

Session 12

The coral reef microbiome and microbial interactions and changes

Session chairs:

Max Teplitski, maxtep@uft.edu

Kim B. Ritchie, ritchie@mote.org

Julie Meyer, juliemeyer@uft.edu

Jennifer Sneed, SneedJ@si.edu

Rebecca Vega Thurber, rvegathurber@gmail.com

Deron Burkepille, deron.burkepille@lifesci.ucsb.edu

Adrienne M.S. Correa, ac53@rice.edu

Shelby E. McIlroy, smcilroy@buffalo.edu

David M. Baker, dmbaker@hku.hk

Ross Cunning, ross.cunning@gmail.com

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Interspecific coral bacterial competition under ocean acidification scenarios

G. Banc-Prandi, K. Imhof, E. Hall, K.B. Ritchie

Abstract Coral microbes have been hypothesized to promote coral health and protect the host against pathogens. Recent outbreaks of coral diseases are correlated with higher sea-surface temperatures and ocean acidification but few studies focus on the effect of these stresses on coral beneficial bacteria's physiology. This study examines the impact of ocean acidification on two coral commensals' ability to prevent infection by the coral pathogen *Vibrio shiloi*. Beneficial bacteria *Pseudoalteromonas sp. ZJ6102* and *Pseudoalteromonas euthinica* were isolated from the surface of healthy *Acropora cervicornis* and, when challenged with *V. shiloi*, resulted in an antibacterial response. Competition experiments were performed by mixing equal amounts of each commensal with the pathogen in dialysis tubing maintained at high (pH=8.1) or low (pH=7.7) pH under stable temperatures (25.5°C). Colony forming units (CFUs) of each strain were counted 24, 48 and 72 hours after inoculation. Results showed that lower pH tends to boost commensals' ability to limit the pathogen's growth. To test commensals' ability to protect the host from bacterial infection, inoculation experiments were performed directly on *A. cervicornis*. Coral health was estimated visually and indirectly using a PAM underwater fluorometer to assess deterioration of the symbiotic dinoflagellates by the pathogen. Results showed higher photosynthetic activity and reduced bleaching in low pH for corals treated with both the pathogen and commensal compared to controls treated with the pathogen only. Studies such as these will help improve our understanding of coral microbial dynamics in response to environmental changes.

Keywords: coral, beneficial bacteria, *Vibrio shiloi*, competition, ocean acidification, antibiotics.

G. Banc-Prandi
Ecole Normale Supérieure de Lyon

G. Banc-Prandi, K. Imhof, E. Hall, K.B. Ritchie
Mote Marine Laboratory

Corresponding author: K.B. Ritchie, kritch@uscb.edu

Introduction

Coral reefs have been shown to be major hotspots of marine biodiversity worldwide. They host thousands of species, from microscopic organisms to large fishes, representing more than 25% of marine life (McClanahan et al. 2016). Recently, serious losses of biodiversity in these ecosystems have been reported and linked to multiple outbreaks of diseases and bacterial infections causing

corals to die (Miller et al. 2015). This emergence of coral diseases is now mostly correlated to higher seawater temperatures, which have been shown to promote the attachment of the bacterial pathogens within the coral mucosal layer (Rosenberg et al. 2002). As a result of these diseases, the coral holobiont, a complex symbiotic organism consisting of the coral host, endosymbiotic zooxanthellae and an array of microorganisms, undergoes severe damages disrupting the mutualistic associations between its members (Cárdenas et al. 2011). In order to understand and predict how corals could react to these frequent infections, multiple studies focused on the coral organism itself, and characterized the coral's immune response as being relatively weak, unable to cope with the high pathogenicity of most coral pathogens (Palmer et al. 2011). More interestingly, an increasing number of studies suggest that coral mucus-associated bacteria would be implicated in promoting the health of their host (Bourne et al. 2009). For example, commensal bacteria of the elkhorn coral *Acropora palmata* are able to inhibit glycosidases critically needed by the coral pathogen *Serratia marcescens* for its growth on coral mucus (Krediet et al. 2012). As a result, colonization of the coral mucus is decreased. Direct inhibition of the pathogen's growth has also been reported as a consequence of antibiotics production by coral's commensals (Mao-Jones et al. 2010). Studies estimated that at least 20% of coral commensal bacteria show antimicrobial properties when challenged with specific pathogens (Ritchie 2006; Reshef et al. 2006). So far, very few studies have examined the impact of global warming on the ability of these commensals to cope with coral pathogens. A well-studied model, tested only in a context of higher seawater temperatures is the opportunistic coral pathogen *Vibrio shiloi* (Ben-haim 2003). *Vibrio shiloi* has been characterized as the causative agent of coral bleaching of the Mediterranean coral *Oculina patagonica* (Kushmaro et al. 2001). Higher seawater temperatures would promote the disruption of the coral's endosymbiotic algae's photosystem II by the pathogen's Zinc-metalloprotease, rapidly causing an irreversible photoinhibition (Sussman et al. 2009). As a result, the coral host loses its main source of energy and after expelling the inactivated algae, bleaches and often dies. Co-culture of *V. shiloi* with the coral commensals, selected for their antimicrobial properties, revealed a domination of the pathogen over the beneficial bacteria at 30°C under stable pH (Frydenborg et al. 2014). To go further in the understanding of the impact of global warming on coral health, we examined in this study the effect of ocean acidification (as the second main impact of global warming with higher temperatures) on the ability of coral's commensals to respond to an infection by *V. shiloi*.

