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ORIGINAL ARTICLE

Corals shed bacteria as a potential mechanism of resilience to organic matter enrichment

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Understanding the mechanisms of resilience of coral reefs to anthropogenic stressors is a critical step toward mitigating their current global decline. Coral–bacteria associations are fundamental to reef health and disease, but direct observations of these interactions remain largely unexplored. Here, we use novel technology, high-speed laser scanning confocal microscopy on live coral (*Pocillopora damicornis*), to test the hypothesis that corals exert control over the abundance of their associated bacterial communities by releasing ('shedding') bacteria from their surface, and that this mechanism can counteract bacterial growth stimulated by organic inputs. We also test the hypothesis that the coral pathogen *Vibrio coralliilyticus* can evade such a defense mechanism. This first report of direct observation with high-speed confocal microscopy of living coral and its associated bacterial community revealed a layer (3.3–146.8 μm thick) on the coral surface where bacteria were concentrated. The results of two independent experiments showed that the bacterial abundance in this layer was not sensitive to enrichment (5 mg l^{-1} peptone), and that coral fragments exposed to enrichment released significantly more bacteria from their surfaces than control corals ($P<0.01$; $35.9 \pm 1.4 \times 10^5$ cells cm^{-2} coral versus $1.3 \pm 0.5 \times 10^5$ cells cm^{-2} coral). Our results provide direct support to the hypothesis that shedding bacteria may be an important mechanism by which coral-associated bacterial abundances are regulated under organic matter stress. Additionally, the novel ability to watch this ecological behavior in real-time at the microscale opens an unexplored avenue for mechanistic studies of coral–microbe interactions.
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Introduction

Coral reef resilience to the suite of anthropogenic stressors they currently face has been an active area of research in recent years. There have been substantial discussions in the literature about how to define resilience (Nystrom *et al.*, 2008), how to manage reefs in ways that encourage it (Hughes *et al.*, 2010), and how to synthesize case studies to elucidate the large-scale mechanisms that determine whether a stony-coral-dominated ecosystem can resist a phase-shift or rebound from one (McClanahan *et al.*, 2002; Norstrom *et al.*, 2009). However, very little attention has been given to the small-scale mechanisms relevant to the microbial processes that determine the resilience of individual corals to a given stressor. Delineating the mechanisms by which individual colonies resist or recover from environmental stress may be a critical link for understanding and predicting larger reef- and region-wide patterns of resilience.

Recently, researchers have begun to examine the potential for climate-driven impacts on coral–microbe associations and the corresponding influence on the function of coral-dominated reef ecosystems (Mouchka *et al.*, 2010; Meron *et al.*, 2011). While climate-driven impacts are of great concern to the longevity of all reefs, excessive organic matter input is another acute global threat degrading reefs worldwide (Fabricius, 2005). Coral–microbe relationships can be sensitive to organic matter inputs (Kline *et al.*, 2006; Smith *et al.*, 2006; Voss and Richardson, 2006; Thurber *et al.*, 2009), and resilience at the single colony level has been observed on short time scales (Garren *et al.*, 2009). Elucidating the underlying mechanisms of resilience to organic pollution may clarify how and why large-scale phase shifts occur on enriched reefs (that is, what triggers the 'tipping point') and could directly inform management actions.

A curious finding has been that corals exposed to chronic enrichment from a wastewater treatment plant did not harbor more bacteria on their surface than corals at reference reefs (Garren and

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Azam, 2010), and the same has been observed for corals exposed to large amounts of fish-pen effluent harboring an order-of-magnitude more bacteria than the typical reef water for the area (Garren *et al.*, 2009). There were differences in the community composition between the samples from the reference and treatment sites but no significant difference in the corals' surface bacterial abundance. We do know that a tipping point can exist in certain disease states where corals become more heavily colonized than in their healthy state (Luna *et al.*, 2007), but the underlying mechanisms are not known.

