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ORIGINAL ARTICLE

The *Vibrio* core group induces yellow band disease in Caribbean and Indo-Pacific reef-building corals

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Abstract

Aims: To determine the relationship between yellow band disease (YBD)-associated pathogenic bacteria found in both Caribbean and Indo-Pacific reefs, and the virulence of these pathogens. YBD is one of the most significant coral diseases of the tropics.

Materials and Results: The consortium of four *Vibrio* species was isolated from YBD tissue on Indo-Pacific corals: *Vibrio rotiferianus*, *Vibrio harveyi*, *Vibrio alginolyticus* and *Vibrio proteolyticus*. This consortium affects *Symbiodinium* (zooxanthellae) *in hospite* causing symbiotic algal cell dysfunction and disorganization of algal thylakoid membrane-bound compartment from corals in both field and laboratory. Infected corals have decreased zooxanthella cell division compared with the healthy corals. *Vibrios* isolated from diseased *Diploastrea heliopora*, *Fungia* spp. and *Herpolitha* spp. of reef-building corals display pale yellow lesions, which are similar to those found on Caribbean *Montastraea* spp. with YBD.

Conclusions: The *Vibrio* consortium found in YBD-infected corals in the Caribbean are close genetic relatives to those in the Indo-Pacific. The consortium directly attacks *Symbiodinium* spp. (zooxanthellae) within gastrodermal tissues, causing degenerated and deformed organelles, and depleted photosynthetic pigments *in vitro* and *in situ*. Infected *Fungia* spp. have decreased cell division compared with the healthy zooxanthellae: 4·9% *vs* 1·9%, ($P \geq 0{\cdot}0024$), and in *D. heliopora* from 4·7% to 0·7% ($P \geq 0{\cdot}002$).

Significance and Impact of the Study: Pathogen virulence has major impacts on the survival of these important reef-building corals around the tropics.

Introduction

Different types of infectious marine diseases leading to coral death have been reported worldwide in the last decade (Antonius 1981; Kushmaro *et al.* 2001; Smith *et al.* 1996; Santavy and Peters 1997; Goreau *et al.* 1998; Richardson 1998; Sutherland *et al.* 2004; Aeby 2006). Coral yellow band disease (YBD) is a highly infectious disease affecting both Caribbean and Indo-Pacific reef-building coral species. YBD is characterized by blotches followed

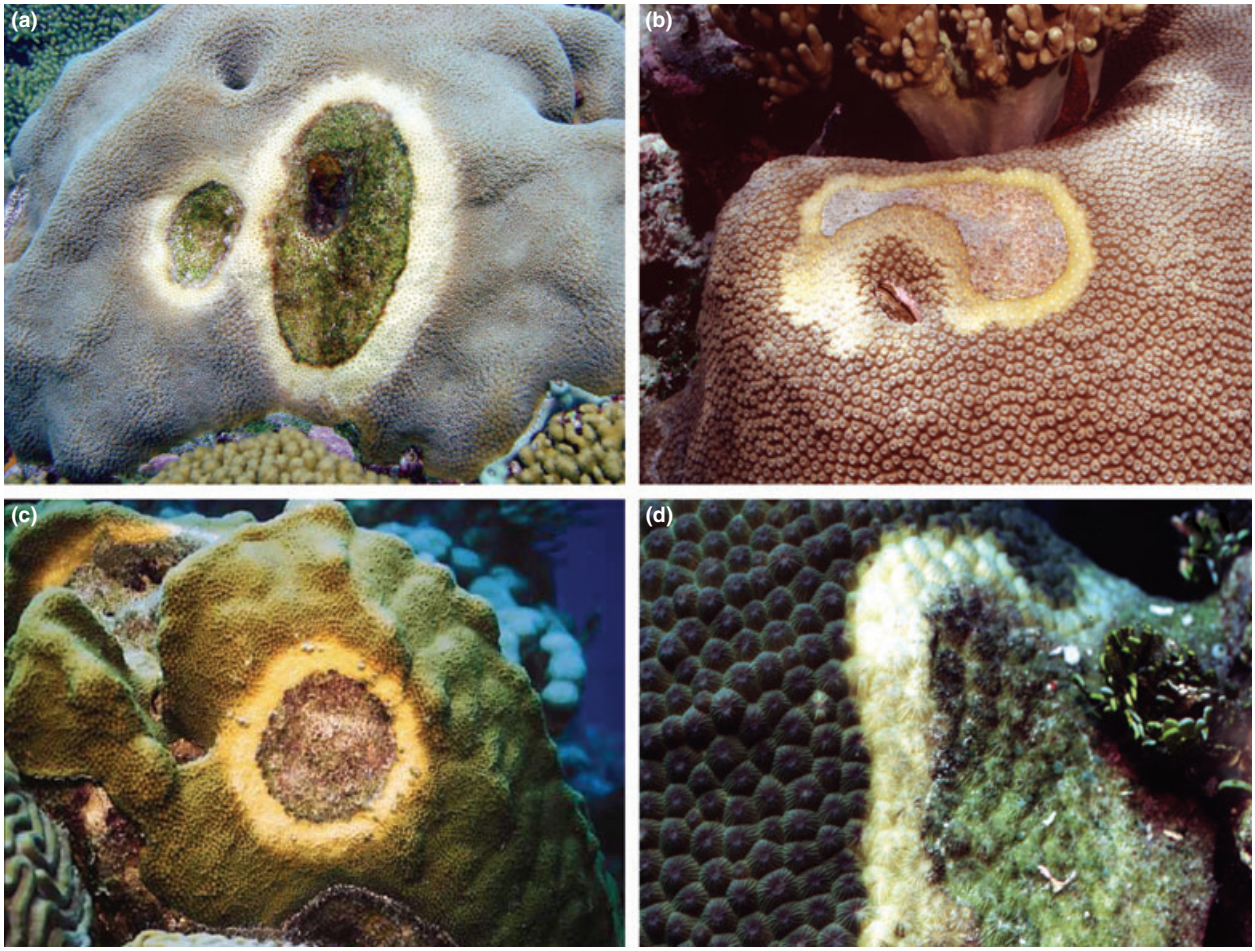

Figure 1 (a) *Montastraea* spp. during spring 2007 in Bonaire. (b) *Diploastraea heliopora* with a classic yellow ring on the upper surface of the colony. (c) A similar yellow band is seen in active stages in Bonaire during a 1998 field survey. (d) Yellow band lesion on the lower portion of *D. heliopora*.

by a circular ring pattern with a pale yellow to white margin (Santavy *et al.* 1999; Cervino *et al.* 2001). In the case of *Vibrio*-induced bleaching of *Oculina patagonica*, a proline-rich P-toxin is triggered by increasing sea water temperature, leading to zooxanthellae lysis and death (Banin *et al.* 2001). A clear link has now been shown between the increased rate of spread of yellow YBD and high sea surface temperatures (SST; Cervino *et al.* 2005). YBD lesions have been previously reported in the Caribbean and can be easily confused with thermal bleaching owing to the paling and coloration changes. This paper provides the first reports of YBD disease in the Pacific, in different genera than those affected in the Caribbean, and shows that the same core vibrio group of pathogens is involved (Fig. 1a–d).

