Environmental Microbiology (2006) **8**(12), 2068–2073 doi:10.1111/j.1462-2920.2006.01148.x

The Coral Probiotic Hypothesis

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Summary

Emerging diseases have been responsible for the death of about 30% of corals worldwide during the last 30 years. Coral biologists have predicted that by 2050 most of the world's coral reefs will be destroyed. This prediction is based on the assumption that corals can not adapt rapidly enough to environmental stress-related conditions and emerging diseases. Our recent studies of the *Vibrio shiloi/Oculina patagonica* model system of the coral bleaching disease indicate that corals can indeed adapt rapidly to changing environmental conditions by altering their population of symbiotic bacteria. These studies have led us to propose the Coral Probiotic Hypothesis. This hypothesis posits that a dynamic relationship exists between symbiotic microorganisms and environmental conditions which brings about the selection of the most advantageous coral holobiont. Changing their microbial partners would allow the corals to adapt to changing environmental conditions more rapidly (days to weeks) than via mutation and selection (many years). An important outcome of the Probiotic Hypothesis would be development of resistance of the coral holobiont to diseases. The following evidence supports this hypothesis: (i) Corals contain a large and diverse bacterial population associated with their mucus and tissues; (ii) the coral-associated bacterial population undergoes a rapid change when environmental conditions are altered; and (iii) although lacking an adaptive immune system (no antibodies), corals can develop resistance to pathogens. The Coral Probiotic Hypothesis may help explain the evolutionary success of corals and moderate the predictions of their demise.

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Introduction

Following our initial reports (Kushmaro *et al*., 1996; 1997) that the causative agent of the bleaching disease (loss of the endosymbiotic zooxanthellae) of the coral *Oculina patagonica* in the Mediterranean Sea is the bacterium *Vibrio shiloi*, we demonstrated that bleaching of the coral *Pocillopora damicornis* on the coral reefs in the Indian Ocean and Red Sea is the result of an infection with *Vibrio coralliilyticus* (Ben-Haim *et al*., 2003). The mechanisms of these diseases have been studied extensively during the last 10 years (reviewed in Rosenberg and Falkowitz, 2004). However, as reported in this article, during the last 2 years *O. patagonica* has developed resistance to the infection by *V. shiloi*; *V. shiloi* can no longer be found on the corals, and *V. shiloi* that previously infected corals are unable to infect the existing corals. To explain these data we propose the Coral Probiotic Hypothesis.

The term 'Probiotic', meaning 'for life', is derived from the Greek language. The following wide definition is primarily used for human and other mammalian hosts, but there is no reason why it should not also be applicable to invertebrates, such as corals: Probiotics are live microorganisms which confer a health benefit on their host (Schrezenmeir and de Vrese, 2001).

The basic concept of the Coral Probiotic Hypothesis is that the coral animal lives in a symbiotic relationship with a diverse metabolically active population of microorganisms (mostly bacteria). When environmental conditions change, e.g. increased seawater temperature, the relative abundance of microbial species changes in a manner that allows the coral holobiont to adapt to the new condition.

Abundance, diversity and specificity of coral-associated microorganisms

Rohwer and colleagues (2001; 2002) were the first to apply culture-independent methods to study coral-associated bacteria. Their data demonstrated a very high species diversity (Shannon index = 4.95), including a majority of novel species. Similar bacterial populations were found on the same coral species that were geographically separated while different populations were found on different coral species. Bourne and Munn (2005) used both culture-based and culture-independent techniques to investigate the microbial community of the reef building coral *P. damicornis*. They found the majority of clones obtained from coral tissue slurry libraries were

γ-*proteobacteria*, whereas the coral mucus was dominated by α-*proteobacteria*. Many of the retrieved clone sequences were conserved between coral colonies, further supporting the hypothesis of specific bacteria–coral associations. Diverse and abundant populations of *Archaea* are also associated with corals (Wegley *et al*., 2004). Direct counts with Archaea-specific peptide nucleic acid probes showed that corals contained ~ 10^8 Archaea per cm^2 (Wegley *et al*., 2004).