Shedding of bacteria from mucosal surfaces is a common occurrence in many animals, for instance the human gut (Neish, 2009); however, the ability to observe and quantify this behavior in reef-building corals had not been possible until recently. The observation of live coral–microbe interactions had not been successful previously for a combination of reasons that include a strong autofluorescence signal from the coral that can overwhelm signal from stains and the performance abilities of the last generation microscopes, which did not offer the speed, detection sensitivity, resolution, and working distance needed to simultaneously image the millimeter scale coral animal and the micrometer scale bacterial associates in real time. The newest generation of confocal microscopes alleviates these barriers with drastically improved sensitivity, resolution and speed (imaging up to 150 frames s^{-1} on living coral).

The goal of this study was to employ novel technology to test the hypotheses that the coral *Pocillopora damicornis* is able to exert some control over the abundance of its associated bacterial communities by releasing ('shedding') bacteria from their surface, and that a known pathogen, *Vibrio coralliilyticus*, may have the ability to overcome such a mechanism. We tested these hypotheses *in situ* on live coral using a high-speed laser scanning confocal microscope to directly observe and quantify the natural shedding rate and the subsequent response to organic enrichment. To the best of our knowledge, this is the first time that the natural assemblage of coral-associated bacteria have been observed *in situ* on live coral, and their ecological interactions documented in real time.

Materials and methods

Coral and bacterial cultures

The reef-building coral *P. damicornis* was used for this study. Colonies were cultured at 28 °C on a 12 h light/12 h dark cycle in flow-through seawater aquaria at Scripps Institution of Oceanography (La Jolla, CA, USA). Small fragments (5–10 mm in length) were clipped from three individual donor colonies and allowed to acclimate for 48 h. For all experiments, fragments were transferred to individual 50 ml closed-system chambers containing 0.2 μm filtered, autoclaved seawater (FASW) with a 0.2-μm filtered air supply. The temperature was maintained by placing the chambers in a 28 °C water bath, and light cycling remained the same.

The coral pathogen *V. coralliilyticus*, known to infect *P. damicornis* (Ben-Haim *et al.*, 2003), was cultured at 28 °C in FASW amended with 0.374 g l^{-1} 2216 Marine Broth (Difco, Franklin Lakes, NJ, USA). Cells were grown for 24 h to a concentration of $\sim 10^8$ cells ml^{-1} before staining with 1 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI), quantifying the cell density (on a 0.2-μm black polycarbonate filter (Millipore, Billerica, MA, USA) (following Porter and Feig, (1980))), diluting the stained liquid culture to experimental concentrations using unamended FASW and checking for motility in a chamber well slide (Nunc, Roskilde, Denmark) on an Olympus IX 51 epifluorescence microscope. The aliquots of culture taken for motility assessment were dual-stained with the lipophilic dye FM-4-64fx (5 μg ml^{-1}; Invitrogen, Carlsbad, CA, USA) to visualize the sheathed flagellum of *V. coralliilyticus*, and they were also observed by dark field microscopy (Supplementary Video 1). The percent motility of the culture was qualitatively observed in comparison with the undiluted culture.

Experimental design

Three independent experiments were conducted. The first quantified the *in situ* effects of short-term (24 h) organic enrichment (5 mg l^{-1} peptone) on the abundance of bacteria naturally associated with the corals. The second quantified the natural rate of particle and bacteria release during a time course through two diel cycles before exposing the corals to enrichment and sampling the time course for an additional 24 h. The third experiment quantified the *in situ* abundance of pathogen cells adhering to the living coral surface after a short (1 h) exposure to three different concentrations of pathogen. All data are reported with their associated standard error.

Influence of organic enrichment on shedding in situ

The natural bacterial population was directly quantified on and around the living coral surface using a high-speed laser scanning confocal microscope (Nikon A1-R) with a temperature-controlled chamber maintained at 28 °C (Supplementary Videos 2–4). Three coral fragments from separate donor colonies were imaged at T_0, whereas one fragment from each donor colony was exposed to 5 mg l^{-1} peptone ($\sim$200 μM dissolved organic carbon; approximately double the concentration of ambient dissolved organic carbon (ranging from 57 to 93 μM on relatively undisturbed reefs (Garren *et al.*, 2009; Tanaka *et al.*, 2011)) to simulate levels observed on heavily polluted reefs (Garren *et al.*, 2009)) in FASW for 24 h, and parallel fragments from the same donor colonies were maintained in FASW without peptone for 24 h as controls. For sampling, an aliquot of water from each experimental chamber was fixed at