Materials & methods

Coral maintenance

The branching coral *Acropora cervicornis* was used as recent studies revealed evidences of bleaching caused by *Vibrio* (Cramer KL et al. 2012). Healthy *A. cervicornis* fragments collected from the staghorn nursery (Lower Florida Keys, USA) were cut into four-centimeters pieces and maintained in flowing seawater at ambient (pH=8.1) or low (pH=7.7) pH, under stable temperature (25.5°C). Salinity, dissolved oxygen concentration, pH and temperature were controlled on a daily basis. Tanks were cleaned once a week, and corals were fed every other day.

Isolation of bacteria from *A. cervicornis*

Wooden applicator sticks were used to swab the mucosal layer of the corals, previously rinsed with filter-sterilized seawater of the right pH. The mucus was directly streaked out on marine agar medium (DIFCO), and the plates were incubated for two days at room temperature (22.5°C) (Kalimutho et al. 2007). 96-wells plate libraries were then prepared and used to store cultures at -80°C.

Detection of antibiotic activity

Antibiotic assays were performed to assess the ability of coral's beneficial bacteria to produce antimicrobials when challenged by *V. shiloi* (Gantar et al. 2011). On day one, libraries of *A. cervicornis*' bacteria were stamped on marine agar plates and incubated at room temperature (RT) for two days. On day two, liquid cultures of *V. shiloi* were made using marine broth (DIFCO) (24 hours incubation at RT). On the third day, batch cultures of *V. shiloi* were made by inoculating 0.8% agar marine broth with the 24 hours-old liquid cultures of *V. shiloi* at a concentration of 120 μ L.mL⁻¹. The mix was kept at 50°C while the 48 hours-old stamped libraries were UV-killed for two hours to avoid cross contamination from coral-derived bacteria during the antibiotic assay. The soft agar plates containing *V. shiloi* were then poured onto the UV-killed bacteria. After 24 hours of incubation at 30°C, the diameter of the zones of inhibition (area where the bacterium produces antimicrobial, inhibiting the growth of *V. shiloi*) were measured.

Identification of the antimicrobial-producing bacteria

The DNA of the antibacterial producing bacteria was extracted from pure cultures using the PowerSoil® DNA isolation kit (MO BIO). PCR amplification was carried out on genomic DNA using 16S rDNA forward primer U9F and the reverse primer U1509R and purified using a Qiagen PCR purification kit followed by directly sequenced using Sanger method at the UIUC Core Sequencing Facility (Shallowater, Texas, USA). Consensus sequences from reverse and forward DNA strands were generated. Percentage identity to known bacteria was obtained using GenBank

BLAST with a 97% cut-off.

