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INVERTEBRATE MICROBIOLOGY

Outcomes of Infections of Sea Anemone *Aiptasia pallida* with *Vibrio* spp. Pathogenic to Corals

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Abstract Incidents of coral disease are on the rise. However, in the absence of a surrogate animal host, understanding of the interactions between coral pathogens and their hosts remains relatively limited, compared to other pathosystems of similar global importance. A tropical sea anemone, *Aiptasia pallida*, has been investigated as a surrogate model to study certain aspects of coral biology. Therefore, to test whether the utility of this surrogate model can be extended to study coral diseases, in the present study, we tested its susceptibility to common coral pathogens (*Vibrio coralliilyticus* and *Vibrio shiloi*) as well as polymicrobial consortia recovered from the Caribbean Yellow Band Disease (CYBD) lesions. *A. pallida* was susceptible to each of the tested pathogens. *A. pallida* responded to the pathogens with darkening of the tissues (associated with an increased melanization) and retraction of tentacles, followed by complete disintegration of polyp tissues. Loss of zooxanthellae was not observed; however, the disease progression pattern is consistent with the behavior of necrotizing pathogens. Virulence of some coral pathogens in *Aiptasia* was paralleled with their glycosidase activities.

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Introduction

Over the last decade, several coral pathogens were characterized. It appears that most of coral pathogens are opportunistic, causing infections in stressed hosts [1, 2]. For example, even though vibrios are ubiquitous in the ocean and are commonly recovered from coral surfaces, they have also been linked to diseases of corals and other marine organisms [3–7]. *Vibrio shiloi* AK1 has long been associated with bleaching in Mediterranean corals through inhibition of photosynthesis and lysis of *Symbiodinium* spp. cells during periods of elevated sea surface temperatures [5]. Interestingly, interactions of *V. shiloi* with its winter host, fireworm *Hermodice carunculata*, appear to be commensal [8]. Infections of corals with *Vibrio coralliilyticus* lead to bacterially induced bleaching [9] and "white syndrome" (a collection of symptoms which include inhibition of the photosystem-II of *Symbiodinium*, paling of coral tissue and the spread of coral tissue lesions culminating in mortality) [10]. Virulence of *V. coralliilyticus* is thought to be due to the temperature-dependent production of a suite of proteases [11]. While at 24–26 °C proteases of *V. coralliilyticus* appear to target the coral symbiotic dinoflagellates, at 27–29 °C coral tissue is the primary target with tissue necrosis as the only observable sign of disease [12]. Therefore, even though bleaching is often associated with coral vibriosis, it is not the sole disease manifestation.

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In addition to being able to cause coral diseases individually, vibrios form polymicrobial consortia described as Caribbean Yellow Band Disease (CYBD). CYBD is well documented in *Montastraea* spp. around the greater Caribbean [13]. It manifests as pale yellow blotches or bands that spread over the surface of the coral [14]. A core *Vibrio* group consistently associated with CYBD in affected colonies has been characterized [14]. However, sequencing of culturable vibrios from both asymptomatic and diseased colonies did not demonstrate the presence of this core group in the diseased colonies and absence from healthy corals [15]. Although differences in the dominant *Vibrio* spp. strains were observed in pooled samples, there was inconsistency between individual samples within groups [15]. Thus, it appears that the outcome of the interactions of corals with environmental vibrios may depend on the numbers of the bacteria, stress status of the host, and integrity of the associated commensal microbiota. This observation is generally true for other coral diseases, as well as other microbial diseases [1, 16, 17].

While the prevalence and frequency of coral diseases are increasing, understanding of the mechanisms by which pathogens infect corals is still fairly limited compared to other host–pathogen interactions. It was the recognition of the need for a useful model system that led Weis *et al*. to nominate glass anemone *Aiptasia* sp. as a model organism for studying various aspects of coral genetics and physiology [18]. While the availability of natural corals for study in the laboratory is rapidly declining, *Aiptasia* can be grown in large numbers. *Aiptasia* is an anthozoan, which is also symbiotic with dinoflagellates. *Aiptasia* lacks the calcium carbonate skeleton, which is characteristic of corals [18]. Clonal populations of *Aiptasia* are available, which allow for genetic studies with greater ease including a recent generation and analysis of the *Aiptasia pallida* transcriptome [19, 20]. Much progress has been recently made in understanding interactions between *Aiptasia* and *Symbiodinium* spp. [21, 22]; however, significantly less is known about interactions of this model polyp with opportunistic coral pathogens. It has been demonstrated previously that the coral white pox pathogen, *Serratia marcescens* PDL100, is capable of infecting *Aiptasia*, and its virulence in this surrogate host depended on the ability to efficiently attach to mucus, degrade it, and compete with the native microbiota [23–25]. To further test the usefulness of this organism in studying coral pathogens and their invertebrate hosts, in the current study, we tested the outcomes of the interactions between *A. pallida* and well-characterized coral pathogens from the genus *Vibrio* and their consortia to determine how closely they mimic those observed in coral diseases. Even though bleaching of the anemones was not observed, *Aiptasia* were vulnerable to the infections with *V. shiloi*, *V. coralliilyticus*, and microbes comprising the CYBD consortium.