Recently, we examined bacteria associated with *O. patagonica* (Koren and Rosenberg, 2006). The mucus layer contained 3×10^8 bacteria per ml, 99.8% of which failed to produce colonies on MB agar. In addition to the mucus bacteria, an even larger number of bacteria was associated with the coral tissue after removing the mucus by centrifugation. The tissue-associated bacteria were quantitatively and qualitatively very different from the mucus bacteria. In considering the diversity of bacteria associated with *O. patagonica*, out of a total of 1088 16S rRNA genes sequenced, *c*. 400 were different operational taxonomic units (OTUs). As most of the 400 OTUs appeared only once, many more clones would have to be sequenced in order to estimate the full diversity of the coral bacteria. In addition to the mucus and tissue-associated bacteria, *O. patagonica* contains a large number of cyanobacteria inside the $CaCO_3$ skeleton (Fine and Loya, 2002).

Changes of coral-associated bacterial populations in response to environmental stress

Several investigators have shown that the bacterial communities of diseased corals are different from healthy ones both qualitatively and quantitatively. For example, Ritchie and Smith (1995; 2004) demonstrated that *Vibrio* populations increased during bleaching and returned to previous levels during recovery. Conversely, pseudomonads decreased during bleaching, and increased during recovery. Although they measured only two metabolic groups, one would expect large changes in the microbial community when the levels of oxygen and photosynthetic products are greatly reduced as a result of the loss of zooxanthellae. In a study of the white plague-like disease of *Favia favus*, it was shown that the culturable bacteria at the site of infection increased more than a thousand-fold (Barash *et al*., 2005). Only a small portion of these bacteria, namely *Thallosomonas loyaeana*, was the causative agent of the disease (Thompson *et al*., 2006). Pantos and colleagues (2003) showed a clear difference in the 16S rRNA gene profiles between corals affected by the white plague (type 1) disease and non-diseased corals.

The bacterial community of *O. patagonica* changes with the seasons (Koren and Rosenberg, 2006). Although the dominant bacterial species in both the summer (28°C) and winter (18°C) was *Vibrio splendidus*, representing *c*. 35% of all clones sequenced (Table 1), the next five most abundant species completely differed between summer and winter. Thus, the bacterial population of apparently healthy corals underwent changes within a period of a few months, probably as a result of temperature changes.

Table 1. The most abundant bacteria associated with the coral *O. patagonica* in the summer and winter.[a]

Summer 2005 (%)[b]	Winter 2005 (%)[c]
Vibrio splendidus (34.6)	*Vibrio splendidus* (34.8)
Prosthecochloris sp. (8.0)	Sulfuroxidizer (2.0)
Cytophaga sp. (5.9)	*Nitrospiras* sp. (1.6)
Proteobacterium sp. (4.5)	*Vibrio litoralis* (1.2)
Vibrio harveyi (2.8)	*Vibrio supersteus* (1.2)
Alcanivorex sp. (1.4)	α-*proteobacterium* sp. (1.2)

a. Summarized from Koren and Rosenberg (2006).
b. Species abundance (% of 253 16S rRNA genes sequenced).
c. Species abundance (% of 425 16S rRNA genes sequenced).

How does the bacterial population associated with a coral change as a function of disease or environmental stress? Certain species in this population must decline and others increase in frequency. In general, if the rate of multiplication of any specific bacterial species is lower than the rate of loss, then that species will become less abundant. Periodic discharge of mucus is one mechanism by which bacteria are removed from corals. Also, because marine bacteria are in dynamic equilibrium with bacteriophages (Wilhelm and Suttle, 1999), it is likely that coral bacteria are constantly being lysed as a result of bacteriophage infection. The source of newly abundant species can be from those that were previously present at low concentrations or from the outside. In addition to seawater serving as an inoculum source, animals that feed on corals can serve as vectors for transmitting bacteria from one coral to another. For example, the marine fireworm, *Hermodice carunculata*, is a winter reservoir and spring/summer vector for transmitting *V. shiloi* to *O. patagonica* (Sussman *et al*., 2003). It has also been shown that the macroalgae *Halimeda opuntia* can serve as a reservoir and vector for bacterial diseases of Caribbean corals (Nugues *et al*., 2004).

