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A novel *Vibrio* sp. pathogen of the coral *Pocillopora damicornis*

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Abstract A coral pathogen was isolated from the diseased tissue of *Pocillopora damicornis* in Zanzibar. The pathogenic bacterium, referred to as *Vibrio coralyticus* YB, was classified as a member of the genus *Vibrio*. Based on its 16S rDNA sequence, *V. coralyticus* is probably a new species. In controlled aquaria experiments at 26–29°C, inoculation of pure cultures of *V. coralyticus* YB either into the seawater or by direct contact onto the coral caused tissue lysis of *P. damicornis* fragments. At 29°C, lysis began as small white spots after 3–5 days, rapidly spreading so that by 2 weeks the entire tissue was destroyed, leaving only the intact bare skeleton. When an infected diseased coral was placed in direct contact with a healthy one, the healthy coral lysed in 2–4 days, further indicating that the disease was contagious. Inoculation with as few as 30 bacteria ml^{-1} was sufficient to infect and lyse corals. Seawater temperature was a critical variable for the infectious process: infection and lysis occurred rapidly at 27–29°C, slowly at 26°C and was not observed at 25°C. The data suggest that the presence of *V. coralyticus* YB, even in low numbers, in seawater surrounding a coral reef will lead to tissue destruction of *P. damicornis*, when seawater temperatures rise.

Introduction

Novel pathologies of coral reef organisms, especially reef-building scleractinian corals, have escalated during the last two decades. These emerging diseases have appeared with progressively greater frequency and wider distribution. Coral reef diseases have altered both total abundance and species diversity (Peters 1997; Richardson 1998). For example, Loya et al. (2001) reported a reduction of 85% in the coral cover at Sesoko Island, Japan, from 1998 to 2000, and a drastic decrease in coral diversity of over 60% in coral species, with no evidence of recruitment, following the 1998 bleaching event. However, relatively little progress has been made in identifying and characterizing the causative agents for these diseases, and essentially nothing is known about their modes of transmission.

Disease is defined as any impairment of an organism's vital functions, systems, organs, or cells. Infectious diseases are characterized both by an identifiable group of signs and the presence of the recognized etiologic or causative agent. There are currently only four known coral diseases, for which both diagnostic coral tissue changes and the presence of a consistent, characteristic microorganism or microbial consortium responsible for the disease are known: aspergillosis (Smith et al. 1996; Nagelkerken et al. 1997; Geiser et al. 1998), black band disease (Antonius 1985; Carlton and Richardson 1995; Richardson et al. 1997), white plague type II (Smith et al. 1996; Richardson et al. 1998) and bleaching of *Oculina patagonica* by *Vibrio shiloi* (Kushmaro et al. 1997).

It is increasingly evident that abiotic factors, such as anthropogenic inputs as well as global climate changes, influence the distribution and impact of infectious diseases (Colwell 1996). A disease outbreak is favored by changing environmental conditions that either increase prevalence and virulence of existing diseases or facilitate new diseases. Two conditions, climate variability and human activity, appear to have played key roles in epidemics by undermining host resistance and/or facilitating pathogen transmission. Recently, there has been considerable attention paid to the fact that climate factors, including seasonal weather changes and large-scale disturbances such as El Niño, influence the occurrence and transmission of infectious diseases through multiple direct and indirect effects upon pathogenic microorganisms, vectors, reservoirs and hosts. Many infectious

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diseases occur cyclically, and the patterns of occurrence often suggest that environmentally dependent factors play a role in outbreaks and epidemics (Harvell et al. 1999; Colwell and Patz 1997; Daszak et al. 2001).

One of the best studied coral diseases from the point of view of the mechanism by which temperature affects the disease process is the *V. shiloi*/*O. patagonica* coral-bleaching model system (Kushmaro et al. 1998; Rosenberg et al. 1999; Banin et al. 2000). A large number of *V. shiloi* virulent factors are produced only at elevated seawater temperature, including adhesins (Toren et al. 1998; Banin et al. 2001a), toxins (Ben-Haim et al. 1999; Banin et al. 2001b), intracellular multiplication and superoxide dismutase (Israely et al. 2001). In addition to coral bleaching, a number of other coral diseases have been correlated with high seawater temperature, including black band disease (Edmunds 1991; Kuta and Richardson 1996).

The present paper describes the isolation and characterization of a novel temperature-dependent bacterial pathogen of the coral *Pocillopora damicornis*. At water temperatures above 27°C, a pure culture of the pathogen, referred to as *Vibrio coralyticus* YB, causes a rapid destruction of the coral tissue within 2 weeks. Furthermore, direct contact between infected tissue and healthy corals caused transmission of the disease, further demonstrating its infectious nature.