In vitro competition experiment

Pseudoalteromonas sp. ZJ6102 and *Pseudoalteromonas euthinica* (two commensal bacteria isolated from *A. cervicornis* and producing antibacterial against *V. shiloi*) as well as the pathogen *V. shiloi* were inoculated in marine broth. After 24 hours of growth (corresponding to mid-exponential phase), bacterial cultures were centrifuged for 10 minutes at 4000 rpm at room temperature. The pellet was washed and suspended in sterile seawater (SSW). The new cultures were then standardized at a concentration of 10^7 colony-forming units (CFU) per milliliter using a spectrophotometer. For the bacteria to compete with each other, co-cultures were made by mixing equal number of cells of each strains (*P. ZJ6102* or *P. euthinica* with *V. shiloi*) into dialysis tubes (SnakeSkin Dialysis Tubing 10K MWCO) and placed into flowing seawater at ambient (pH=8.1) or low (pH=7.7) pH and stable temperature (25.5°C) (Anand et al. 2013). Both ends of the dialysis tubing were tied and clamped just above water level to avoid contamination. Monocultures as well as tubes inoculated with SSW were used as controls for the co-cultures. For each condition, six tubes were inoculated (three time points in duplicates). CFUs of each strain were determined at 24, 48 and 72 hours after inoculation of the tubes. A non-parametric repeated measures ANOVA known as the Friedman test was performed to determine the significance of the results. The null hypothesis H_0 was the equality of the means of the $\text{CFU}\cdot\text{mL}^{-1}$. P-values lower than 0.05 enabled to reject H_0 meaning that the means of the growth rates were significantly different.

In vivo competition experiment

Fifteen *A. cervicornis* coral fragments were used, eight acclimated to ambient pH (pH=8.1) and seven acclimated to low pH (pH=7.7). Bacterial liquid cultures were prepared similarly to the ones used for the in vitro competition experiment. Three coral fragments from the ambient pH condition and two from the low pH condition were incubated with 10^7 CFUs of both *P. ZJ6102* and *V. shiloi* (co-culture) for 3 minutes, and then put back into their tanks. Inoculations of corals with one strain only (two corals with *P. ZJ6102* and two with *V. shiloi*), or SSW for each pH condition were used as controls for the co-cultures. Every day for five days, pictures of every fragments were taken. In addition, direct non-invasive measurements of the effective quantum yield of photosystem II were carried out using a Pulse amplitude modulation underwater fluorometer (PAM). Good correlations between quantum yield and photosynthetic rate have been reported for algae (Sundberg et al. 1997). These values were used as indirect indicators for the progression of the infection by *V. shiloi*. A Friedman statistical test was used again to analyze the results.

Results

Antibiotic assays against *Vibrio shiloi*

The antibiotic assays performed on *A. cervicornis*' commensal bacteria challenged with *V. shiloi* revealed two bacteria producing antimicrobials against the pathogen (Figure 1). Diameters of their respective zones of inhibition were similar (1.5 mm in average). The blast of the 16S rRNA sequences of these two coral commensals allowed to identify them as *P. sp.* ZJ6102 and *P. euthinica* with a 97% cut-off (resp. Figure 1 A and B).

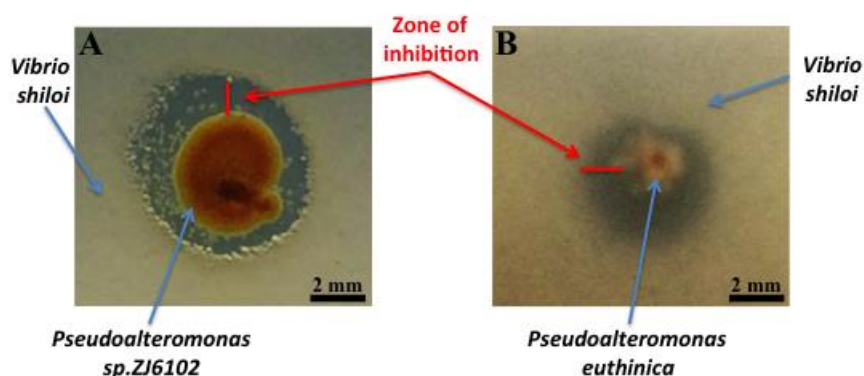


Fig 1 Antimicrobial activity of *Pseudoalteromonas sp.* ZJ6102 and *P. euthinica* against *Vibrio shiloi*. Antibiotic assays were performed by challenging beneficial bacteria isolated from *Acropora cervicornis*' mucus with coral pathogen *V. shiloi*. **A**, *P. ZJ6102*. **B**, *P. euthinica*. Zones of inhibition are clear area on which *V. shiloi*'s growth is inhibited by antimicrobial produced by the beneficial bacteria