Methods

A. pallida Husbandry

An initial stock of *A. pallida* clonal strain CC7 (originally purchased from Carolina Biological Supply Company) was provided by Dr. John Pringle (Stanford University). For some experiments, as indicated in text, *Aiptasia* spp. were acquired from Carolina Supply Company (Burlington, NC, USA). Polyps were maintained in 10-gallon aquaria with artificial seawater (ASW; Red Sea Coral Pro Salt, Eilat, Israel). Tank salinity was maintained between 32 and 34 ppt. Aquaria were equipped with activated carbon filters and were maintained at ambient temperature (~22 °C) under blue actinic (460 nm) and super daylight white 6,500 k fluorescent bulbs on a 12:12-h light/dark cycle. Polyps were fed weekly with freshly hatched *Artemia* (brine shrimp) nauplii; no feeding occurred after the infection. Water quality parameters (pH, salinity, nitrate, and ammonium levels) were monitored with kits for measuring aquarium water quality.

Bacterial Strains and Culture Conditions

V. coralliilyticus (ATCC BAA-450) and *V. shiloi* AK1 (ATCC BAA-91) were from the American Type Culture Collection (ATCC; Manassas, VA, USA). Environmental samples of the CYBD were collected from diseased colonies of *Montastraea* (*Orbicella*) *faveolata* at Looe Key Reef in the Florida Keys (24° 33′ 75″ N, 81° 24′ 05″ W). Bands were aspirated from the advancing disease front using a needleless 60-cc syringe. Samples were kept on ice during transport to the lab; aliquots were stored as glycerol stocks at −80 °C and were used in experiments without excessive subculturing. A laboratory consortium consisting of an equal mix of individual *Vibrio*-like isolates (strains 1B4, 3B7, 1H5, and 2H12) was previously isolated from the CYBD-affected *M. faveolata* [13, 15]. An α-proteobacterium 45A11, isolated from surfaces of *Symbiodium* clade D2 from the Red Sea Forams [25], was used as a control. Bacteria were routinely cultured in marine broth or on marine agar plates (Becton, Dickenson, Franklin Lakes, NJ, USA) at 30 °C, with shaking at 250 rpm for broth cultures or statically for plates.

A. pallida Infections with Opportunistic Coral Pathogens

Individual polyps (stalk length approximately 1 cm) were transferred from the stock tanks into wells of six-well plates (Corning Scientific, Corning, NY, USA) with 10 ml of filter-sterilized (0.22 μm) ASW, where they were acclimated for 24 h at 22 °C on a tabletop rotary shaker at 70 rpm. For inoculations with individual coral pathogens, overnight cultures grown in marine broth were washed three times in filter-sterilized ASW and diluted to approximately 10^8, 10^7, and

10^6 cfu ml^{-1}; the water in the wells was replaced with 10 ml of these suspensions. For un-infected polyps, the water in the well was replaced with 10 ml of filter-sterilized ASW.

For the infections with the environmental samples of the CYBD, individual polyps were prepared as above. Environmental CYBD consortium samples were diluted 10^{-1}, 10^{-2}, and 10^{-3} in filter-sterilized ASW, and six polyps were infected with 10 ml of each diluted suspension. In parallel, individual overnight cultures of strains *Vibrio* spp. 1B4, 3B7, 1H5 and *Photobacterium* sp. 2H12 were mixed in equal volumes to generate a reconstituted consortium. Cell suspensions were then diluted to 10^5 and 10^6 cfu ml^{-1} and inoculated onto individual polyps. Polyps were incubated in the wells with diluted bacterial suspensions for 7 days. Upon completion of the incubation period, the surviving polyps were homogenized using a Tissue Master 125 (Omni International, Kennesaw, GA, USA) in approximately 250 μl ASW. Tissue homogenate was dilution plated onto thiosulfate citrate bile salts sucrose (TCBS) agar plates to recover *Vibrio* species.

The polyps were monitored for disease signs and were photographed daily with a Canon Eos Rebel Xsi digital camera. Images of polyps presented were corrected in Adobe Photoshop using default settings. Survivorship analyses were performed for each infection using Kaplan–Meier product-limit estimator: $S(t_i)-\prod_{t_i \le t}\left(1-\frac{d_i}{n_i}\right)$ where $S(t_i)$ is the estimated survival probability for any one of the listed time periods, n_i is the number of subjects at risk at the beginning of time period t_i, and d_i is the number of subjects that died during time period t_i [26]. Survivorship analyses were conducted using JMP 9.0 Pro statistical software (SAS Institute Inc., Cary, NC, USA), and mortality across treatments was compared with a Cox proportional hazards chi-square (χ^2) model [27].