T_{24} with a final concentration of 2% 0.2 μm-filtered formaldehyde to enumerate bacterial concentrations following Porter and Feig (1980). Coral fragments were removed from the experimental chambers, briefly rinsed in fresh 28 °C FASW and then stained with 1 μg ml^{-1} DAPI in FASW for 15 min in cover glass bottom chamber slides (Nunc, USA). A total of 10 different locations on each fragment were imaged in three dimensions (approximately 215 μm × 215 μm × 150 μm) using a ×60 water-immersion objective (Supplementary Video 3). Individual planes of focus were also imaged at video rate (up to 150 frames s^{-1}) to record the release of bacteria through time (Supplementary Video 2). Nikon Elements software program was used for both the acquisition and analysis of images. All surface areas and volumes were calculated by thresholding on the DAPI signal and using the area and volume calculation functions of the Elements software (Supplementary Video 4). A Student's *t*-test was used to evaluate the significance of the difference between the mean abundances and frequency of dividing cells of bacteria found on and shed from corals with and without exposure to peptone for both experiments.

Natural rate of particle and bacteria release through two diel cycles

To quantify the rates of bacteria and particle release from corals over time, a larger volume than the chamber slides could accommodate was needed, and thus counting was done *ex situ* (that is, by counting fixed samples placed on filters). Four fragments from the same donor colony were kept in 25 ml of FASW in 50 ml experimental chambers for 72 h. Ninety percent of the water (22.5 ml) was replaced with fresh FASW at 1, 3, 6 h, and then every 6 h following, for a total of 48 h. At T_{48}, the FASW was amended with 5 mg l^{-1} peptone to expose the corals to organic enrichment. Ninety percent of the water was again removed and replaced with fresh FASW plus peptone after 1 h (T_{49}), 3 h (T_{51}), 6 h (T_{54}) and then every 6 h after that for a total of 24 h (T_{72}). The controls consisted of two experimental chambers that never had a coral introduced (the 'blanks') to control for possible environmental contamination during the course of the experiment, and a set of parallel controls for each coral where 2.5 ml of the removed water at each time point was inoculated into a sterile experimental chamber with 22.5 ml of FASW (or FASW + peptone for T_{48} through T_{72}) to detect *in situ* growth of bacteria in the water column.

Sampling procedure

Ten milliliters of removed water from each chamber were fixed with a 2% final concentration of 0.2 μm-filtered formaldehyde at each time point. A portion was filtered onto 0.2 μm white polycarbonate filters (Millipore), stained with 1 × Alcian Blue for mucus-derived transparent exopolymers (TEP; Alldredge *et al.*, 1993), and 2 μg ml^{-1} DAPI for bacteria and counted on an Olympus IX-51 epifluorescence microscope. TEP abundance was quantified at ×100 magnification, whereas total bacterial abundances and the number of bacteria attached to TEP particles were quantified at ×1000. The number of bacteria counted in each of the controls was subtracted from the number in the corresponding treatment chamber to account for *in situ* growth of bacteria in the water column during each time period. The dilution culture approach was necessary to keep growth in the water column low enough to detect the bacteria being released from the corals.

Mucus samples were collected from each fragment after a rinse in FASW at T_0 and T_{72} by air exposure for 3 min over a sterile 1.5 ml tube. The mucus was fixed with 2% final concentration 0.2 μm-filtered formaldehyde and bacteria were counted following a previously published protocol (Garren and Azam, 2010).

A portion of unfixed water from each of the 10 chambers was sampled at T_0 and T_{72} for total organic carbon. Briefly, water samples were acidified and analyzed on a Shimadzu TOC-V high temperature combustion instrument fitted with an autosampler (Aluwihare Laboratory, Scripps Institution of Oceanography, La Jolla, CA, USA). The total organic carbon concentration of each sample was calculated from an average of four 100 μl injections using a five-point potassium hydrogen phthalate standard curve and consensus reference materials (courtesy of Dr Wenhao Chen, Rosenstiel School of Marine and Atmospheric Science).