Development of resistance of corals to pathogens

In studies carried out in 1995, it was reported that all eight bleached *O. patagonica* examined contained *V. shiloi*, whereas none of the 14 healthy corals tested contained the pathogen (Kushmaro *et al*., 1996). From April to September 2000, 21 bleached *O. patagonica* examined contained $4–80 \times 10^6$ *V. shiloi* per cm^2 coral surface (Israely *et al*., 2001). However, since 2004 we have been unable to detect *V. shiloi* in either healthy or bleached corals:

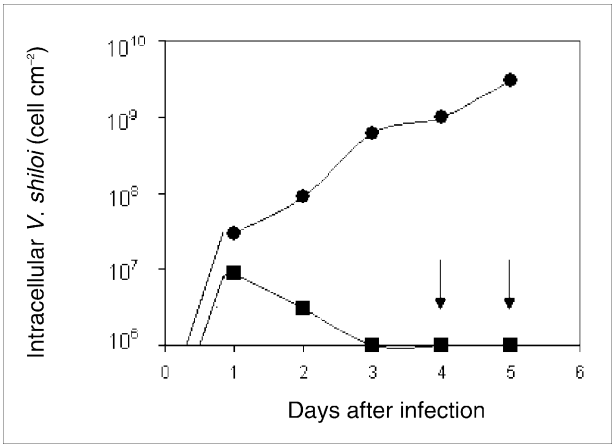

Fig. 1. Infection of *O. patagonica* with *V. shiloi*. The number of internal *V. shiloi* was determined by the gentamicin invasion assay (Isberg and Falkow, 1985) using fluorescent anti-*V. shiloi* antibodies (Banin *et al*., 2000). Data from Israely and colleagues (2001) (●) are compared to results obtained with corals collected in 2006 (■). The arrows indicate that the values were below the limit of detection (10^6 cells cm^{-2}).

approximately 20 bleached corals were examined by fluorescence microscopy using specific anti-*V. shiloi* antibodies and culturing methods (L. Reshef, unpubl.), and 240 16S rRNA clones from three bleached corals collected in 2005 were sequenced (Koren and Rosenberg, 2006).

From 1995 to 2002, laboratory stocks of *V. shiloi* infected healthy corals and caused the bleaching in controlled aquaria experiments. Starting in 2003, all laboratory stocks failed to cause bleaching of fresh healthy corals taken from the field. Moreover, a culture deposited in a National Collection (NCIMB) failed to bleach the corals. The stocks had been stored at –70°C in 20% glycerol or lyophilized.

In an attempt to understand the basis for the coral resistance to the pathogen, an infection experiment was performed and compared to an experiment performed in 2001 (Fig. 1). When the experiment was performed in 2001, *V. shiloi* adhered to the coral, penetrated into the exoderm and multiplied intracellularly, reaching over 10^9 cells per cm^2 coral surface. The concentration of intracellular *V. shiloi* remained over 10^8 cells/cm^2 for at least 25 days. Currently, the infection pattern is different. The bacteria still adhere to the coral and penetrate into the tissues, but after 24 h there is a decline and by the fourth day after inoculation, *V. shiloi* can no longer be detected in the tissue (Fig. 1). By some unknown mechanism, the coral is now able to lyse the intracellular *V. shiloi* and avoid the disease. Corals lack the ability to produce antibodies and have no adaptive immune system (Nair *et al*., 2005). The Coral Probiotic Hypothesis would predict that one of the properties of the bacterial community which is currently established in *O. patagonica* is the ability to produce materials which bring about the lysis of *V. shiloi*.

There are other less well documented examples of infectious diseases of corals that have changed over time. For example, the white plague disease was first observed off Key Largo, Florida, by Dustan (1977). A more virulent form of the disease occurred on the same reefs in 1995 (referred to as white plague type II). A bacterial pathogen was isolated from the type II disease and characterized as a novel bacterial genus (*Aurantimonas coralicida*, *gen. nov*. sp. nov.; Denner *et al*., 2003). Although the 1995 outbreak of the type II disease was severe (Richardson *et al*., 1998), it has had a minimal long-term impact on these reefs. Interestingly, healthy corals are no longer susceptible to the pathogen (Richardson and Aronson, 2002). As discussed above, the Coral Probiotic Hypothesis could explain these data.

