetrate into

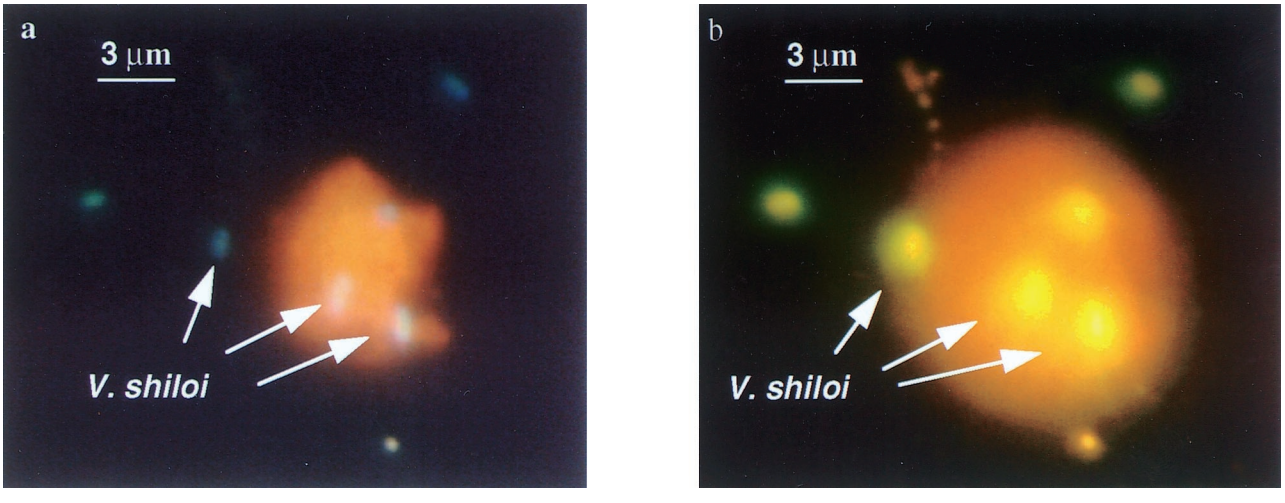

FIG. 4. Viability of *V. shiloi* in coral tissue. The experiment was performed as described in the legend to Fig. 3. After incubation with the bacteria for 24 h, the washed coral was crushed and examined by fluorescence microscopy for *V. shiloi* by staining with anti-*V. shiloi* antibodies (blue) (a) and viable cells (green) by staining with the Live/Dead BacLight Bacterial Viability Kit (b).

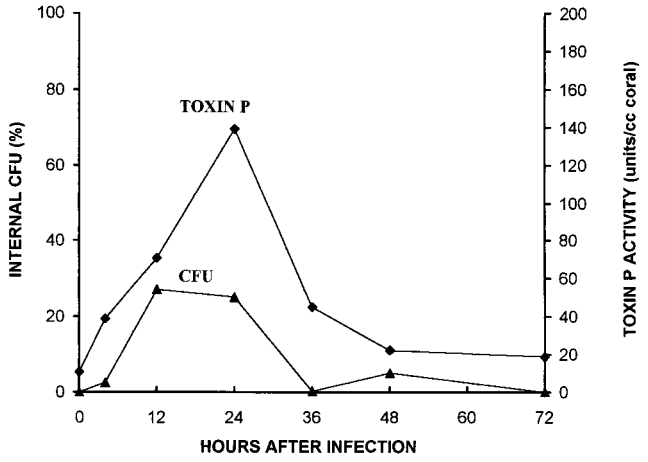

FIG. 5. Production and degradation of *V. shiloi* photosynthesis inhibitor toxin (toxin P) in coral tissue (◆) compared to internal viable counts of the bacterium (▲). The experiment was performed as described in Table 1, footnote *a*. Levels of toxin P activity were determined on extracts of coral tissue.

the coral during the first 24 h of infection, it was interesting to determine the amount of toxin P activity inside the coral at different times after infection (Fig. 5). The rise and fall of toxin P activity in the coral tissue followed kinetics similar to those of the intracellular CFU. The toxin P activity in the coral tissue 24 h after infection was more than 10 times higher than the level that was produced in flask cultures.

DISCUSSION

The data presented here demonstrate that the coral pathogen *V. shiloi* penetrates into the epidermis of its coral host following adhesion to the coral surface. Then, once inside the coral, the intracellular *V. shiloi* bacteria multiply and are transformed into a state that does not form colonies on media that normally support the growth of extracellular *V. shiloi*. These results have significant implications with regard to both the mechanism of coral bleaching and the difficulties that may be encountered in isolating other potential coral-bleaching pathogens from different species of corals.

With regard to the mechanisms of pathogenesis, we have previously demonstrated that *V. shiloi* produces extracellular heat-resistant and heat-sensitive toxins that cause inhibition of photosynthesis and that bleach as well as lyse zooxanthellae isolated from the coral host (1, 20). The fact that *V. shiloi* penetrates into the coral tissue would facilitate contact between these toxins and their target algae. The high tissue activity of toxin P following infection, shown in this article, supports this hypothesis. Since toxin P activity requires the presence of ammonia to inhibit zooxanthella photosynthesis, it is unlikely that the toxin would function when *V. shiloi* is outside of its host because the concentration of ammonia in seawater is extremely low (21).

Entry of bacteria into a state described as viable but not culturable (VBNC) has been reported repeatedly with a large number of bacterial species, including several *Vibrio* species, *Vibrio vulnificus* (18), *Vibrio parahaemolyticus* (3), *Vibrio cholerae* (4), and *Vibrio fischeri* (16). A bacterium in the VBNC state has been defined "as a cell which 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" (17). Intracellular *V. shiloi* cells fit that definition precisely, but unlike most cases of VBNC that have been studied, this is not brought about by starvation or low temperatures. Rather, the entry of *V. shiloi* into the VBNC state occurs inside the coral epidermis, where nutrients are abundant and, in fact, *V. shiloi* multiplies. It is possible that the intracellular differentiated *V. shiloi* becomes dependent on one or more nutrients present in the coral cell. This hypothesis could be tested by attempting to plate intracellular *V. shiloi* on media containing coral tissue homogenate.

The gentamicin invasion assay (11) was developed for measuring the penetration of *Yersinia pseudotuberculosis* into human epithelial cells and has been found to be generally useful for determining the invasion of animal cells by different bacteria. Since *V. shiloi* was efficiently killed only by relatively high concentrations of gentamicin (200 μg/ml), it was necessary to demonstrate that the antibiotic did not kill *V. shiloi* present in coral cells. Whereas the gentamicin treatment killed bacteria in the coral mucus with more than 99% efficiency, there was no killing of intracellular bacteria. In both cases, the samples were treated with 0.01% methyl-β-galactopyranoside as well as the antibiotic to desorb *V. shiloi* (23) that had not been internalized.

The VBNC state of intracellular bacteria in corals could make it difficult to demonstrate that they are the causative agents of coral bleaching. In the case of *V. shiloi*, it was possible to isolate the bacterium from the coral mucus and then apply Koch's postulates to prove that it is the causative agent of bleaching of the coral *O. patagonica*. However, culturing potential pathogens from other corals may present a problem if they exist in the VBNC state. It would therefore be desirable to develop a general method for culturing intracellular coral bacteria. Further studies on the VBNC state of intracellular *V. shiloi* may be useful in this regard.

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