During the past decade, the documentation of coral disease outbreaks owing to bacterial infections have increased significantly (Santavy and Peters 1997; Richardson 1998; Goreau *et al.* 1998; Ben-Haim *et al.* 2003; Green and Bruckner 2000; Cervino *et al.* 2000; Rosenberg *et al.* 2007). This is concurrent with an increased frequency of thermal coral reef bleaching events (Goreau and Hayes 1994, 2005; Goreau *et al.* 2000) and coral disease events (Harvell *et al.* 1999; Kushmaro *et al.* 2001; Ben-Haim *et al.* 2003a,b; Cervino *et al.* 2004b). Despite their apparent coincidence, there are morphological and physiological differences between thermal coral bleaching and coral diseases (Cervino *et al.* 2004a). Until recently, the correlation between thermal stress and coral diseases was unknown (Goreau *et al.* 1998; Harvell *et al.* 1999; Cervino *et al.* 2001). However, a clear link has now been shown between the increased rates of spread of YBD once infection has been established either in normal conditions (Cervino *et al.* 2004b) or during high SST (Cervino *et al.* 2005). During thermal bleaching (Hoegh-Gulberg *et al.* 1987), corals eject or expel their symbiotic zooxanthellae from the gastrodermal cavity owing to increased exposure to high temperatures or ultraviolet

radiation leaving the tissue clear exposing the skeleton (Mayor 1918; Jokiel and Coles 1990; Glynn 1993; Cervino 1996; Lesser 1997; Vermeulen *et al.* 2003; Tu and Bassler 2007). Unlike thermal bleaching (Mayor 1918; Wilkerson *et al.* 1988; Jokiel and Coles 1990; Lesser 1997; Richardson 1998), some of the known coral diseases seem to have different cell death mechanisms associated with the loss of the symbiotic algae (Cervino *et al.* 2004a; Ainsworth *et al.* 2007). *Vibrio*-induced diseases such as YBD-infected corals, show *in hospite* destruction of the symbiotic algae residing in the animal gastroderm in contrast to thermal bleaching where symbiotic algae are ejected from the tissues of corals during stress (Glynn 1984; Gates *et al.* 1992; Cervino *et al.* 2004a,b). Corals may also expel their symbionts in response to a decrease in salinity (Goreau 1964; Lesser 1997), exposure to chemicals from cyanide fishing (Cervino *et al.* 2004a) or sedimentation (Peters 1984; Vargas *et al.* 2007). Large *Montastrea* spp. colonies, centuries old, have been decimated throughout the Caribbean in the last decade owing to thermal stress, YBD and other diseases such as black band disease. A consortium of *Vibrio* species has been found in YBD (Cervino *et al.* 2004b). This paper provides the first reports of YBD disease in the Pacific, in different genera than those affected in the Caribbean, and shows that the same group of pathogens is involved (Fig. 1a–d). The aims of this study were to document their distribution in southeast Indo-Pacific, taxonomically characterize the *Vibrios* associated with YBD from the Caribbean and Indo-Pacific and evaluate the effect of these *Vibrios* on the cell cycle of symbiotic zooxanthellae as revealed by the mitotic index formula (Wilkerson *et al.* 1988; Jones and Yellowlees 1997).

Materials and methods

Pacific YBD was documented in Bali, Indonesia (8·65°S; 115·22°E) and in the Tukang Besi island chain of southeast Sulawesi, Indonesia (6°S; 124°E), in Ko Tao, Thailand, in Sagay, Negros Occidental, Philippines, and in Gili Trawangan, Lombok, Indonesia where 80–90% of the *Fungia* spp. are currently infected owing to a massive outbreak. Reefs were surveyed in Wakatobi Island Chain (southeast Sulawesi, Indonesia) at Roma, Lorenz's, Mari Mubk, Inka's, House Reef, Pastel and Onamobaa dive sites. Surveys were conducted using belt transects, each covering an area measuring 15 m × 1 m. Five horizontal transects between 10 and 12 m were completed at 7–8 dive locations. Study sites were chosen based on reef visitation frequency, selecting sites that were both heavily visited and less frequently visited. Transect sites were run beginning at the shore-dive entry point, and were continued at increasing depth and distance from the shore. To conduct the survey, a waterproof, tape-measured line was stretched horizontally at each coral reef location. All transects were between depths of 3 and 16 m. Visual counts of YBD-infected corals were compared with those of the healthy corals of the affected species. Colonies were photographed during August 2004 using an Underwater Nikon RS (SLR System).

Sample collection of YBD- infected corals

Total numbers of zooxanthellae and mitotic indices were determined from the 2·5-cm diameter tissue samples taken using a stainless steel hole punch from the control (healthy) and affected colonies in Wakatobi. Coral tissue samples were collected by SCUBA diving. The samples were taken outside the band lesion (healthy tissue), from tissue bordering healthy and actively spreading YBD and directly from the centre of the band or blotch for analysis. At each survey site, three samples of each from normal and affected tissue of *Diploastrea heliopora* and *Fungia* spp. were taken. These were placed in 100-ml polyethylene bottles. Samples were kept in ice coolers, in fresh filtered seawater (FSW) and transferred into 10% glutaraldehyde seawater solution for mitotic index determination (Jones and Yellowlees 1997; Wild *et al.* 2004).

Mitotic index analysis, TEM and cytology

Coral tissue samples were stored in a freezer (−4·0°C). The tissues were removed from all specimens using a Water Pik following the methods of Johannes and Wiebe (1970). The liquid extract containing tissue and zooxanthellae were homogenized and centrifuged at 5000 rev min^{-1} for 5 min, in order to separate host tissue from zooxanthellae. Liquid extract was discarded and the pellet was re-suspended in FSW/10% gluteraldehyde and 17% phosphate-buffered saline (PBS) solution and re-centrifuged for counts. Zooxanthellae abundance and mitotic indexes were determined by direct examination under a Zeiss Apo-Tome fluorescence microscope at 100–400× magnification, using a Neubauer ruling haemocytometer as detailed in Wilkerson *et al.* (1988). Three 2·5-cm plug samples were collected from different coral colonies of healthy tissue and YBD regions of *D. heliopora* and *Fungia* spp. Zooxanthellae counts were conducted in healthy and YBD-infected tissues. Transmission electron microscopy (TEM) was conducted at the Marine Biological Lab in Woods Hole; details are outlined in Cervino *et al.* (2004a).