In vitro competition experiment under ocean acidification scenario

The growth of both beneficial bacteria *P. ZJ6102*, *P. euthinica* and *V. shiloi* was monitored by taking absorbance measurements ($\lambda = 600$ nm) over 20 hours, and by calculating specific CFUs for each bacteria at each time point by dilution plating. Both beneficial bacteria show strong ability to outcompete *V. shiloi* (see Figure 2, A and B). Pictures of the plated co-cultures taken after 48 hours of incubation revealed a clear domination of both *Pseudoalteromonas* strains over the coral pathogen in low pH especially (very few white colonies of *V. shiloi* compared to the orange colonies of both *Pseudoalteromonas*). The estimation of the CFU.mL⁻¹ for both beneficial bacteria and *V. shiloi* in either mono or co-culture in both pH conditions revealed the dominance of the pathogen by both commensals (see Figure 3). *P. sp.* ZJ6102 grew significantly better than *V. shiloi* in both ambient pH after 24 (p-value = 0.0013) and 48 hours post inoculation (p-values = 0.00134) and in low pH at the same time points (p-values respectively of 0.007 and 2.10⁻⁶). The same result was observed for *P. euthinica* but only after 48 hours of co-culture (p-values of 0.008 and 0.002 in ambient and low pH,

respectively). In ambient conditions with both coral commensals, the CFUs.mL⁻¹ for *V. shiloi*, both in mono and co-culture, were significantly the same (see Figure 3 A and C : p-values of 1 and 0.08326 for respectively *P. ZJ6102* and *P.euthinica*). Interestingly, in low pH, *V. shiloi* grew significantly better in monoculture as compared to the co-culture with each beneficial bacteria (see Figure 3 A and B : p-values of 0.0455 for both beneficial bacteria in co-culture). The presence of the commensals was therefore responsible for the inhibition of *V. shiloi*'s growth. More interestingly, in low pH condition, *V. shiloi* declined faster in the presence of *P. ZJ6102* as compared to the co-culture with *P. euthinica* (see Figure 3B as compared to Figure 3D). *P. ZJ6102* seems more efficient in outcompeting *V. shiloi* in low pH condition than *P. euthinica*.

In vivo competition experiment on *A. cervicornis*

Corals inoculated with the coral pathogen *V. shiloi* only in both pH showed signs of bleaching around dark polyps (almost black, see Figure 4, B and F). On the contrary, coral fragments inoculated with SSW were apparently healthy (Figure 4, A and E) as well as the coral fragments inoculated with *P. ZJ6102* (see C and G). Corals inoculated with both bacteria in ambient pH had dark polyps and few white tissues around them, similar to fragments inoculated with the pathogen only (Figure 4B). Interestingly, in low pH, corals inoculated with both bacteria were apparently healthy, similar to the fragments inoculated with SSW (see Figure 4H compared to the seawater control in low pH condition in Figure 4E).

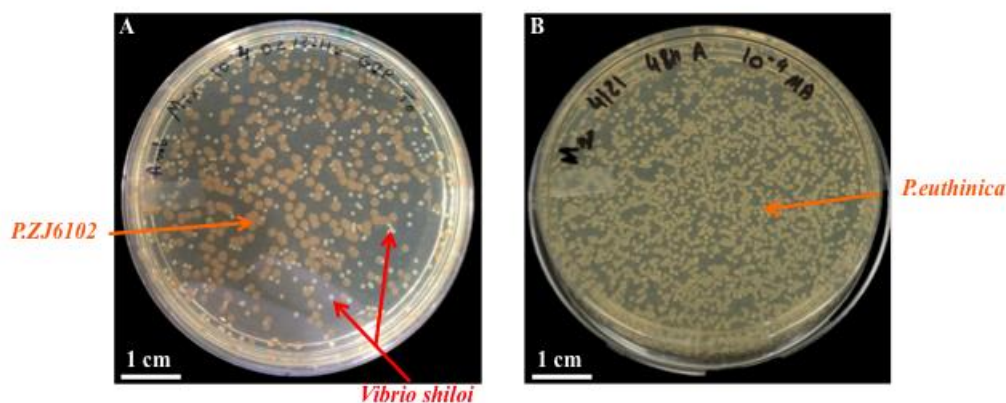


Fig. 2 Domination of two coral commensals over *Vibrio shiloi*. Cocultures of two coral commensals, either *Pseudoalteromonas sp. ZJ6102* (A) or *P. euthinica* (B), with *V. shiloi* were made by mixing each bacteria in a dialysis tube incubated in seawater at pH=8.1 or low pH=7.7 at 25.5°C. Each tube was plated on marine agar after 24, 48 and 72 hours of incubation (dilution to the thousands). **A, B:** 48 hours post inoculation, pH=7.7