Pathogen challenge

A fragment from each of two separate donor colonies was exposed in individual wells of four-chamber well slides maintained at 28 °C to 1 ml of prestained *V. coralliilyticus* (described above) for 1 h at one of three concentrations: 5×10^5, 5×10^6, or 5×10^7 cells ml^{-1}. After 1 h, fragments were briefly rinsed in 28 °C FASW, placed in a new chamber slide containing 2.5 ml of FASW, and the prestained pathogen cells were directly counted on the live coral surface using confocal microscopy (as described above).

Results and discussion

Influence of organic enrichment on shedding in situ

Direct observation with high-speed confocal microscopy of living coral and its active associated bacterial community revealed a layer on the coral surface above and through the ectoderm where bacteria were concentrated (Figure 1). The thickness of this layer ranged from 3.3 to 146.8 μm. It was thinnest on the tentacles and thickest on the coenosarc between polyps (Figure 1b). Exposure to

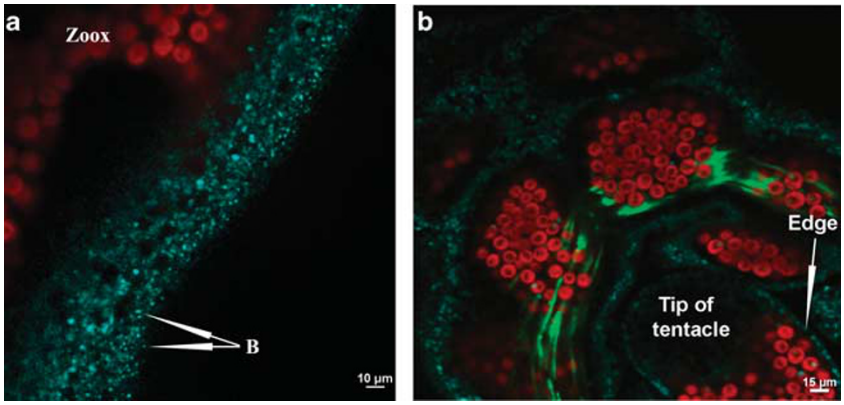

Figure 1 Laser-scanning confocal images of DAPI-stained *P. damicornis*. (**a**) An optical slice through the coenosarc of a fragment from the control treatment showing the inner layer of symbiotic dinoflagellates (zooxanthellae (Zoox)) with an outer crust of abundant DAPI-stained bacterial cells on the coral surface. Arrows point to individual bacterial cells (B) and the zooxanthellae appear in red due to the autofluorescence of their chlorophyll pigments. (**b**) A view into a polyp. The tentacle is covering the oral cavity. This image depicts the varying thickness of the bacteria-laden layer. The arrow points to the edge of the tentacle where this layer was thinnest on all corals imaged. The green in natural autofluorescence from the coral animal.

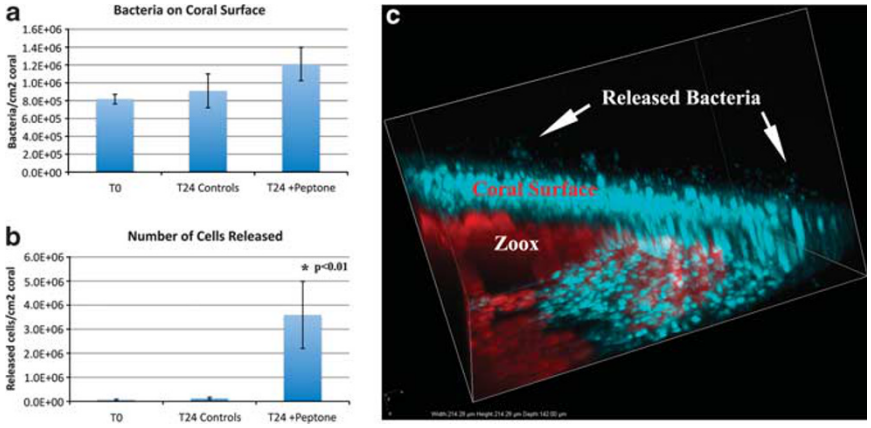

Figure 2 (**a**) The number of bacteria counted directly on the coral surface at T_0 and T_{24} with and without peptone enrichment. No significant difference was observed. (**b**) The average number of bacteria releasing from the surface of the corals at each time point. (**c**) A representative three-dimensional rendering of a z-stack from a coral in the peptone treatment after 24 h of exposure showing bacteria, some of which are attached to mucus-like particles, being released into the water just above the coral surface.