Bacterial taxonomic characterization

Eight 10 ml sterile syringe samples were drawn from the mucous surface layers of YBD-infected and healthy corals

from the dive locations at Wakatobi reef. This method has been shown to be most effective owing to collecting important microbial populations not seen within the tissues. Syringe samples were immediately inoculated onto glycerol artificial sea water (GASW) agar plates and incubated at 26°C. Colony morphology was examined using a standard stereomicroscope. The different bacterial colonies were selected from the plates and purified on marine agar 2216 (Difco). Pure cultures were maintained in vials with 10% glycerol at −80°C. The 72 strains obtained in this study are listed in Fig. 4. All pure cultures were characterized by rep-polymerase chain reaction (PCR) analysis as described previously (Gomez-Gil *et al.* 2007). Briefly, genomic DNA was extracted with the Promega Wizard DNA extraction kit (Promega) according to the manufacturer's instructions. The DNA obtained was adjusted to 50 ng ml^{-1} spectrophotometrically. DNA fingerprinting of all the strains was performed with rep-PCR using the $(GTG)_5$ primer. rep-PCR products were amplified with the AmpliTaq® DNA polymerase enzyme (Applied-Biosystems, CA, USA) and the products underwent electrophoresis in 2·25% 20 × 20 cm agarose gels for 18 h at 55 V and 4–8°C. The gels were stained with ethidium bromide and visualized after integration in a gel documentation system (UVP). TIFF files obtained were analysed with the GelCompar II software (ver. 4·5; Applied Maths, Belgium), a similarity matrix was calculated with the Jaccard coefficient and the dendrogram constructed with Ward (optimization 0·35, position tolerance of 0·59%). Type and references strains of all valid species of the *Vibrionaceae* were incorporated in the analysis, as well as many strains from other origins, allowing clear species identification. Ten representative isolates were also characterized by 16S rRNA gene sequencing (Thompson *et al.* 2001). DNA isolation from the 10 bacterial isolates was followed by PCR amplification of the 16S rRNA gene using universal primers: 27F (5′-AGAGTTTGATCCTGGCTAG-3′) and 1522R (5′-AAGGAGGTGATCCARCCGCA-3′). PCR is performed using an Eppendorf thermocycler. The thermal programme consisted of: 5 min at 95°C, three cycles of 1 min at 95°C + 2 min and 15 s at 55°C and 1 min, 15 s at 72°C, 30 cycles of 35 s at 95°C + 1 min, 15 s at 55°C and 1 min, 15 sec 72°C and a final 7 min at 72°C. The PCR products were purified with a solution of PEG8000 and NaCl (2 mol l^{-1}). Purified PCR products were eluted in 30–200 μl of sterile MilliQ water. Subsequently, 3·0 μl of the purified PCR product was mixed with 1·0 μl ET Terminator™ Mix (Amersham, UK), 3·0 μl sequencing primer (4 μmol l^{-1}), 1·5 μl dilution buffer (5×) and 1·5 μl MilliQ water. The sequence of the sequencing primer is 5′-ACC AGG GTA TCT AAT CCT GT. The thermal programme consisted of 30 cycles of 15 s at 96°C + 1 s at 35°C + 4 min at 60°C. The sequenced products were purified using ethanol and acetate. Sequencing of the DNA fragments was performed in Megabace 1000 (Amersham). Raw sequence data were transferred to the Gene Builder module within Kodon package 2·03 (Applied Maths) where consensus sequences were determined using two reads. Similarity matrices and phylogenetic trees were constructed in the software Mega version 3·0 (69). Trees were drawn using the Neighbour-Joining method. A simple gram stain was applied *in situ* to determine the presence of gram-negative bacteria in all diseased and healthy specimens.

Inoculation experiments

Fragments of *Montastraea faveolata* (*c.* 300 cm^2) were collected using SCUBA from the NOAA coral nursery in Key West 24·32·7N–81·24·5W and transported to seawater-holding tables at the Mote Tropical Research Laboratory. After 24 h, the colonies were sectioned into replicates of 25 cm^2 in size. Fragments were held in an open seawater table under 70% light-reducing shade cloth for 5 weeks prior to initiation of the experiment. Four 10-gallon aquaria were placed in an open seawater bath providing temperature regulation and filled with filtered seawater. Each tank contained an immersion heater for temperature control and an airline with a small airstone bubbler to provide water circulation. These systems were allowed to equilibrate overnight to reach a constant temperature of 25°C prior to introduction of coral fragments. The temperature experiments were conducted between 26 and 32°C. Each aquarium contained six 5–7-cm coral fragments. Six *Vibrio* strains were used in the pathogenicity challenges. These were R-1191 (=YBFLG2A), R-1207 (=YB36), R-1216 (=YBFL3122), R-1257 (=YB23), R-1249 (=H11), and R-370 (=YBD-K). Each *Vibrio* isolate was resuspended in 4 ml of sterile sea water solution (SSS) individually. All of the suspensions were mixed in a beaker to create an inoculum with a density of *c.* 10^8 CFU ml^{-1}. The coral fragments were dipped in this inoculum for 1 min and subsequently returned to the aquaria. The control preparations were treated following the identical protocol, with 250 ml of seawater suspension. The observations were recorded on a daily basis.

Results

Field surveys clearly suggest that the morphological characteristics of the YBD found on *D. heliopora* of the Indo-Pacific were identical to the yellow band lesions of the Caribbean (Fig. 1b,d). The YBD in *Fungia* spp. appeared as a blotch that forms an expanding circle but never seemed to form circular rings (Fig. 2c). In the advanced stages

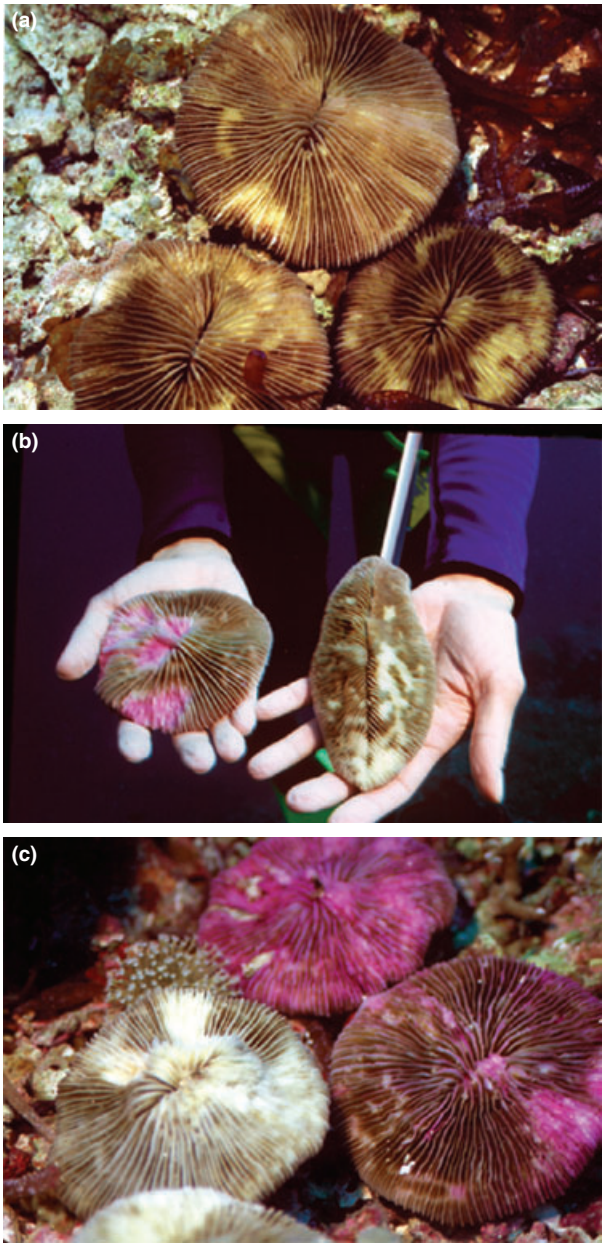

Figure 2 (a) Yellow blotch lesions in the later stages of the disease. The three lower colonies of the image and the upper portions is an indicator of what happens when the host tissue dies after the zooxanthellae are completely lysed inside the endoderm. (b) Early stages of yellow band blotches on *Herpolitha* spp. and in later stages of the *Fungia* spp. (right). (c) *Fungia* spp. showing the early stages of yellow blotch lesions on the surface of the colony.