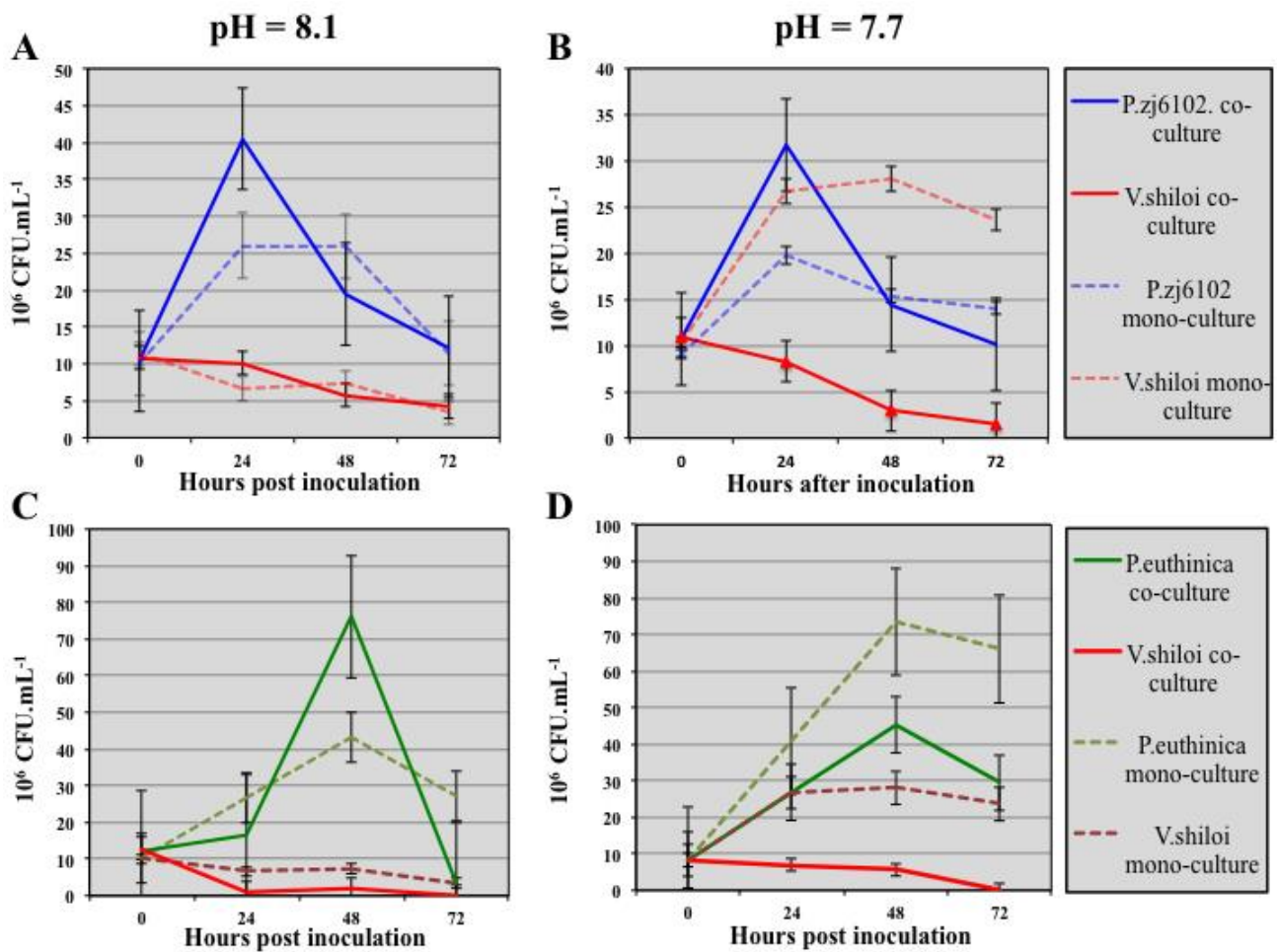


Fig.3 Outcompetition of coral pathogen *Vibrio shiloi* by two coral commensals boosted in low pH. *Pseudoalteromonas sp.* ZJ6102 (A,B) and *P. euthinica* (C,D) were isolated from *Acropora cervicornis*' mucus, and selected for their ability to produce antimicrobials against *V. shiloi*. 10⁷ colony forming units (CFU) of each bacteria were mixed in cocultures in dialysis tubes, and incubated in seawater at pH=8.1 (A,C) or pH=7.7 (B,D) at 25.5°C. Monocultures were used as controls for the cocultures. CFU.mL⁻¹ values were calculated 24, 48 and 72 hours after inoculation of the tubes. Error bars represent standard error of the mean