Materials and methods

Collection and maintenance of the corals

Bleached, partially whitened and healthy coral samples were collected in March 1999 from the reef of Mawi Island, located 7 km east of the main island of Zanzibar, in the Indian Ocean. Corals were collected (*Pocillopora damicornis*, *Acropora formasa*, *Acropora* sp. and *Cycloseries* sp.; Fungiidae) from depths of 3–5 m, while seawater temperature was 29.5°C. Fragments of ca. 5 cm^2 of partially whitened and healthy colonies were picked from a depth of 3–5 m. Each coral fragment (from different colonies) was kept separately in aerated seawater for 1–2 h until bacteria were isolated as described below. Corals collected for laboratory inoculation experiments were picked by SCUBA diving off Eilat in the Gulf of Aquaba, Red Sea, from depths of 3–6 m during 1999–2001. The corals were fragmented into 1–2 cm^2 pieces, and then allowed to recover and regenerate in 3- to 10-l aerated aquaria at the same seawater temperature (22–26°C) at which they were collected. After tissue recovery the corals were slowly adjusted to the experimental temperature by elevation of 0.5–1°C day^{-1}. Then, the corals were maintained at the experimental temperature for a further 7–14 days prior to initiating the experiment. If any fragment failed to heal (complete cover of damaged skeleton by new tissue, and dark pigmentation), it was not used in the experiments. The corals were maintained in freshly prepared artificial seawater (Instant Ocean, USA) adjusted to a salinity of 35 ppt. Water was replaced every 3–6 days. The aquaria were aerated and illuminated with fluorescent lamps (Sylvania, Aquastar 10,000 K) at 12 h light:12 h dark intervals.

Isolation of bacteria from coral samples

Small pieces of diseased and healthy corals, collected as described above, were washed twice in filter-sterilized seawater (ssw) and drained. Samples of the mucoid layer of each coral were obtained by gently touching the coral surface with a sterile loop and streaking onto marine broth (MB) agar plates (1.8% MB, Difco MA 2216, 0.9% NaCl and 1.8% Difco Bacto agar) and on *Vibrio*-selective TCBS agar (4.5% Difco TCBS agar, 0.9% MB, 0.45% NaCl and 1.2% Difco Bacto agar). Mucus samples were streaked from different parts of the same diseased coral, i.e. whitened or bleached parts, and the interphase between the healthy and diseased parts. Mucus was also streaked from apparently healthy colonies. In addition, mucus was dripped off coral fragments into sterile 1.5 ml tubes, then diluted, and 0.1 ml was spread onto MB and TCBS agar. Bacteria were isolated from within the coral tissue by the following procedure: coral fragments were rinsed twice in 20 ml ssw, crushed in 5 ml ssw using a mortar and pestle, and finally vortexed in 10 ml tubes for 3 min. Samples were taken after 3 min from the upper phase of the liquid, after allowing the crushed skeleton to sediment. The samples were diluted 10^{-1}–10^{-5} in ssw, and 0.1 ml was spread on MB and TCBS agar. Plating was used both for comparison of bacterial colonies obtained from diseased and healthy corals, and in order to quantify the number of *Vibrio* spp. bacteria and total bacteria in the mucus and the tissue. Estimation of bacterial concentration was done by plating triplicate samples of appropriate dilutions in ssw on each medium. *V. coralyticus* YB was obtained in pure culture by picking up the colony from the relevant TCBS agar plate after 48 h of growth, and restreaking three times in order to purify the strain.

Characterization of *V. coralyticus* YB

Strain *V. coralyticus* YB was routinely cultivated on MB agar or MBT liquid medium (1.8% MB, 0.9% NaCl and 0.5% tryptone; Difco) at 30°C. Liquid cultures were prepared in 125 ml flasks containing 10 ml MBT, inoculated with one colony, and incubated at 30°C with shaking (160 rpm) for 24–48 h. In order to preserve the strain, stock cultures containing 15% glycerol were stored at –70°C. Cell morphology and motility were determined by phase microscopy. Biochemical classification tests were carried out by using two identification kits for Gram-negative bacteria: Biolog GN2 MicroPlate (Biolog, Hayward, Calif., USA) and Api-20 NE (Bio Mérieux SA, Marcy-Ietoil, France). For examination with the Biolog kit, the cells were grown on MB agar for 12–18 h, then suspended in the inoculation fluid (supplemented with NaCl to a final concentration of 3%) to a density of between 0.130 and 0.143 at A_{600} as recommended by Ritchie and Smith (1995). The standard Api-20 NE method was used except that the media were adjusted to 3% NaCl (Kushmaro et al. 1997). Extracellular proteolytic and cellulytic activities were determined by plating the strain on Davis minimal medium supplemented with 0.5–1% casein or 0.5% cellulose, respectively. Acid production was tested on TCBS agar. Exponential growth rates were calculated from growth curves at 20°C, 25°C and 30°C. Bacterial growth was followed by determination of turbidity in a Klett–Summerson photometer. Salt tolerance was determined in 2 ml MBT medium containing 1–15% NaCl. Sensitivity to antibiotics was examined using either the minimal inhibition concentration (MIC) method for erythromycin (24 $\mu g\ ml^{-1}$), tetracycline (20 $\mu g\ ml^{-1}$), chloramphenicol (6 $\mu g\ ml^{-1}$) and kanamycin (50 $\mu g\ ml^{-1}$), or by the paper disc method for penicillin (10 $\mu g\ disc^{-1}$) and ampicillin (10 $\mu g\ disc^{-1}$). The growth was also tested on MB agar plates containing gentamycin (200 $\mu g\ ml^{-1}$).