(after the yellow lesions disappear), complete tissue degradation occurred and a pink colouration under the tissue residing in the skeleton was evident (Fig. 2a,b). *Diploastraea heliopora, Herpolitha* spp. and *Fungia* spp. suffered from YBD infections in the different locations at Wakatobi. Yellow band affected *Fungiidae* seen in Bali in 2005, but in the following 2 years there were few active cases but many dead *Fungia*. In Thailand and Philippine sites, both active cases and many dead corals were seen. In contrast in Gil Trawangan, Indonesia in December 2007 almost all *Fungiidae* were actively affected, but there were few dead corals. This suggests that the epizootic is very virulent, but local outbreaks eventually burn themselves out.

Gram stain of diseased specimens revealed 100% gram-negative bacterial species. Healthy samples showed a mixed population of gram(−) and gram(+) varieties. A total of 80 isolates were obtained from the coral samples (Table 1). The isolates originated from Antigua (n = 22), Bonaire (n = 4), Florida Keys (n = 6) and Wakatobi (n = 48). Only 11 isolates (13·7%) could not be identified, from the rest, 57 isolates (71·2%) were identified as belonging to the genus *Vibrio*, 6 isolates (7·5%) as *Bacillus*, 4 isolates as *Halomonas* (5·0) and 2 isolates (2·5%) as *Psychrobacter*. The bacterial isolates identified as *Vibrio* were all allocated to the core group of *Vibrio*, specifically within the species *Vibrio alginolyticus* (n = 17), *Vibrio harveyi* (n = 5), *Vibrio rotiferianus* (n = 16), *Vibrio proteolyticus* (n = 14) and a putative novel *Vibrio* species (n = 5) (Fig. 3). Of the *Bacillus* isolates, five were identified as *Bacillus pumilus* and one as *Bacillus* sp. The *Halomonas* and *Psychrobacter* could not be identified to the species level.

The rep-PCR analysis of the isolates identified as *Vibrio* permitted us to differentiate strains and clonal groups (as defined by isolates having around 90% pattern similarity; Gomez-Gil *et al.* 2007) within the species identified to be *Vibrio*. *Vibrio alginolyticus* formed the largest group with 17 isolates, but almost all belonged to only one clonal group (16 isolates, Fig. 3 and Table 2; clonal group Va). Group Va consists of isolates obtained from all localities but not from Bonaire and from all sources (Table 2), and from healthy and diseased corals. *Vibrio harveyi* consisted of two strains with two isolates each (Vh1 and Vh2): Vh1 isolated from diseased *Montastraea* corals in Antigua and Vh2 from healthy corals in Wakatobi. *Vibrio proteolyticus* consists of three strains: clonal group Vp1 with 2 isolates, Vp2 with 11 isolates and a single isolate strain (R1209). Vp1 and Vp2 were isolated from diseased corals whereas R1209 was isolated from a healthy one; all from Wakatobi. *Vibrio rotiferianus* was the most heterogeneous and widespread group. The rest of the isolates were determined to belong to a potentially novel *Vibrio* species forming only one clonal group (V1). This group was found in both Bonaire and the Florida Keys in diseased *Montastraea* corals, suggesting the wide geographic distribution of a highly successful clone. The 16S rDNA sequences of this putative novel species revealed that this group is a close phylogenetic neighbour of *V. alginolyticus*.

Four of the isolates used in the pathogenicity experiments were identified as the putative novel species of

Table 1 List of isolates used in this study

Isolation site (region)	Species	R number	Source	Remarks
Antigua	*Bacillus pumilus*	1264	Coral (*Montastraea* sp.)	Healthy coral
	Bacillus sp.	1261	Coral (*Montastraea* sp.)	Healthy coral
	Halomonas sp.	1252	Coral (*Montastraea* sp.)	Healthy coral
	No id.	1215	Coral (*Montastraea* sp.)	Healthy coral
		1222, 1229, 1271	Coral (*Montastraea* sp.)	Yellow band lesion
		1234	Coral (*Montastraea cavernosa*)	Yellow band lesion
	Psychrobacter sp.	1275	Coral (*Montastraea* sp.)	Healthy coral
	Vibrio alginolyticus	1193, 1232, 1260	Seawater	
	Vibrio harveyi	1223, 1231	Coral (*Montastraea* sp.)	Yellow band lesion
	Vibrio rotiferianus	1196, 1200, 1202, 1226, 1266	Coral (*Montastraea* sp.)	Yellow band lesion
		1199, 1213	Coral (*Montastraea* sp.)	Healthy coral
		1267	Coral (*Diplostraea* sp.)	Yellow band lesion
Karpata, Bonaire	*V. rotiferianus*	1195, 1227	Coral (*Montastraea* sp.)	Yellow band lesion
	Vibrio sp. nov. 1	1207, 1257	Coral (*Montastraea* sp.)	Yellow band lesion
Looe Key, Florida Keys, Florida	*B. pumilus*	1263	Seawater	
	No id.	1274	Coral (*Montastraea* sp.)	Yellow band lesion
	V. alginolyticus	1240	Coral (*Montastraea* sp.)	Yellow band lesion
	Vibrio sp. nov. 1	1191, 1216, 1246	Coral (*Montastraea* sp.)	Yellow band lesion
Wakatobi, Sulawesi	*B. pumilus*	1205	Seawater	
		1276, 1277	Coral (*Diplostraea* sp.)	Healthy coral
	Halomonas sp.	1214, 1251	Coral (*Fungia* sp.)	Healthy coral
		1221	Coral (*Diplostraea* sp.)	Healthy coral
	No id.	1206, 1217, 1235	Coral (*Fungia* sp.)	Yellow band lesion
		1211	Coral (*Diplostraea* sp.)	Yellow band lesion
		1236	Coral (*Diplostraea* sp.)	Healthy coral
	Psychrobacter sp.	1253	Coral (*Fungia* sp.)	Healthy coral
	V. alginolyticus	1189, 1190, 1208, 1237, 1249	Coral (*Fungia* sp.)	Yellow band lesion
		1197	Coral (*Diplostraea* sp.)	Yellow band lesion
		1201, 1244, 1245, 1247, 1254	Seawater	
		1212	Coral (*Diplostraea* sp.)	Healthy coral
		1243	Seawater	Plankton bloom
	V. harveyi	1210	Coral (*Diplostraea* sp.)	Yellow band lesion
		1224	Coral (*Diplostraea* sp.)	Healthy coral
		1225	Coral (*Fungia* sp.)	Healthy coral
	Vibrio proteolyticus	1203, 1204, 1228, 1230, 1233, 1238, 1239, 1241, 1242, 1248, 1250, 1256	Coral (*Fungia* sp.)	Yellow band lesion
		1209	Coral (*Fungia* sp.)	Healthy coral
		1218	Coral (*Diplostraea* sp.)	Yellow band lesion
	V. rotiferianus	1192, 1220	Coral (*Fungia* sp.)	Yellow band lesion
		1194	Seawater	
		1198, 1219	Coral (*Diplostraea* sp.)	Yellow band lesion
		1265	Coral (*Diplostraea* sp.)	Healthy coral

Vibrio. These were: R-1191 (=YBFLG2A), R-1207 (=YB36), R-1216 (=YBFL3122) and R-1257 (=YB23) (Fig. 3). All species also formed part of the clonal group V1. The other isolate (R-1249, =H-11) was identified as *V. alginolyticus*, part of the clonal group Va (Fig. 3) as well as isolate R-370 (=YBD-K), which has been identified as *V. alginolyticus* (isolate not shown, personal communication, Dr Kim Ritchie).