The yield (Y-value) for corals inoculated with *P. ZJ6102* only, *V. shiloi* only or both bacteria was significantly not different than for the fragments inoculated with SSW in ambient conditions (see Figure 4I, p-value = 0.179). Surprisingly, in low pH condition, while the yield of the corals inoculated with both bacteria was significantly the same as the yield for fragments inoculated with SSW (control), the Y-values for the corals inoculated with *V. shiloi* only tended to be lower, especially from three to five days after inoculation (p-value = 0.083) (see Figure 4J). In ambient conditions, the yield for the “*V. shiloi* only” treatment was not significantly different from the yield of both the SSW control and the mix (p-value = 0.1797). Interestingly, corals inoculated with the

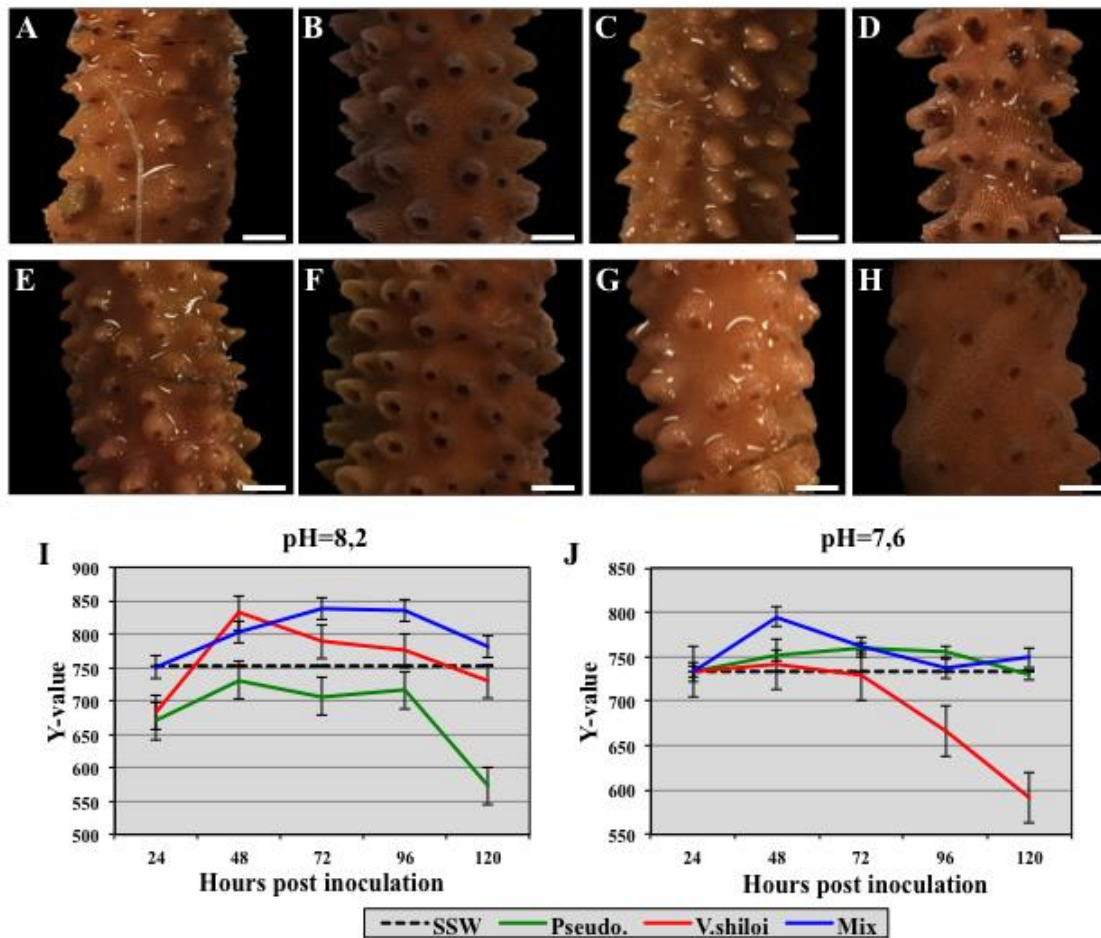


Fig. 4 Inoculation of corals *Acropora cervicornis* with its own beneficial bacteria reduces infection by *Vibrio shiloi* in low pH condition. *Pseudoalteromonas sp.* ZJ6102 was isolated from *A. cervicornis* mucus and produced antimicrobial against *V. shiloi*. 10^7 CFU of both the beneficial bacteria and *V. shiloi* were inoculated directly on 4 centimeters pieces of coral for three minutes. Inoculations with each strain or sterile seawater (SSW) were used as controls. Corals were maintained in either ambient (pH=8.1) or low (pH=7.7) pH under stable temperature (25.5°C). **A to H:** pictures of corals five days after inoculation (**A, B, C, D** respectively inoculation in ambient pH with SSW, *V. shiloi* only, *P. ZJ6102* only, both bacteria. **E,F,G,H:** same treatments but in low pH). White lines: 5mm scale. **I, J:** quantum yield (Y-value) of endosymbiotic zooxanthellae measured 24, 48, 72, 96 and 120 hours after inoculation. “Pseudo” refers to *P. ZJ6102*, and “Mix” to the inoculation with both bacteria. Error bars represent standard error.

beneficial bacteria showed lower yield values than the other treatments in ambient condition, and also started declining after 72 hours post inoculation in low pH condition.

Discussion

Production of antimicrobials by coral commensals

The two coral commensals isolated from healthy *A. cervicornis* and selected for their ability to produce antibacterial activity against *V. shiloi* belong to the gammaproteobacteria class. It has been shown that this class in particular is the most abundant in healthy coral mucus (Cárdenas et al. 2011).