The Coral Probiotic Hypothesis and the future of coral reefs

Coral reefs are a joy to behold, critical for the health of the sea and of major economic benefit to many countries. The value of resources and services derived from coral reefs has been estimated at $375 billion per year (Costanza *et al*., 1997). Coral reefs are clearly a resource that should be conserved.

Based on conservative estimates of increased seawater temperatures in the near future and the effects that increased temperatures have had on corals during the past 20 years, Hoegh-Guldberg has predicted that coral reefs will have only remnant populations of reef-building corals by the middle of this century (Hoegh-Guldberg, 1999; 2004). This prediction is based on the assumption that corals can not adapt rapidly enough to the predicted temperature increases in order to survive. This assumption was first challenged by the 'adaptive bleaching hypothesis (ABH)' (Buddemeier and Fautin, 1993). The ABH proposes that stressed corals first lose their dinoflagellate symbionts (bleach) and then regain a new mixture of zooxanthellae that are better suited to the stress conditions. Although some evidence has been reported in support of the ABH (reviewed by Buddemeier *et al*., 2004), it is not clear if changing algal partners is sufficiently rapid or extensive enough to survive the challenge of temperature stress.

The Coral Probiotic Hypothesis is more encompassing than the ABH. First, it incorporates the ABH if one considers the symbiotic dinoflagellates as microorganisms. More importantly, it includes the much larger number and greater diversity of bacteria associated with the coral. The hundreds of different bacterial species associated with the coral give the coral holobiont an enormous genetic potential to adapt rapidly to changing environmental conditions. As examples, let us consider three environmental stress conditions in which the coral bacteria may serve as

probiotics, i.e. benefit the health of the coral: changing nutrient availability, infection with a pathogen and temperature-induced bleaching.

Corals satisfy their nutritional requirements by exploiting two separate systems: sharing photosynthetic products produced by their symbiotic algae and capturing particulate organic material with the aid of their tentacles. Photosynthetic products provide them with carbon and energy, but not with the nitrogen that is required for synthesizing essential building blocks, such as amino acids, purines, pyrimidines and aminosugars. The oligotrophic waters where corals flourish contain very little utilizable sources of nitrogen. As nitrogen fixation is a process carried out exclusively by prokaryotes, it is reasonable to assume that some coral bacteria will benefit the coral holobiont by fixing nitrogen. Symbiotic nitrogen-fixing bacteria have been found in corals (Shashar *et al*., 1994; Lesser *et al*., 2004). Regarding the second method by which corals obtain nutrients, i.e. capturing particulates, bacteria probably play a key role in the catabolism of these materials. Three of the most abundant organic compounds in the sea, cellulose, agar and chitin, are degraded by bacteria but not by eukaryotes. After the bacteria degrade these compounds and multiply, some of these bacteria may serve as food for the coral animal (Kushmaro and Kramarsky-Winter, 2004). Thus, coral bacteria can allow corals to obtain energy from a complex mixture of polymers. In addition to having an enormous genetic potential to produce degradative enzymes, the relative amounts of different coral degradative bacteria can change rapidly as the nutrient source changes. Another nutritional consideration is that many of the coral bacteria are facultative anaerobes. During the day, the coral tissue and boundary layers are highly aerobic due to the production of oxygen by the endosymbiotic algae (Shashar *et al*., 1993; Kuhl *et al*., 1995). At night when the tissues, including the mucus, become anaerobic, we suggest many of the coral bacteria can carry out anaerobic metabolism, producing products which may serve as nutrients for bacteria and corals during the day (aerobic conditions).

By analogy with other animal systems, indigenous bacteria may help prevent infection by pathogens by occupying niches on the coral surface and tissues, and by producing antibacterial materials (Koh, 1997). Although little research has been carried out in this area with stony corals, it has been shown with sponges (Thakur *et al*., 2004) and soft corals (Kelman *et al*., 2006) that associated bacteria produce antibiotics that are active against planktonic bacteria, but not those bacteria associated with the animal. Castillo and colleagues (2001) have reported antibacterial substances produced by various marine organisms. Recently, Shnit-Orland and Kushmaro (unpublished) found that 25–30% of culturable bacteria from several hard corals produce antibiotics against indicator bacteria.