organic enrichment did not influence the number of bacteria colonizing the coral surface despite ~sevenfold higher numbers of bacteria in the surrounding water in the peptone-enriched experimental chambers at T_{24} compared with the control chambers ($P<0.01$, an average of $35.2 \pm 1.6 \times 10^5$ versus $5.2 \pm 0.4 \times 10^5$ cells ml^{-1} in the control chambers). There were no significant differences in coral-associated bacterial abundance among any of the fragments from the T_0 (6.8–9.7 $\times 10^5$ cells cm^{-2} coral; 2.6–5.1 $\times 10^8$ cells cm^{-3} coral surface layer) and T_{24} controls (4.8–15.6 $\times 10^5$ cells cm^{-2} coral; 1.5–2.2 $\times 10^8$ cells cm^{-3} coral surface layer) or the T_{24} peptone treatment (7.8–19.0 $\times 10^5$ cells cm^{-2} coral; 1.1–4.3 $\times 10^8$ cells cm^{-3} coral surface layer) (Figure 2a). However, the fragments exposed to peptone released significantly more bacteria from their surfaces than the control fragments in any given moment ($P<0.01$; an average of $35.9 \pm 1.4 \times 10^5$ cells cm^{-2} coral versus $1.3 \pm 0.5 \times 10^5$ cells cm^{-2} coral; Figures 2b and c). These findings underscore the potential importance of shedding bacteria as a mechanism for regulating the abundance of cells on the coral surface in the face of organic enrichment and substantial increases in bacterial abundance in the surrounding water.

Another notable observation was that the frequency of dividing cells in each coral-associated bacterial community was extremely high, ranging from 25 (T_{24} controls) to 30% (T_{24} peptone treatment) of the total observed population, and not significantly different among treatments. Previous studies of coral mucus found the frequency of dividing cells to range from 3 to 7% (Garren and Azam, 2010). This observation suggests that the coral-associated bacterial communities may grow rapidly under these experimental conditions. However, we cannot rule out (indeed we consider it likely) that the frequency of dividing cells values in this study were overestimated: some non-dividing

bacteria may go uncounted in our procedure developed to obtain reliable minimum estimates of bacterial abundance in the dense mucus layer matrix, and therefore these data may underestimate the total abundance and/or preferentially count dividing cells because of their distinctly identifiable cell morphology. In either case, these measurements give minimum estimates of the actual bacterial abundances.

Natural rate of particle and bacteria release through two diel cycles

Over the course of 72 h, the abundance of bacteria in the surrounding water increased by more than four orders of magnitude (from $1.2 \pm 0.6 \times 10^2$ cells ml^{-1} at T_0 to an average of $9.1 \pm 0.6 \times 10^6$ cells ml^{-1} at T_{72}), however, the number of bacteria in the coral mucus did not change significantly (an average of $2.2 \pm 0.4 \times 10^5$ cells ml^{-1} mucus at T_0 versus $2.3 \pm 0.6 \times 10^5$ cells ml^{-1} mucus at T_{72} (Figures 3a and b)). The rate at which bacteria were released increased during the course of the experiment (from an average of $2.8 \pm 0.5 \times 10^4$ cells cm^{-2} of coral per h during day 1, to $86.7 \pm 12.4 \times 10^4$ cells cm^{-2} of coral per h during day 2, to $541.7 \pm 90.0 \times 10^4$ cells cm^{-2} of coral per h during day 3 with the peptone enrichment; $P<0.01$; Figure 3c) as did the number of bacteria associated with TEP particles ($P<0.05$; Figures 3d and 4). The rate of TEP release was not significantly different over the course of the first 2 days; however, after the addition of peptone, it did increase significantly ($P<0.05$) (from an average of $2.0 \pm 0.5 \times 10^3$ TEP particles per cm of coral per h during day 2 to $6.7 \pm 0.6 \times 10^3$ TEP particles cm^{-2} of coral per h during the day with peptone). These numbers implicate the shedding of bacteria as an important component of the mechanism by which corals maintain a stable concentration of bacteria on their surface.