Gammaproteobacteria were also found to be involved in the production of antimicrobials in coral mucus of many *Acropora* corals (Shnit-Orland et al. 2009). As the culture dependent methods used to isolate bacteria are biased toward a small percentage of bacteria successfully cultivated in the laboratory (Kalimutho et al. 2007), additional putative beneficial bacteria with the potential to produce antimicrobials against coral pathogens are hypothesized to be present.

Coral mucus-associated bacteria are now known to play a role in protecting their coral host from bacterial infection (Reshef et al. 2006). The production of antibiotic compounds by coral commensals in the mucus would prevent bacterial diseases by either killing or inhibiting the growth of the specific pathogen or contributing to the antimicrobial activity associated with the coral host (Ritchie 2006). Some of these commensals have been shown to have antibiotic properties only if cultured in mucus-based media (Shnit-Orland et al. 2009). Therefore, enrichment media may enable the detection of more antibiotic-producing coral bacteria for future studies related to coral beneficial bacteria.

Outcompetition of *V. shiloi* by coral commensals is boosted in low pH conditions

The results obtained for the in vitro competition experiment between both beneficial bacteria and *V. shiloi* showed that *Pseudoalteromonas* spp. out competed *V. shiloi* at low pH in experimental conditions. Interestingly, it has been shown that higher seawater temperatures boost *V. shiloi*'s virulence, leading to an out competition of the coral *Oculina pathagonica*'s commensals (Sussman et al. 2009). Indeed, at 30°C compared to 25°C, the ability of *V. shiloi* to utilize glycoproteins from the coral mucus is significantly higher than for the coral's beneficial bacteria. Performing the same in vitro competition experiment combining both pH and temperature variations would help in understanding the dynamic of bacterial competition in the field in a context of global warming.

The quantity of bacteria used for each strain was fixed at 10^7 CFUs to be close to their concentrations in the environment. Indeed, studies show that the density of *V. shiloi* on diseased *A. cervicornis* and *A. palmata* corals reaches up to 10^7 CFU.cm⁻² (Rosenberg et al. 2002). Therefore, the results of this in vitro competition experiment may yield insights into the dynamic of bacterial interactions in the environment. Future studies using multiple concentrations of beneficial bacteria could help determine the minimal concentration of commensal needed to most efficiently outcompete the pathogen.