DNA isolation, amplification and sequence analysis

Genomic DNA was isolated from a 2 ml overnight bacterial culture, using Wizard genomic DNA purification kit (Promega, Madison, Wis., USA). 16S rDNA was amplified through the polymerase chain reaction (PCR) in a 50 ml reaction mixture consisting of 5 ml of 10× PCR buffer, 5 ml of 2.5 mM total dNTPs mixture, 1 mM of each primer, 1 mg template DNA and 2.5 U of ex *Taq* DNA polymerase (Takara Shuzo, Japan). The primers used

were general prokaryotic 16S rDNA primers: forward 5′AGAGTTTGATYMTGGCTCAG and reverse 5′ACCTTGTTACGACTT (Y=C/T, M=A/C), manufactured by GenSet. Amplification conditions for the PCR included denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 45 s. The final extension step was at 72°C for 5 min. Reaction products were purified and sequenced using the chain termination method in the ABI PRISM (model 377, version 3.3) automatic sequencer. Finally, the *V. coralyticus* YB 16S rDNA sequence was compared with homologous published sequences.

Infection experiments of healthy corals with *V. coralyticus* YB

Bacteria for inoculation were grown in MBT medium for 30–48 h at 30°C with aeration (160 rpm). Then, 10-ml cultures were centrifuged at 7,000 rpm (Sorval, SS-34) for 5 min at 4°C, washed in 10 ml ssw, and resuspended to a final volume of 0.5–1 ml ssw. Viable counts on MB and TCBS agar were performed in order to estimate the precise size of the inoculum in each experiment. Infection experiments were all performed under controlled conditions of illumination and temperature, in 2- to 3-l aerated and covered aquaria. Each aquarium contained one to six coral fragments. Two types of infection experiments were conducted: localized and diffused. In the localized experiments, the corals were removed from the water into a sterile petri dish and immediately inoculated with 10–30 μl of *V. coralyticus* YB. After 1 min, the corals were carefully returned to the aquaria. The control experiments were treated in the same manner except that inoculation was with 10–30 μl ssw. In the diffused inoculation experiments, 1 ml of the resuspended bacteria was added directly to the water of the aquarium containing the corals. Corals were not removed from the aquaria for this latter procedure. In some experiments, 1 ml samples of the aquaria seawater were diluted and plated on TCBS agar at 0, 24 and 72 h after the inoculation. Observations on the corals were recorded every day. Percentage of tissue lysis was determined visually, by estimating the relative lysing area compared to intact tissue on the coral fragment.

Electron microscopy

Scanning electron microscopy was performed on an overnight culture of *V. coralyticus* YB, after negative staining with 1% uranyl acetate. Experimentally infected corals were fixed in 2.5% glutaraldehyde in filtered seawater (0.2 μm), coated with gold, and examined under a scanning electron microscope (Jeol 840A).

Results

Isolation of *Vibrio coralyticus* YB from *Pocillopora damicornis*

Streaking crushed tissue samples of *P. damicornis* on TCBS agar yielded relatively high concentration of *Vibrio* spp. compared to the other crushed coral samples. Comparison of colony morphologies obtained from the seven healthy and diseased corals, led to isolation in pure culture of 15 strains of Vibrionaceae, which seemed to be dominant in diseased corals but not in healthy ones. These strains were subsequently tested for their pathogenic effect on corals. Infection experiments on healthy corals with the suspected strains led to one strain, originally isolated from *P. damicornis*, which was highly pathogenic to *P. damicornis* in controlled experiments. This strain, referred to as *V. coralyticus* YB, was isolated from a colony of *P. damicornis* that showed partially degrading tissue and white patches. *V. coralyticus* YB, was isolated from the crushed tissue that covered a partially lysed branch, with apparently recent tissue lysis.

Characterization of *V. coralyticus* YB

V. coralyticus YB yielded characteristic cream-colored colonies and yellow colonies on MB and TCBS agar, respectively. The growth characteristics of *V. coralyticus* YB, as well as a comparison of its 16S rDNA sequence with closely related *Vibrio* species are summarized in Table 1 . *V. coralyticus* YB is a Gram-negative, rod-shaped, motile bacterium (1.2–1.5×0.8 μm) that contains a single polar-sheathed flagellum (Fig. 1). Growth rates during exponential phase in MBT medium, calculated from the growth curves at 20°C, 25°C and 30°C were 2.4, 2.0 and 0.42 generations h^{-1}, respectively. Growth occurred over a wide range of salinity conditions (1–7% NaCl); the bacterium failed to grow in media containing 8% or higher NaCl. Biochemical characterization and partial classification were performed using the Biolog GN2 MicroPlate microbial identification kit and the Api-20 NE micromethod tests for identification of Gram-negative bacteria (Table 1). No definitive identification of the microorganism could be obtained by either of the two kits. According to the Biolog examination test, the bacterium was found to be close to *Vibrio harvei*, *Vibrio carcharia*, *Vibrio alginolyticus*, *Vibrio splendidus* and *Aeromonas encheleia* (similarities of 0.31–0.44), and, according to the Api-20 NE examination test, it was found to be close to *Vibrio fluvialis* or *Aeromonas hydrophyla*, but with significant differences in carbon utilization and enzymatic activities.

The 16S rDNA sequence of *V. coralyticus* YB was determined and compared to the NCBI sequence analysis database. The data demonstrate that this strain is clearly a member of the Vibrionaceae. The closest similarity was to an unidentified marine bacterium PP-14 (98%). The closest known *Vibrio* species were *V. vulnificus* (97%), *V. shiloi* (96%), *V. mediterranei* (96%) and *V. orientalis* (96%). Since strains of the same species have 16S rDNA sequence similarities of >99% (Fox et al. 1992; Kita-Tsukamoto et al. 1993), *V. coralyticus* YB is clearly a new species.