The inoculation experiments carried out in the laboratory using healthy *Montastraea* spp. showed the same morphological gross signals observed in the YBD in the Pacific and Caribbean after 48 h. Yellow blotch lesions occurred in aquaria with temperatures of 26–28°C. No lesions were observed in aquaria with temperatures of 30–32°C. It is important to note that during this experiment as well as in the past experiments (Cervino *et al.* 2004b), infectivity

Figure 3 Rep-polymerase chain reaction-amplified 16S rDNA gene sequences of all strains located in the Caribbean, Pacific and Florida, USA. *Vibrio* species of novelty 1 are indicated.

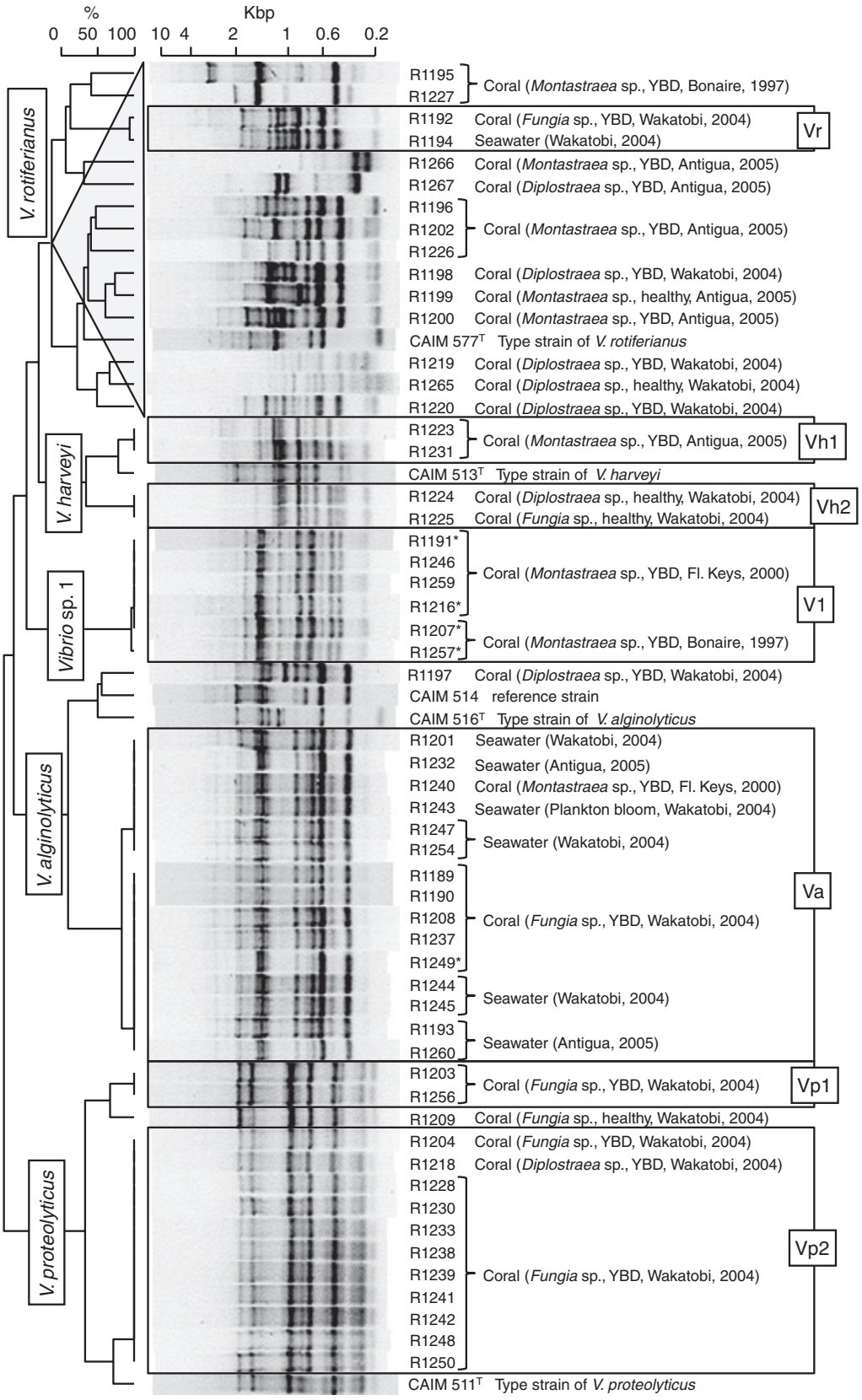

Table 2 Source of the *Vibrio* clonal groups found

Clonal group	Isolation site (region)	Source	Remarks	No. of isolates
V1	Karpata, Bonaire	Coral (*Montastraea* sp.)	Yellow band lesion	2
	Looe Key, Florida Keys, Florida	Coral (*Montastraea* sp.)	Yellow band lesion	3
Va	Antigua	Seawater		3
	Looe Key, Florida Keys, Florida	Coral (*Montastraea* sp.)	Yellow band lesion	1
	Wakatobi, Sulawesi	Coral (*Diplostraea* sp.)	Healthy coral	1
		Coral (*Fungia* sp.)	Yellow band lesion	5
		Seawater		5
		Seawater (plankton bloom)		1
Vh1	Antigua	Coral (*Montastraea* sp.)	Yellow band lesion	2
Vh2	Wakatobi, Sulawesi	Coral (*Diplostraea* sp.)	Healthy coral	1
		Coral (*Fungia* sp.)	Healthy coral	1
Vp1	Wakatobi, Sulawesi	Coral (*Fungia* sp.)	Yellow band lesion	2
Vp2	Wakatobi, Sulawesi	Coral (*Diplostraea* sp.)	Yellow band lesion	1
		Coral (*Fungia* sp.)	Yellow band lesion	10
Vr	Wakatobi, Sulawesi	Coral (*Fungia* sp.)	Yellow band lesion	1
		Seawater		1

V1, *Vibrio* sp. nov. 1; Va, *Vibrio alginolyticus*; Vh1, *Vibrio harveyi* clonal group 1; Vh2, *V. harveyi* clonal group 2; Vp1, *Vibrio proteolyticus* clonal group 1: Vp2, *V. proteolyticus* clonal group 2; Vr, *Vibrio rotiferianus*.

Figure 4 (a) Healthy *Montastraea* spp. (b) A closer image of an early developing lesion after 24 h of being inoculated with the *Vibrio* culture during normal ambient temperatures.

happened during normal ambient temperatures. Once infectivity occured and if the temperatures have increased, this higher thermal stress intensified the virulence of this disease. The inoculation experiments on healthy *Montastraea* spp. showed morphological similarities to yellow blotch lesions seen in both the Pacific and Caribbean. Inoculation experiments indicate an infectious agent when inoculated as a consortium, but are less virulent when inoculated separately. They did not induce YBD when alone; the lesion only appears with the Caribbean and Florida R groups together as a consortium as published in Cervino *et al.* (2004b). However, here we show that when using Pacific strains isolated from diseased Pacific corals similar disease signs appear. Figure 4a shows a healthy *Montastraea* sample used in the aquaria infection experiments conducted at Mote Marine Laboratory (Summerland Key, FL). Figure 4b indicates a yellow lesion on the surface of *Montastraea* spp. after 48 h when inoculated with Pacific strains, *V. alginolyticus* (R-370) and R-1249 in combination. It is important to note that when adding R-1249 and

R-370 with any two or three combinations of the Caribbean or Florida V-1 R groups, the YBD lesion would appear similar when only using Pacific strains R-1249–R-370 together (R-370 is not represented in the dendrogram; was re-sequenced by Dr Kim Ritchie to be *V. alginolyticus*).