Surprisingly, for both co-cultures in low pH conditions the two beneficial bacteria significantly outcompeted *V. shiloi*. Observations show that growth of *P. ZY6102* decreased after a day of incubation whereas *P. euthinica* grows for two days before declining. So far, the nature of the interaction between the pathogen and the two coral commensals is unknown. Some studies define

two main mechanisms that could enable the beneficial bacteria to outcompete *V. shiloi*, either directly or indirectly. The commensals could secrete growth inhibitors in the enzymatic activities involved in mucus layer colonization preventing the pathogen from multiplication in mucus (Krediet et al. 2012), or could inhibit quorum sensing and surface spreading. A more direct mechanism limiting the infection by the pathogen is the production of antimicrobials by some coral commensals (Mao-Jones et al. 2010). Given the design of the in vitro competition experiment, it would be difficult to detect the production of antibiotics in vitro. Indeed, isolating the beneficial bacteria after the three day incubation with *V. shiloi* may modify genes expression, and potentially alter their ability to produce antibiotics. Surprisingly, only 6-12% of opportunistic *V. shiloi* would be outcompeted directly by antimicrobials (Ritchie 2006). Therefore, indirect mechanisms, such as inhibition of growth factors or limitation of the pathogen's motility, would be more likely to take place in the mucus of infected corals. As this study only focuses on the effect of two beneficial bacteria on the growth of *V. shiloi*, it would be interesting to determine potential changes in levels of virulence gene expression in the pathogen. It is known that higher temperatures up-regulate virulence genes in *V. shiloi* such as genes implicated in motility, adherence and tissues degradation (Anand et al. 2013). Lower pH may also impact gene expression. Transcriptomics analysis will help better understand the dynamic of these interactions in future experiments.

Beneficial bacteria, potential line of defense against *V. shiloi*

The results of the in vivo competition experiment showed a slight reduction of bleaching for the corals inoculated with both the pathogen and *P. ZJ6102* in low pH only estimated using PAM fluorometry. Based on the yield measurements, the photosynthetic activity started decreasing only after 96 hours after inoculation of the corals with *V. shiloi* in low pH compared to the ones inoculated with the mix (see Figure 4J). However, it has been shown that *V. shiloi* causes photoinhibition of the endosymbiotic dinoflagellates after only 6 hours of incubation (Ben-haim et al. 2003). This difference of timing could be due to the conditions in which corals were maintained during the experiment. Air pumps were used to provide oxygen to the corals. Potentially, excessive airflow could have limited the attachment of the pathogen on the coral surface, disturbing its interaction with the coral's beneficial bacteria.

For this experiment, two parameters were monitored to assess the progression of the infection by *V. shiloi*. Daily visual assessments of coral fragments gave direct evidence of signs of bleaching, whereas measurements of the photosynthetic activity of the coral's endosymbiotic algae were used as indirect indicators of the progression of the disease. It has been shown that, with a more concentrated inoculum of *V. shiloi*, 25% of the algae were lysed after six hours of incubation (Sundberg et al.

1997). Investigation of the concentration and health of the algae present in the coral tissues at the end of the experiment (more than just the photosynthetic activity) could help better understand the dynamics of outcompetition of *V. shiloi* by the beneficial bacteria on the endosymbiotic algae. For instance, *V. shiloi* invasion of coral mucus and tissue has been correlated with high concentrations of ammonia, which might be the principal cause for algal bleaching, triggering the disruption of the photosystem II and the lysis of the algae (Sundberg et al. 1997; Brown et al. 2005). As inoculating corals with both the pathogen and the coral's commensal seem to prevent the loss of photosynthetic activity in low pH, it can be hypothesized that *P. ZJ6102* may produce compounds buffering the ammonia burst, or preventing its production in low pH conditions. Another interpretation is that the low pH, itself, is buffering the effect of the ammonia. Moreover, the diminution of the yield value of corals inoculated with the beneficial bacteria only could be due to the fact that the inoculated coral commensal might disturb the dynamics of the holobiont when being largely dominant over the rest of the members of the coral microbiota. Optimizing the concentrations of inoculum for the in vivo competition experiment to maximize the outcompetition of the pathogen while reducing the potential side effects of the commensal's transplantation should be attempted in future experiments.

This method of inoculating diseased corals with beneficial bacteria able to outcompete coral pathogens could be used for other coral species and in studies addressing the feasibility of direct applications for coral disease management.

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