Infection of *P. damicornis* with *V. coralyticus* YB

Infection of healthy *P. damicornis* corals, collected from the Red Sea (Eilat) with a pure culture of *V. coralyticus* YB resulted in tissue lysis of the corals within 1–2 weeks (Fig. 2). Seven days after inoculation of healthy corals with *V. coralyticus* YB, 11/21 infected corals showed at least 10% tissue lysis. After 14 days, 20/20 infected corals showed 50–100% tissue lysis.

Table 1. Description of *Vibrio coralyticus* YB. For sequences, *numbers in parentheses* refer to the deposition in the NCBI database

Source of the bacterium	Zanzibar, Indian Ocean; tissue of a partially whitened *Pocillopora damicornis*colony (March 1999)
Bacterial colony morphology	On marine broth agar: cream–beige, smooth-edged, 3 mm diameter after 3 days On TCBS agar: yellow, smooth-edged, 3 mm diameter after 3 days
Cell morphology	Rod, 1.2–1.5×0.8 μm
Gram stain	Negative
Motility	Positive, polar-sheathed flagellum
Salinity	Growth in marine broth containing 1–7% NaCl
Growth rate (doubling time in MBT medium)	20°C – 140 min 25°C – 36 min 30°C – 25 min
Biochemical tests	
Gelatinase, protease	+
Urease	+
Oxidase	+
Nitrate reduction	+
Indole production	–
Acidification of glucose medium	+
Acidification of sucrose medium	+
Arginine dehydrolase	–
ß-Galactosidase	+
Utilization of carbon source	Positive: D-mannose, D-galactose, D-fructose, D-mannitol, maltose, m-inositol, D-trehalose, D,L-serine, L-leucine, D-gluconic acid, propionic acid, D,L-lactic acid Negative: L-arabinose, cellobiose, lactulose, α-D-lactose, gentiobiose, L-fucose, L-rhamnose, maltonic acid, urocanic acid, D-glucoronic acid, D-galacturonic acid
Antibiotic sensitivity	Sensitive to: erythromycin, tetracycline, chloramphenicol, gentamycin Resistant to: kanamycin, ampicillin, penicillin
16S rDNA sequence analysis	*V. coralyticus*–*Vibrio shiloi* (AF007115.1) 96% similarity (15 Kbp) *V. coralyticus*–*Vibrio mediterranei* (X74710.1) 96% similarity (15 Kbp) *V. coralyticus*–marine bacterium PP-14 (AJ296157.1) 98% similarity (13 Kbp) *V. coralyticus*–*Vibrio orientalis* (X74719.1) 96% similarity (15 Kbp) *V. coralyticus*–*Vibrio vulnificus* (X76333.1) 97% similarity (15 Kbp) *V. coralyticus*–*Vibrio splendidus* (AB038030.1) 96% similarity (14 Kbp)

After 17 days, 19/20 infected corals were 100% lysed. In the control experiments, 26/31 uninoculated corals remained completely healthy for at least 21 days under identical conditions; 2/31 control coral fragments died after 6 days and 3/31 control corals died 12 days after the experiment began.

The pathogen was reisolated in high concentrations from the experimentally infected corals. At different stages of tissue lysis, infected corals were washed twice in sterile seawater and crushed. The crushed tissue was diluted and plated on MB and TCBS agar. Uninoculated control corals were examined similarly. *V. coralyticus* YB was found to be present in all diseased corals examined, and absent in all control corals that were tested. Furthermore, over 95% of the bacteria isolated from the diseased tissue were *V. coralyticus* YB. Infected corals undergoing 20–80% tissue lysis, 6–13 days after inoculation, yielded 3.6×10^3–2.5×10^4 *V. coralyticus* YB cm^{-2} crushed coral tissue. Control corals, crushed and plated similarly, did not contain the pathogen (<10 *Vibrio* sp. cm^{-2} crushed coral tissue). In addition, the pathogen could be reisolated from aquarium seawater containing the diseased corals, but not from water samples plated from the control aquaria. Interestingly, *V. coralyticus* YB was not detected (<10 cells ml^{-1}) in the water during the early stages of tissue lysis. Biochemical and genetic identification tests (Biolog, Api-20 and 16S rDNA sequence analysis) confirmed that, in fact, the bacteria reisolated from the infected corals were *V. coralyticus* YB.

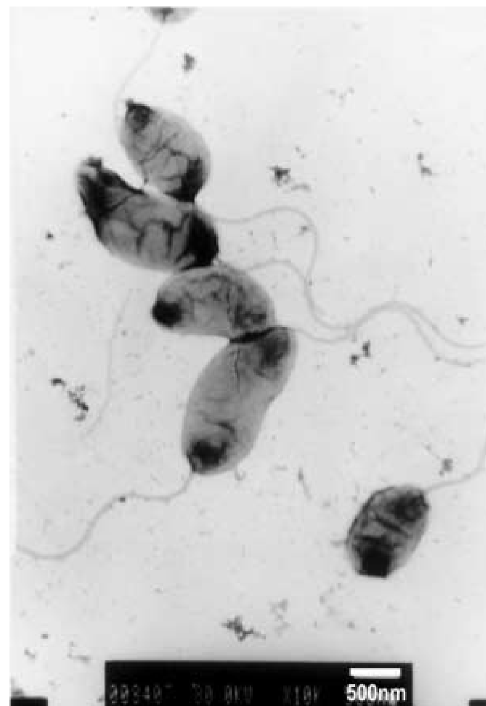

Fig. 1. *Vibrio coralyticus* YB. Scanning electron micrograph of negative-stained cells. *Scale bar*: 500 μm