As some components of coral mucus stain as TEP (Goldberg, 2002), the release of heavily colonized TEP particles suggests that these coral fragments can shed substantial numbers of bacteria as they shed their mucus. Not all mucus components stain with Alcian Blue, so there are likely to be many more bacteria attached to mucus particles not observed with this method. Additionally, the coral polyps were filter feeding throughout the experiment and likely removing bacteria and particles from the water column. Given that the corals were the only source of TEP particles in these experimental chambers filled with 0.2 μm-filtered sterile seawater, these data provide a minimum estimate of the number of bacteria released on mucus particles.

It is also possible that some members of the bacterial community alter their physiology to take advantage of the enriched water environment. Some of the observed bacteria released from the coral surface could have been initiated by the bacteria themselves. However, given the number of bacteria that were released attached to TEP particles, our observations are consistent with the hypothesis that the release of mucus is a part of the mechanism(s) by

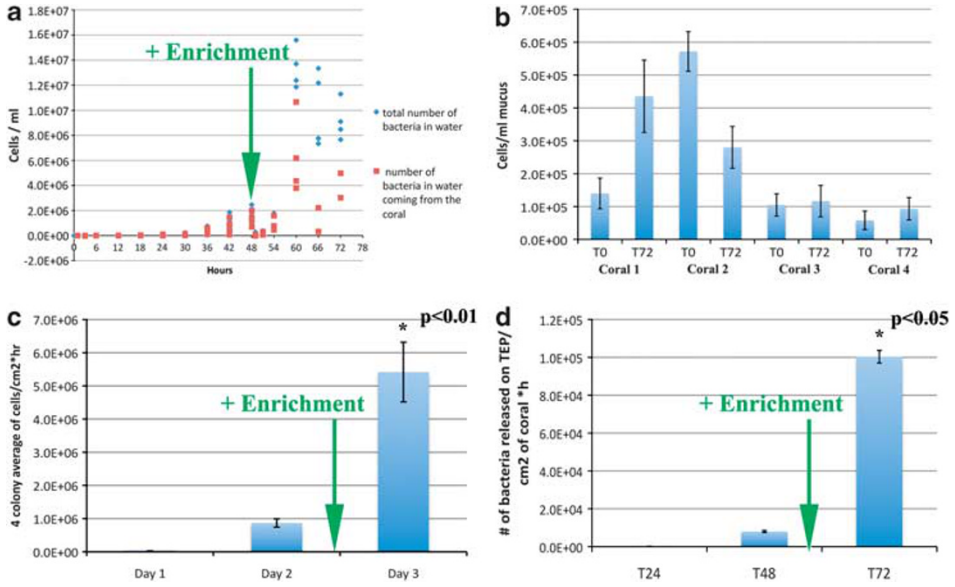

Figure 3 These data are from the diel cycle experiment where bacterial abundances were enumerated *ex situ*. (**a**) The total number of bacteria present (from DAPI counts on filters) in the water of the experimental chambers at each time point for each of the four replicates (blue diamonds) as well as the number of bacteria coming from the coral surface at each time point (red squares). The coral contributed the majority of the bacterial cells to the water until the enrichment addition at T_{48} when *in situ* growth of bacteria in the water increased. (**b**) The abundance of bacteria in the mucus of each coral colony at T_0 and T_{72}. (**c**) The average rate of bacterial cell release per cm^2 of coral per h for each day. The rate of release increased by ~fivefold after the addition of peptone. (**d**) The average rate of bacteria released on TEP particles per day. The rate at which bacteria attached to TEP particles were released increased by ~twelvefold after the addition of peptone.