Cytological analysis, cell densities and per cent mitotic index of the symbiotic algae

Total cell counts of zooxanthellae in the healthy endoderm of *D. heliopora* were much higher than those of YBD-infected lesions. Six specimens showing signs of yellow lesions or disease-infected cells had 95% less symbiotic algae than the healthy specimens ($P \leq 0{\cdot}0001$; $n = 6$; Fig. 5a). The mitotic index of healthy and diseased *D. heliopora* and *Fungia* spp. were *c.* 4·7% and 0·7%, respectively ($P < 0{\cdot}002$; Fig. 5b). The zooxanthellae cell density of YBD-diseased *Fungia* spp. was 47% lower compared with the healthy specimens ($P \leq 0{\cdot}0007$; Fig. 6a). The zooxanthellae close to the edge of the dying tissue showed almost complete cessation in cell division. However, it is important to note that the primary mode of attack is the symbiotic algae. Subsequent to the death of the symbiotic algae, the tissue appears to die back for reasons yet to be determined. The mitotic index of healthy and diseased *Fungia* spp. were 4·9% and 1·9%, respectively ($P < 0{\cdot}0024$; $n = 6$; Fig. 6b).

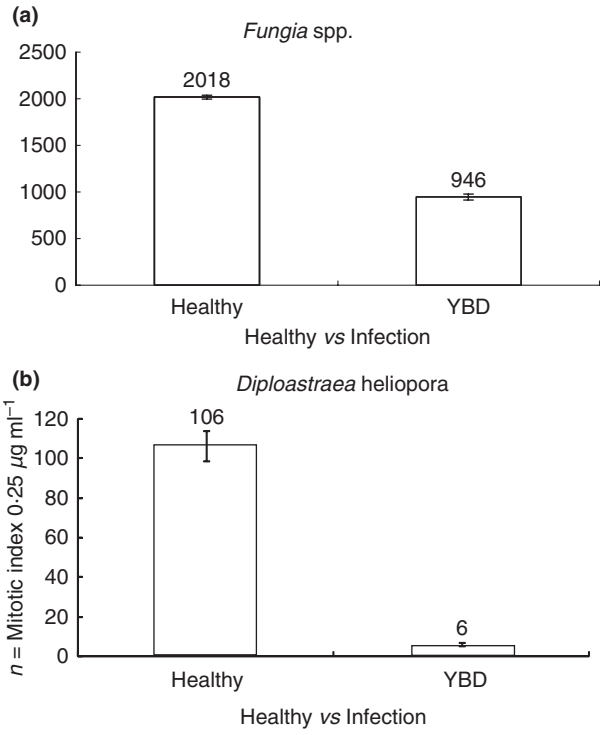

Figure 5 (a) Total cell counts of zooxanthellae found in the healthy and diseased endoderm of *Diploastraea heliopora*. Specimens showing signs of yellow lesions or disease-infected cells next to the healthy tissue; 95% less symbiotic algae seen within the host tissue than in the healthy specimens ($P \leq 0{\cdot}0001$). (b) The mitotic index of *D. heliopora* in healthy samples was 4·7% compared with the symbiotic algae collected in areas directly in the centre of the yellow lesion, which was 0·7% ($P < 0{\cdot}002$).

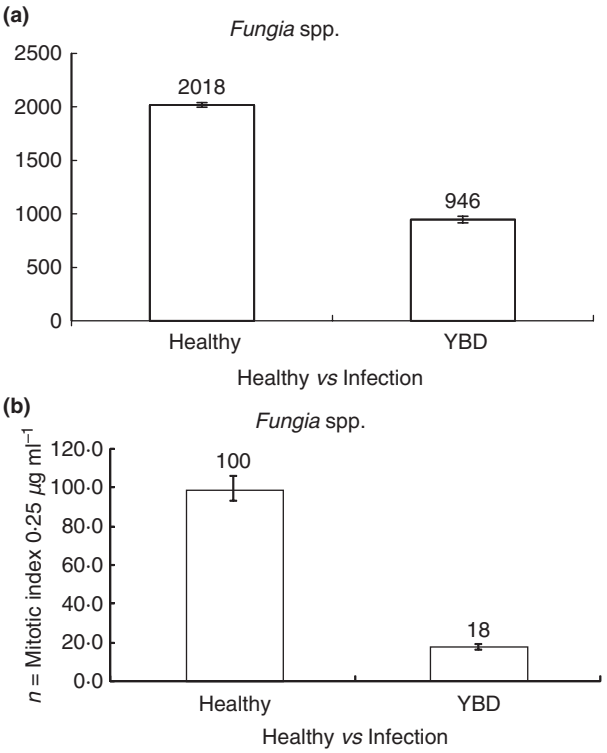

Figure 6 (a) Yellow band disease (YBD)-infected *Fungia* spp. showing a similar trend to Caribbean YBD and *Diploastraea heliopora*. Zooxanthellae cell densities of YBD-infected coral tissue were 47% lower compared with the healthy specimens ($P \leq 0{\cdot}0007$). (b) Mitotic index of *Fungia* spp. in healthy samples was 4·9% compared with the symbiotic algae from YBD samples, which was 1·9% ($P \leq 0{\cdot}0024$).

Microscopic examinations were conducted on symbiotic zooxanthellae that were isolated from healthy coral tissues of *D. heliopora* and directly from the centre of the yellow lesion. The healthy tissue reveals dark pigmented zooxanthellae, dividing cells in the mitotic phase, as well as the clear presence of distinct organelles (Figs 7a and 8). However, symbiotic zooxanthellae collected from the centre of the yellow lesion appear to be degenerative, vacuolated and depleted of organelle structures as shown in Steele and Goreau (1977) and Titlyanov *et al.* (1996) (Fig. 7b). Cell structures were swollen while the cell size was shrunken to about 34% compared with the healthy samples. In YBD-infected alga the thylakoid membrane-bound compartment inside the chloroplasts shows disorganized grana (Fig. 8). The thylakoid membranes contain