Tissue lysis typically started in small spots, usually on the verrucae of the branch, which slowly united into white patches, progressing until the entire tissue

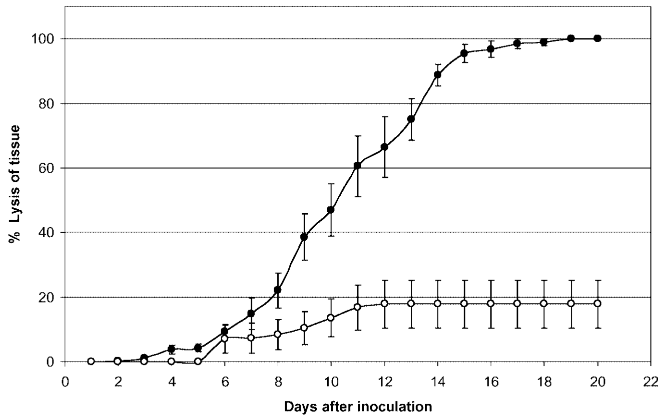

Fig. 2. *Pocillopora damicornis*. Tissue lysis as a function of time after infection with *Vibrio coralyticus* YB. Average percentage of tissue lysis (±SE) of corals inoculated with *V. coralyticus* YB (localized: 10^7 coral^{-1}; diffused: 10^5 ml^{-1}) (*filled circles*) compared to uninoculated control corals (*open circles*) in 2- to 3-l aquaria at 29°C. Percentage of tissue lysis of each coral was measured every day, for 20 days. Average percent lysis was calculated ($n = 21$ corals in seven different experiments, $n = 31$ uninoculated control corals in ten different experiments). Data for localized and diffused infections were pooled because there was no significant difference between them

was degraded, leaving only the intact bare skeleton. A typical example showing macroscopically the progressive tissue lysis of infected corals is shown in Fig. 3. Scanning electron micrography of an infected *P. damicornis* coral, undergoing 50% tissue lysis, showed a high number of rod-shaped bacteria, on the surface and underside of the lysing tissue (Fig. 4b). Figure 4a is an electron micrograph showing the degrading tissue of the infected coral and the zooxanthellae that are held inside the torn tissue.

Infection by direct contact

Additional experiments were performed to examine whether or not the pathogen could be transmitted directly from a diseased coral to a healthy one. Healthy corals were placed in direct contact with infected corals undergoing tissue lysis and then incubated under conditions (temperature, illumination, etc.) similar to those under which the original infection occurred. Contact with infected corals, undergoing 20–70% tissue lysis resulted in degradation of approximately 50% of the tissue of the healthy corals within 2 days at an incubation temperature of 29°C, and 90–100% tissue lysis within 4 days, in 7/7 corals examined. The tissue of the newly infected coral, lysed in a similar way to the originally infected corals, i.e. small white dots of lysing tissue, which combined into white patches, followed by degradation of the entire tissue, leaving behind bare skeleton. Furthermore, the corals infected by direct contact could infect and cause tissue damage to a third fresh healthy coral, thereby propagating the disease and the pathogen. Transmission of the pathogen from infected corals to healthy corals, with similar symptoms of tissue degradation, indicated that the disease was contagious. In control experiments, when a healthy coral was placed in direct contact with another healthy coral and incubated under similar experimental conditions, the corals remained healthy for at least 12 days, in 6/6 corals examined. Figure 5 demonstrates the progression of the tissue lysis of corals in direct contact.