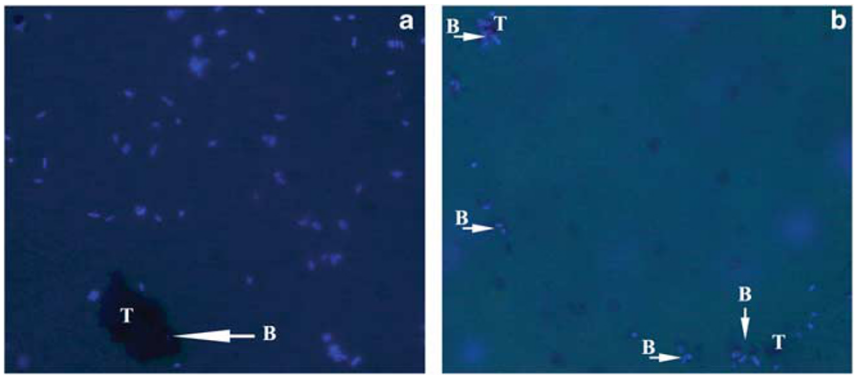

Figure 4 A comparison between TEP particles sampled at T_{48} versus T_{72} during the diel cycle experiment. (**a**) An overlay at $\times 1000$ magnification of a bright field image showing Alcian-stained TEP particles (T) in dark blue and the same field of view imaged with epifluorescence showing DAPI-stained bacteria (B) in bright blue. These two TEP particles from T_{48} were not heavily colonized. (**b**) An overlay of bright field and DAPI images acquired in the same way as part (**a**) depicting heavily colonized TEP particles (T) from T_{72}.

which coral-associated bacterial population abundance may be controlled. The amount of TEP released increased in response to organic enrichment, suggesting that the coral animal may have a role in regulating bacterial abundance through this mechanism.

Pathogen challenge

After 1 h of exposure to 5×10^5 *V. coralliilyticus* cells ml^{-1}, the coral fragments had $1.4–3.5 \times 10^6$ pathogen cells cm^{-3} of coral surface layer. The fragments exposed to 5×10^6 cells ml^{-1} had $0.9–1.5 \times 10^6$ pathogen cells cm^{-3} of coral surface layer and the fragments exposed to 5×10^7 cells ml^{-1} had $10.8–29.6 \times 10^6$ pathogen cells cm^{-3} of coral surface layer. The order of magnitude increase in the number of *V. coralliilyticus* associated with the surface of the *P. damicornis* fragments with the highest exposure suggests that this pathogen may have the ability to overcome the coral's shedding mechanism. The bacterial abundance in the water surrounding the coral fragments during the diel cycle time course reached levels above 10^7 ml^{-1} starting at T_{60}, and yet the abundance of bacteria in the coral mucus at T_{72} did not differ from T_0, when the bacteria in the water were $\sim 10^2$ ml^{-1}. The fairly consistent abundance of the pathogen on the coral surface between the 10^5 and 10^6 cells ml^{-1} treatments also suggests that there is behavioral regulation of *V. coralliilyticus*' association, likely by both the bacterium and the coral. The pathogen quantifiably associates with the corals at both lower dose levels, but it is only at the highest dose that the number of coral-associated cells increases substantially. The coral may be able to minimize the pathogen's association up to a certain threshold, beyond which the pathogenic bacterium can overcome the control mechanisms. This relationship and mechanism underlying it merit further study. In preliminary experiments, we observed that fluorescent beads (used as a passive control for a bacterial cell) did not accumulate on the coral surface; however, the responses of a larger suite of bacterial strains will be an important component of future work.

Conclusions

These experimental results highlight the ability of corals to regulate the abundance of bacteria on their surface in the face of organic enrichment stress and drastic increases in water-associated bacterial abundances. Our results suggest that shedding bacteria into the water column is an important component of the mechanism by which corals regulate their associated bacterial abundance, and that they have the ability to use that pathway to cope with organic matter perturbations. By increasing the amount of bacteria and mucus (as TEP) released following exposure to organic enrichment, the corals were able to maintain a relatively stable number of bacteria in their surface-associated community. It is possible that this mechanism may also assist corals in warding off pathogens, though our results using *V. coralliilyticus* suggest that at least one pathogen may be able to evade or overcome such a regulation mechanism. This raises further questions regarding what constitutes an infectious dose in the natural environment, and whether particulate-laden organic matter inputs (such as sedimentation or sewage effluent) might increase the opportunity for pathogens to reach such a dose in microscale hotspots of enrichment. In addition to identifying one potential mechanism of resilience, the novel ability to watch this ecological behavior in real time at the scale at which it actually occurs opens an unexplored avenue for studying coral–microbe interactions. Such a capability makes it possible to distinguish the *in situ* behaviors of symbiotic versus pathogenic bacteria toward corals, and begin creating an integrated mechanistic understanding of coral health and pathogenesis.

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