The bleaching disease of corals has been correlated with high seawater temperatures and high levels of solar irradiance (reviewed by Jokiel and Brown, 2004). At least, until now, most corals that bleach recover when the temperature drops. In considering the future of corals as a function of global warming, two aspects of the Coral Probiotic Hypothesis should be considered: the role of bacteria in preventing bleaching and other infectious diseases and in allowing the coral to survive a bleaching episode. Two mechanisms for causing bleaching have been posited: infection by a pathogen that targets the zooxanthellae (Rosenberg, 2004), and photoinhibition leading to damage of photosystem II (Jones *et al*., 1998; Warner *et al*., 1999). Coral-associated bacteria can play a role in preventing microbial-induced bleaching diseases by inhibiting colonization of the pathogen or killing it as described above. If the cause is damage resulting from photoinhibition, then it is possible that the damaging agents, presumably oxygen radicals, can be detoxified by bacterial enzymes, such as superoxide dismutase. Precisely those conditions which can lead to bleaching, i.e. high irradiance and temperature, will select for a bacterial population that produces oxygen free radical scavengers. This aspect of the Coral Probiotic Hypothesis could explain the observation that coral exposed to high light prior to high temperature avoided bleaching (Brown *et al*., 2000). Bacteria may help bleached corals avoid death by providing nutrients to replace the algal photosynthetic products. Fine and Loya (2002; 2004) have shown that cyanobacteria become prominent in bleached *O. patagonica* and that photosynthetic products flow from the cyanobacterium to the coral.

Generalizations and predictions

Corals are sessile organisms living in warm oceans containing very low concentrations of organic material and 10^5–10^6 bacteria per ml. To maximize uptake of nutrients, corals contain a high surface to volume ratio (the coral living tissue is only two layers thick). The price corals pay for their high surface to volume ratio is that they are highly exposed to potentially pathogenic microorganisms. In spite of their lack of a sophisticated immune system, corals are no less healthy nor suffer higher morbidity than organisms possessing a combinational adaptive immune system. Previous attempts to explain these findings (e.g. Janeway and Medzhitov, 2002) have been unsatisfactory (McFall-Ngai, 2005).

Based on recent studies in coral microbiology, we have presented the Coral Probiotic Hypothesis to help explain the evolutionary success of corals. One novel aspect of the hypothesis as presented is that the coral holobiont can

'adapt' to changing environmental stress conditions by changing the relative amounts of certain bacterial species that are present in the diverse pool of coral bacteria. As discussed above, this change takes place in *O. patagonica* from summer to winter. It is not unreasonable to predict that under appropriate selection conditions, the change could take place in days or weeks, rather than decades required for classical Darwinian mutation and selection. These rapid changes may allow the coral holobiont to use nutrients more efficiently, prevent colonization by specific pathogens and avoid death during bleaching by providing carbon and energy from photosynthetic prokaryotes.

The Coral Probiotic Hypothesis makes several predictions that can be tested, such as:

i. Corals that become resistant to a specific pathogen should show an increase in abundance of one or more coral bacterial strains that prevent the colonization of the pathogen or inhibit its growth.
ii. Germ-free or antibiotic-treated corals should be highly susceptible to infection and have reduced metabolic capacity.
iii. Corals shifted slowly to higher temperatures should change the relative amounts of different bacterial species and adapt to the temperature stress more readily than when the temperature is raised rapidly.
iv. Inoculation of bacteria taken from a coral that has adapted to a stress condition, e.g. high temperature, should accelerate the rate at which a coral of the same species will adapt to the stress condition.

Finally, it should be stressed that although discussions of probiotics in animal health are generally concerned with humans and other mammals which have a complex and adaptive immune system in addition to constant body temperature, probiotics are particularly relevant to corals and other marine invertebrates just because they lack these important characteristics. Regardless of the validity of the Coral Probiotic Hypothesis, it is now clear that corals must be considered as symbiotic organisms consisting of the coral animal, the endosymbiotic zooxanthellae and a metabolically active, diverse pool of prokaryotes.

Acknowledgements

This research was supported by grants from the Israel Center for the Study of Emerging Diseases, Israel Science Foundation (ISF), the World Bank Coral Disease and Bleaching Groups and the Pasha Gol Chair for Applied Microbiology.

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