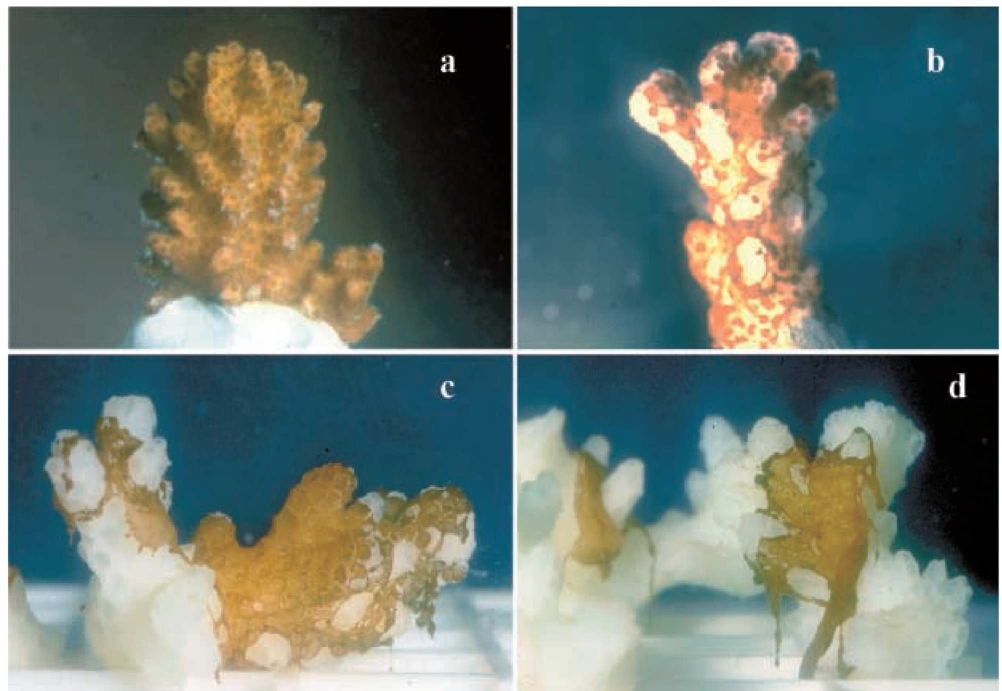

Fig. 3a–d. *Pocillopora damicornis*. Infection with *Vibrio coralyticus* YB (10^5 cells coral^{-1}, in a 2 l aquarium). Series of photographs showing the progressive tissue lysis of the infected corals at 29°C. **a** Healthy uninoculated coral, **b** 15–20% lysis, 7 days after inoculation, **c** 40% lysis after 10 days and **d** 80% tissue lysis 14 days after inoculation

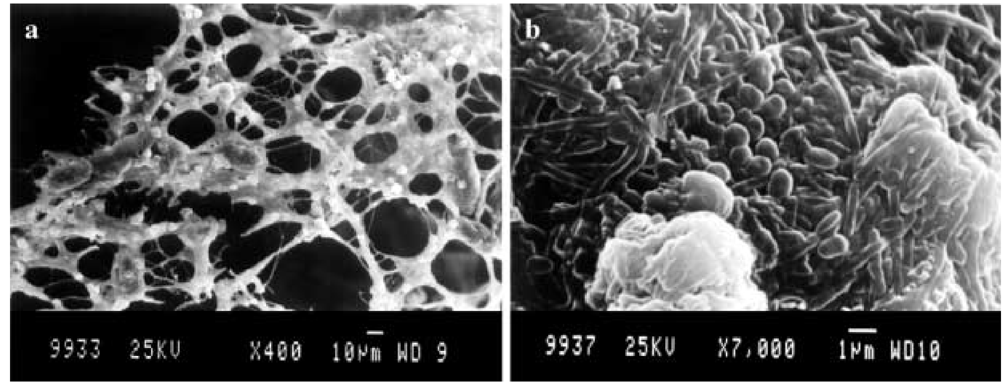

Fig. 4a, b. *Pocillopora damicornis*. **a** Scanning electron micrographs of lysing coral tissue, after infection with 10^7 *Vibrio coralyticus* YB. **b** Close-up of the infected tissue revealed a high number of bacterial cells which adhere to the lysing and underside of the tissue surface. *Scale bars*: 10 µm (a), 1 µm (b)

Effect of temperature on the infection process

In order to examine whether *V. coralyticus* YB infection of *P. damicornis* is temperature regulated, a series of controlled aquarium experiments was performed at different temperatures (Fig. 6). Corals infected and maintained at 29°C showed the fastest rate of tissue lysis. Three days after inoculation, tissue lysis started in 2/5 corals, with small dots of degrading tissue. After 6 days all the infected corals (5/5) showed 15–30% tissue lysis, and after 11 days 5/5 corals were lysed completely. At 27°C the infection progression was a little slower compared to 29°C, with a delay of ca. 2 days in the onset and completion of lysis. The infection progress at 26°C was significantly slower than at the higher temperatures examined. Six days after the inoculation, 1/6 corals showed small lysis dots on the branch, and after 9 days the same coral was about 35% lysed, while the other corals looked healthy. After 14 days, 1/6 corals was completely degraded, 4/6 corals were 10–30% lysing and 1/6 corals looked healthy; after 16 days, all the infected corals were completely degraded. At 25°C and 20°C, no visual signs of disease were observed in any of the inoculated corals for at least 20 days, at which times the experiments were terminated. In control aquaria, where corals were held at each of the temperatures from 20°C to 27°C without inoculation of *V. coralyticus* YB, no observable change in the corals was seen. At 29°C, 26/31 of the uninfected corals showed no change for at least 20 days.

Effect of inoculum size on the infection process

An additional set of infection experiments was conducted at 29°C in order to examine the effect of inoculum size on *V. coralyticus* YB infection of *P. damicornis* (Fig. 7). Uninoculated corals served as controls, and showed no signs of disease. Inoculation of the corals with 10^8 cells coral^{-1}, resulted in rapid tissue lysis, with complete lysis of 5/5 corals after 9 days. By comparison, corals inoculated with 10^6 bacteria coral^{-1} showed somewhat slower lysis, with 100% tissue lysed after 12 days; with 10^4 bacteria coral^{-1} (ca. 30 bacteria ml^{-1}