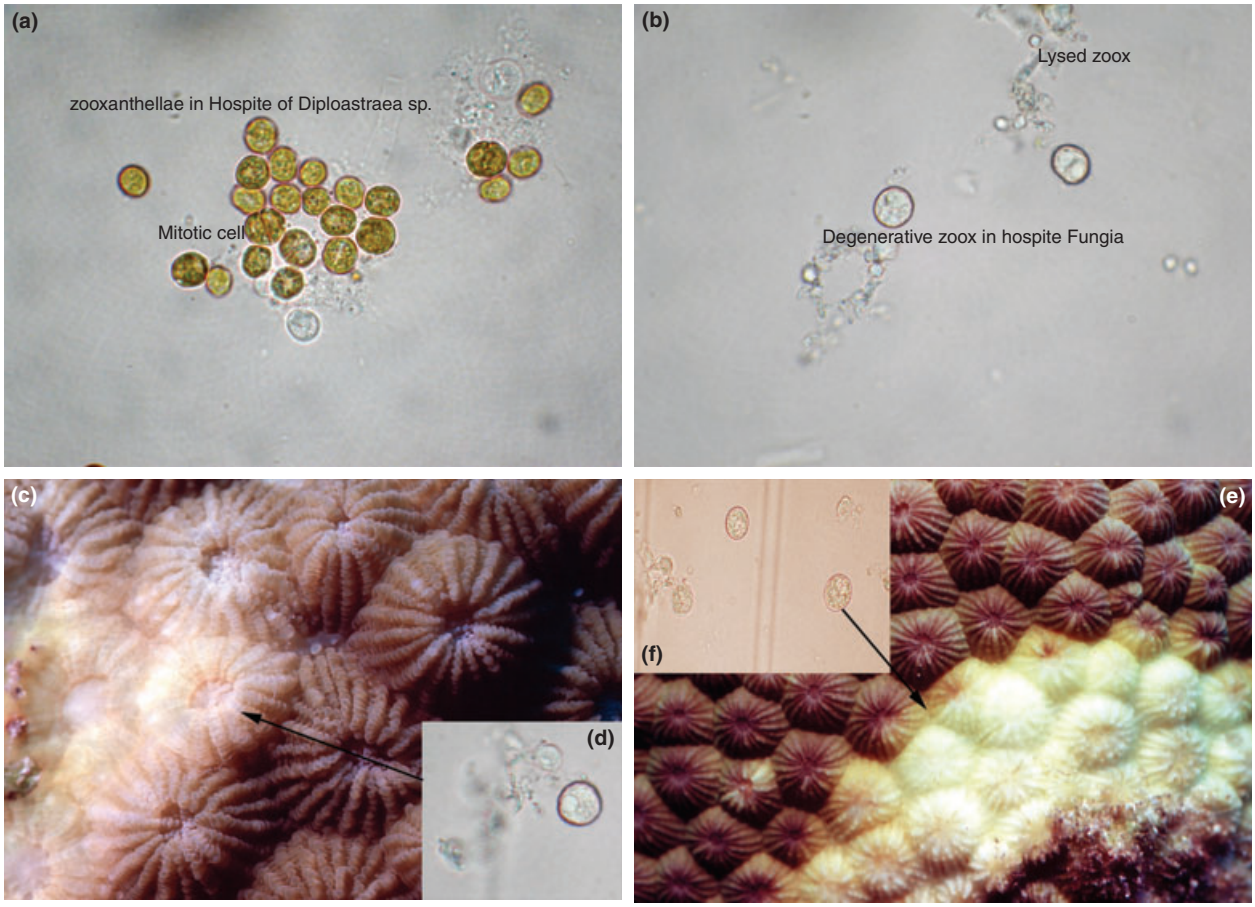

Figure 7 (a) Healthy symbiotic algae from *Diploastrea* spp. Two cells are in the stages of mitosis. (b) Degenerative cell from *Fungi* spp. from Yellow band disease (YBD)-infected tissues. (c) Active stages of vibrionic attack on the zooxanthellae *in hospice* of *Diploastrea* spp. (d) Degenerative ghost zooxanthellae cell taken from inside the infected tissue of *Diploastrea* spp. [taken from the sample in (c)]. (e) Close-up images of *Diploastrea heliopora* under water. The tissue is intact and algal pigments that remain during early infection with YBD pathogens are evident. (f) Late stages of atrophied and lysed degenerative zooxanthellae as a result of *Vibrio* spp. colonizing each and every algal cell. All YBD-infected cells show cytopathology and a reduction in size of the zooxanthellae of approx. 34%, respectively. Cells are swollen, membranes disrupted and cytoplasmic organelles are congealed (or opacified) compared with the controls. All images are at magnification 1000×.

integral membrane proteins that play an important role in light harvesting and the light-dependent reactions of photosynthesis (Warner *et al.* 1996). All YBD specimens are devoid of pigment (Fig. 7c,d) and in later stages complete lysis is observed within a bacterial trap (Figs 7b,f and 8). Zooxanthellae isolated from YBD-affected lesions have significantly less pigments than the healthy samples. Yellow band specimens in the field, of both species, exhibit polyp retraction and a visible increase in mucous strands acting as a surface substrate for bacterial species to proliferate (Waters and Bassler 2006). Similar to YBD of the Caribbean, expulsion of zooxanthellae in mucous during *in situ* observations was not evident in YBD in all Pacific specimens tested. The symbiotic zooxanthellae in affected Pacific corals are dying inside the host tissues as seen in Caribbean YBD-infected corals. During the active stages of YBD, one can observe the paling yellow tissue, an identical pathology and prognosis as seen in the Pacific corals mentioned here (Fig. 7c–f).

Field data

At all six dive locations in Wakatobi the frequency of yellow lesions infecting *D. heliopora* and *Fungia* spp., found in transects were different. The first dive site had a higher percentage of yellow lesions on the surfaces of *D. heliopora*, at 49%, compared with the *Fungia* spp., at 19% (Table 3). In contrast, the second dive site had a higher percentage of infected *Fungia* spp., at 32% compared with the 14% yellow lesions infecting *D. heliopora* (Table 3). This is due in part to the species dominance at each site; the second dive site was dominated by *Fungia*

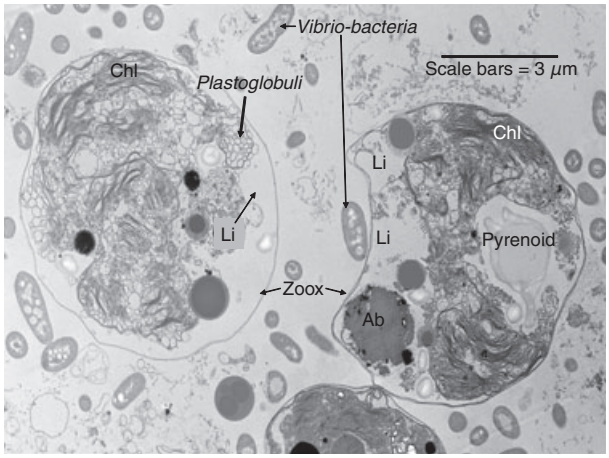

Figure 8 Transmission electron microscopy images showing the presence of *Vibrio* surrounding the zooxanthellae when subjected to thermal stress at 96 h. Scale bar = 3 μm. Chl, chloroplast; Ab, accumulation body; Li, lipid vacuole.