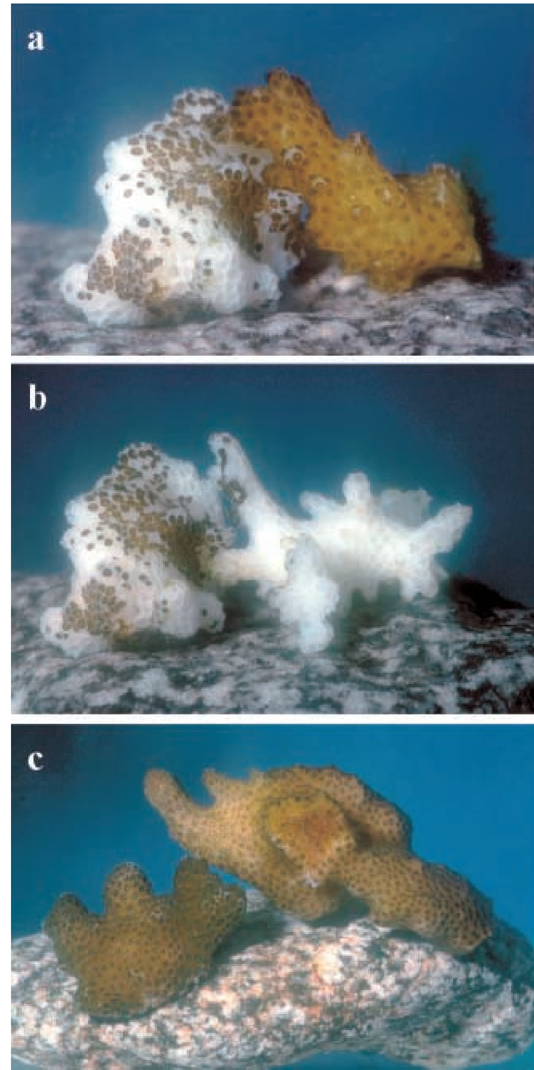

Fig. 5a–c. *Pocillopora damicornis*. Transmission of the pathogen *Vibrio coralyticus* YB from diseased corals to healthy corals by direct contact. The series of photographs shows progressive coral tissue lysis of healthy *P. damicornis* placed in direct contact with a diseased coral. Transmission of the infection, at 29°C, from the diseased coral to a healthy one is demonstrated: **a** 2 days and **b** 4 days after contact. Two uninoculated healthy corals in direct contact did not show tissue damage (**c**)

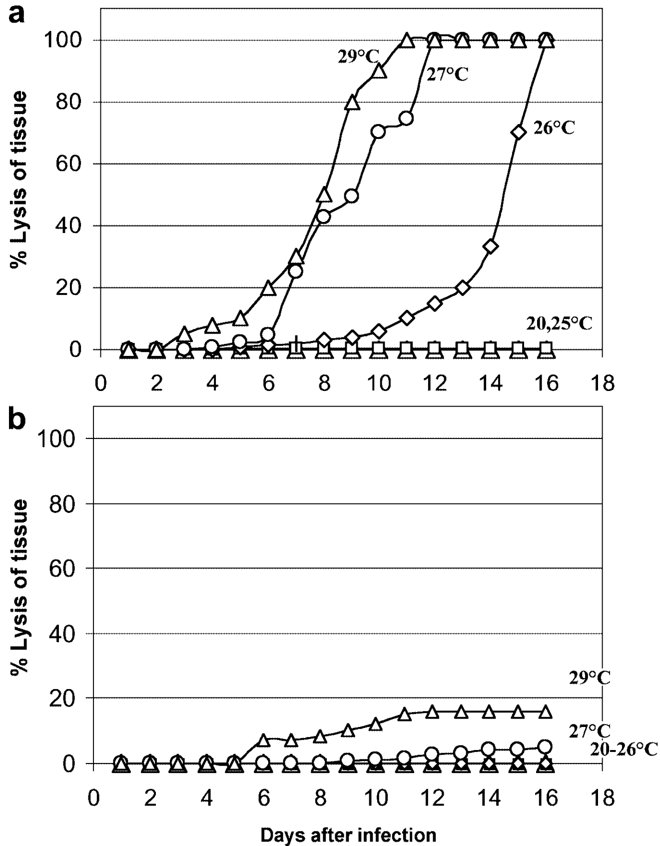

Fig. 6a, b. *Pocillopora damicornis*. Effect of temperature on infection by *Vibrio coralyticus* YB1. Average percentage of tissue lysis of *P. damicornis*, inoculated with *V. coralyticus* YB, during the 16 days following inoculation (**a**), compared to uninoculated control corals (**b**). The corals were inoculated with ca. 10^7 bacteria coral^{-1}, in 2- to 3-l aquaria, and maintained at 20, 25, 26, 27 and 29°C ($n = 2$, 13, 6, 9 and 5 inoculated corals, and $n = 2$, 16, 9, 10 and 31 uninoculated corals control corals, respectively)

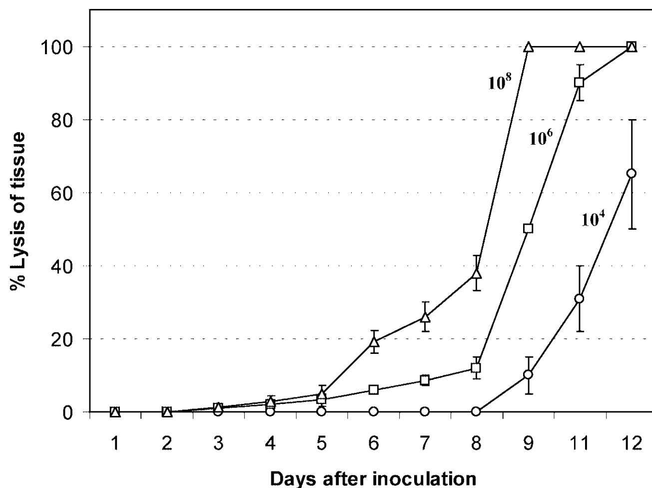

Fig. 7. *Pocillopora damicornis*. Effect of inoculum size on infection by *Vibrio coralyticus* YB. Corals were inoculated with $1{\times}10^4$, $1{\times}10^6$ and $1{\times}10^8$ *V. coralyticus* YB coral^{-1}, in 2.5-l aquaria at 29°C. The percentage of tissue lysis was measured during 12 days following inoculation. Average percentages of tissue lysis (±SE) were calculated and plotted: $1{\times}10^4$ (*circles*, $n = 3$), $1{\times}10^6$ (*squares*, $n = 3$) and $1{\times}10^8$ (*triangles*, $n = 5$)

of aquarium seawater), about 60% tissue lysis was achieved in 12 days. Thus, even with a relatively small inoculum size, *V. coralyticus* YB is able to infect and lyse *P. damicornis* at 29°C.

Discussion

The data presented in this report demonstrate that pure cultures of *Vibrio coralyticus* YB infect and completely lyse the tissues of healthy *Pocillopora damicornis* in controlled aquarium experiments. The pathogen could be transmitted from a diseased coral to a healthy one by direct contact, further indicating that the disease is contagious. In considering the potential ecological significance of this finding, two critical parameters were studied: seawater temperature and inoculum size. Inoculum size affected the rate of coral lysis and death, but not the final outcome. As few as 30 bacteria ml^{-1} were able to lyse the coral within 2 weeks at 29°C. It follows that *V. coralyticus* YB must grow and multiply on the mucus and tissue of the coral. With a generation time of <30 min at 29°C, a single *V. coralyticus* YB cell, if given the right conditions, would give rise to $>10^9$ bacteria in 15 h. Clearly, the key in predicting the outcome of the infection is to be found in the "right conditions", not the inoculum size. Since it is well documented that bacterial pathogens, such as *Vibrio cholera*, can be transported great distances in the ballast water of oil tankers and by other means (Colwell 1996), it is unlikely that *V. coralyticus* YB is restricted to the coral reefs of Zanzibar. We are currently testing for the presence of the strain in the Red Sea.

Seawater temperature was an important factor in determining the outcome of an infection of *P. damicornis* with *V. coralyticus* YB, with 25–27°C being the critical temperature range. At 27°C, complete lysis occurred in <2 weeks, whereas at 25°C, no lysis occurred for at least 20 days. The effect of this 2°C rise in temperature on infection and lysis could, in principle, be due to greater susceptibility of the coral and/or greater virulence of the bacterium. In the case of the well-studied *Vibrio shiloi*/*Oculina patagonica* bleaching disease, increased temperature has a profound effect on the expression of virulence genes (Rosenberg et al. 1999; Banin et al. 2000). For example, the *V. shiloi* adhesin, which is required for binding the bacterium to a ß-galactose-containing receptor on the coral surface, is only produced at the higher temperature, whereas the coral receptor is produced both at the higher permissive temperature and lower restrictive temperature (Toren et al. 1998). Other *V. shiloi* virulence factors, such as the peptide toxin PYPVYAPPPVVP (Banin et al. 2001b), lytic proteins (Ben-Haim et al. 1999) and superoxide dismutase (Israely et al. 2001), are also expressed at much higher levels at the elevated seawater temperatures. Similar to several other pathogenic marine *Vibrio* spp., *V. coralyticus* YB produces a potent extracellular proteinase (M. Keren and Y. Ben-Haim, unpublished

data), which may play a role in coral tissue lysis. This proteinase is expressed at much higher levels at 27°C than at 25°C.

Although the data presented here on bacterial-induced and temperature-dependent coral tissue lysis relate to a single species of coral and one bacterial strain, the conclusions may have some general implications. In this regard, we have recently isolated from diseased *P. damicornis* in the Red Sea, strains of *Vibrio* that are very similar to *V. coralyticus* YB. Preliminary infection experiments indicate that these new isolates are pathogenic to *P. damicornis*. Lysis of coral tissue is a widespread phenomenon, occurring in different species of corals, in many parts of the world, and is often correlated with increased seawater temperature (e.g. WCMC Global Coral Disease Database 2001; Coral Disease Page 2001). In several cases where systematic observations have been carried out over a period of time, tissue lysis is initially localized and then spreads over the coral. A spreading pattern is highly symptomatic of an infectious disease.

As pointed out by Richardson (1998), to demonstrate that a particular disease is caused by a specific microorganism, one must fulfill Koch's postulates. With regard to coral diseases, this can be a difficult task for both theoretical and practical reasons that have been discussed elsewhere (Banin et al. 2001a,b). However, one of the experimental procedures used in this study, placing the diseased coral in direct contact with a healthy coral of the same species (Fig. 5), is a relatively simple technique for demonstrating an infectious disease. The procedure can give positive results even if the pathogen is in a viable but not culturable (VBNC) state, as has been shown for *V. cholera* (Colwell et al. 1996) and *V. shiloi* (Israely et al. 2001).

The mechanisms by which *V. coralyticus* YB causes tissue damage to *P. damicornis* are, at present, unknown. Preliminary data indicate that the bacterium adheres to the coral surface and then penetrates into the tissue. We are currently examining the specificity of this adhesion and the effect of temperature on the process. In addition, we are studying the host specificity of the infection and the production of potential *V. coralyticus* YB virulence factors, such as the extracellular proteinase and toxins.

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