Table 3 Distribution of yellow band disease (YBD) in Wakatobi, Indonesia

Wakatobi dive sites	Healthy *Diploastraea*	YBD lesions	Total % YBD	Healthy *Fungia*	YBD lesions	Total % YBD
#1	34	15	49	246	133	19·6
#2	29	5	14	280	133	32·2
#3	17	3	15	197	84	30
#4	31	1	3·0	257	90	26
#5	34	7	17	179	56	26
#6	36	3	8·3	108	35	24

spp., and the first one was dominated with *D. heliopora*. YBD indices infecting *Herpolitha* spp. were not included in these quantitative transects from Wakatobi; however, qualitative observations were made at these sites and in Bali. *Herpolitha* spp. seems to show a similar rate of YBD lesion trend when compared with the *Fungia* spp., and these habitats had roughly equal numbers of these two species. It appears that towards the final stages of the lesion advancement, when complete tissue degradation occurs, a pink colouration under the tissue residing in the skeleton is evident for a short period before macroalgal overgrowth sets in on the inner portions of the exposed skeletons. While transect counts were not made in Thailand, Philippines, Bali or Lombok sites, the frequency of YBD was much higher in *Fungiidae* than in *D. heliopora*, and the characteristic yellow ring surrounding the dead coral was only frequent at the Thailand site. The possibility of onshore–offshore and/or depth gradients in disease abundance is suggested by a decrease in YBD with depth in Bonaire (T.J. Goreau, personal observation; A.R. Dona, personal communication). It is also suggested by recent observations in Barbados (T.J. Goreau) where over 80% of the *Montastrea* in the fringing reefs had active YBD lesions, compared to 20–30% in offshore bank reefs 15–20 m deep. In contrast, YBD was very rarely noted in a study of coral diseases in the Turks and Caicos Islands during 2006 (T.J. Goreau). In addition, other genera of reef-building corals in both the Caribbean and the Indo-Pacific show paler yellow abnormal areas that may be attributable to YBD-type pathogens; but this remains to be investigated.

Discussion

This study indicates that there is a very close genetic relationship, as assessed by the rep-PCR methodology that interrogates the bacterial genome, between the *V. alginolyticus* isolates found in coral infections in the Indo-Pacific, Caribbean and Florida. The data provided here shows that the YBD of the Indo-Pacific and Caribbean are identical at the morphological and cellular levels and seem to be caused by a consortium of *Vibrio* species. To speculate how part of this bacterial consortium was found in distant and separated geographic locations such as the Pacific and Caribbean, is beyond the scope of this study; however, it certainly is an interesting topic that involves ongoing research. The consortium of vibrios is allocated into the core group for which some members have been recognized as pathogenic for a variety of marine animals (Thompson *et al.* 2004). Another strain of *V. harveyi* (AK2, =LMG 20977) has also been implicated in causing vibrionic coral bleaching in the Mediterranean (Kushmaro *et al.* 2001; Gomez-Gil *et al.* 2007) and is related, by rep-PCR (>65%), to some of the strains of this species found in this study. One group of strains was not identified to any known species, although closely related to *V. alginolyticus*, and possibly represents a new species or subspecies. The *Vibrio* strains obtained in this study may be closely related to strains associated with mortality outbreaks of *Crassostrea gigas* and other marine invertebrates (Thompson *et al.* 2007). The consortium of virulent bacteria associated with YBD infections appears to target the symbiotic zooxanthellae in both the Caribbean and Pacific; however, we cannot yet pin-point the exact mechanism of infection, i.e. receptor site recognition as shown in Banin *et al.* (2001). The fact that YBD may be initiated at 25°C suggests that exposure to high temperature is not always necessary for initiation of infection; however, it is clear from the present study that the expansion of the yellow lesion increases rapidly with rising seawater temperatures (29–30°C). As shown in Cervino *et al.* (2004a), lesion expansion accelerates during warmer temperatures. The Wakatobi Marine Park did experience a higher temperature trend thereby breaching the thermal

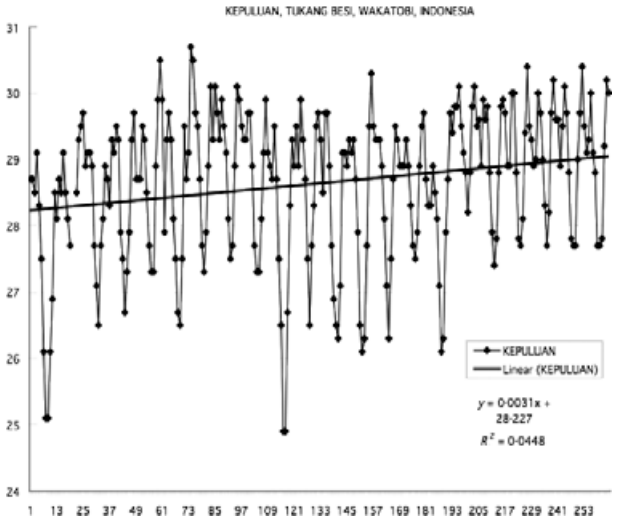

Figure 9 Monthly average sea surface temperature data for Wakatobi from 1982 to 2003 derived from satellite infrared measurements (Goreau and Hayes 2005) shows a clear increase in average temperatures.

bleaching thresholds for a short time period (Fig. 9). It is possible that given the time of infection, warm water formation lagged during the summer months, further exacerbating this outbreak in the Indo-Pacific region.

During YBD infection, the coral–zooxanthellae symbiosis is disrupted possibly because of the presence of a *Vibrio* binding to a receptor site found on the cell wall of the symbiotic zooxanthellae. Lysis of the zooxanthellae occurs. Nevertheless, before lysis, during adhesion, the infection impairs the mitotic cell division of zooxanthellae by an unknown pathogenic mechanism (Fig. 8). Molecular analyses on the pathogenesis of the *Vibrios* on the symbiotic zooxanthellae will shed light on the genes and toxins (proteins) responsible for its cell death. Any cell cycle impairments as a result of environmental conditions can result in changes in cell division and can be used as a biomarker to determine stress (Wilkerson *et al.* 1988; Suharsono and Brown 1992; Titlyanov *et al.* 1996; Jones and Yellowlees 1997; Muscatine *et al.* 1998). This can give researchers an understanding of how pathogens and temperature impair or decouple the symbiotic association (Cervino *et al.* 2001, 2004a). The analyses of infected corals may suggest that the vibrios cause a decrease in the cell cycle, particularly at 48 h after thermal stress. This may be explained by premitotic checkpoints taking place in cells, either allowing or preventing the damaged cell to go into the mitotic phase. Check points are evolutionarily conserved signalling mechanisms that stop cell division and change stress resistance in response to DNA damage (Torres *et al.* 2007). One possibility is that the toxins of these *Vibrio* pathogens may also be obstructing replication fork progression thereby not allowing for normal algal cell replication during YBD infection.

Conclusions

This research indicates a high genomic similarity between the pathogens associated with YBD of the Caribbean and Indo-Pacific. YBD-infected corals show severe thylakoid membrane-bound compartment damage, which can impair light harvesting and the light-dependent reactions of photosynthesis. Current research is underway using pulse-amplitude-modulation (PAM) measuring the relative chlorophyll fluorescence quantum yield in YBD-infected corals. A consortium of different *Vibrio* species of the core group causes the YBD in both oceans. The consortium caused a clear reduction in the mitotic index of zooxanthellae and ultimately the killing of the coral. It seems that the *Vibrios* intimately interact with the zooxanthellae and kill this organism. It is also clear that reef-building scleractinian corals need not be in a 'thermally stressed weakened state' to become infected by *Vibrio* pathogens. *Vibrios* may initiate the YBD independently of thermal stress, but increases in sea water temperature induce a more rapid spreading of this disease. If global warming-induced thermal stress and YBD infections continue to proliferate in the tropics, this will contribute significantly to the collapse of the remaining reef habitats in the next decade.

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