Symbiodinium cells within *Aiptasia* tissues were counted as described previously [28, 29]. Briefly, at each time point, individual animals were transferred into 500 μl of dH_2O containing 0.01 % SDS (Sigma-Aldrich) and flash frozen. For processing, animals were thawed and then homogenized using a two-step protocol. First, a PowerGen125 rotor stator (Fisher Scientific) was used at its highest setting (30,000 rpm) for 8–10 s. The sample was then needle sheared by passage five times through a 25-gauge needle affixed to a 1-ml syringe. Using this protocol, no visible anemone tissue remained, but the algal cells remained intact and were well dispersed. The sample was then further diluted 11-fold (25 μl into 250 μl) in dH_2O containing 0.01 % SDS prior to counting. A Guava easyCyte™ HT 2-laser flow cytometer (Millipore) was used with excitation by the blue (488-nm) laser, and the gain controls at their default settings (yellow and green fluorescence) or set to 9.93 (forward scatter), 4.0 (side scatter), and 3.51 (red fluorescence). Samples of 275 μl were analyzed in 96-well round-bottom plates (Corning Life Sciences) with automatic mixing of each well for 7 s at high speed before sampling. Algal cells were separated from host cells and debris by their combination of side scatter (a measure of granularity) and red fluorescence using the InCyte v2.2 software (Millipore). Algal counts were normalized to total protein of the corresponding homogenates as determined using the Thermo Scientific™ Pierce BCA™ assay (Fisher), which is compatible with buffers containing SDS. The data generated in each experiment were imported into Prism 6.0a (GraphPad Software). Replicates were averaged and the standard errors of the mean or standard deviations were calculated.

Enzymatic Assays

Vibrio-like isolates (strains 1B4, 3B7, 1H5, and 2H12) were grown individually in marine broth overnight at 30 °C. Cells were pelleted and washed three times in sterile ASW. They were then suspended in ASW buffered in 10 mM HEPES, in which they were starved overnight. A sample of CYBD was similarly starved in 10 mM HEPES-buffered ASW. A volume of cell suspensions was then mixed with two volumes of coral mucus in seawater. Mucus was collected from apparently asymptomatic colonies of *M.* (*Orbicella*) *faveolata* at Looe Key Reef, FL, USA (24° 32.764″ N: 81° 24.304″ W as previously described [7]. Mucus was filter-sterilized and assays of enzymatic activities were carried out using *p*-nitrophenyl substrates as described previously [23, 30].

Denaturing Gradient Gel Electrophoresis Assessment of Changes in Microbiota of *A. pallida* Infected with the YBD Consortium

A. pallida was infected with the reconstituted CYBD consortium (*Vibrio* spp. 1B4, 3B7, 1H5 and *Photobacterium* sp. 2H12) as above at 10^6 and 10^7 cfu mL^{-1}, incubated for 3 days until early signs of the disease appeared. Three infected and three un-infected polyps were rinsed in filter-sterilized ASW, and then bacterial DNA from the polyps was extracted with Qiagen QIAamp Stool Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The extracted DNA was used as a template for PCR using CGCCCGGG GCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAA CGC GAAGAACCTTAC and CGGTGTGTACAAGACCC to amplify V6–V8 region of the 16S rRNA gene [31, 32]. In parallel, DNA from the mix of the bacterial strains (*Vibrio* spp. 1B4, 3B7, 1H5 and *Photobacterium* sp. 2H12) grown overnight was treated similarly and used as a control. Denaturing gradient gel electrophoresis (DGGE) was conducted as described previously [32].

Heat Shock Assays

To determine how temperature stress will affect susceptibility of polyps to infections with the culturable isolates from the CYBD consortium, polyps of similar size were removed from the stock aquarium (which was maintained at 22 °C) and transferred to the individual wells of a six-well plate and acclimated at 22 °C, for ~24 h. Half of the experimental plates were then transferred to an incubator at 34 °C, where they were kept on a moving platform (70 rpm) for 24 h. Upon completion of the treatment, artificial seawater in all wells was replaced with freshly prepared ASW, and animals were infected with serially diluted suspensions of the reconstituted CYBD consortium containing *Vibrio* spp. 1B4, 3B7, 1H5 and *Photobacterium* sp. 2H12, as indicated in text and figures. Controls included un-infected, heat-shocked polyps.

Melanin Production

Melanin production was estimated indirectly by measuring the prophenoloxidase activity as previously described with a minor modification [33]. Briefly, individual polyps were flash frozen in liquid nitrogen and ground with a mortar and pestle into a fine powder. The ground powder was extracted with 0.2 M phosphate buffer with 5 mM 2-mercaptoethanol in a microcentrifuge tube on ice for 45 min, and tissue debris were pelleted at 405×*g* for 10 min. The supernatant was centrifuged at 14,000×*g* for 5 min to pellet cellular debris, and the final supernatant was transferred to a new tube. The total protein concentration was determined by Bradford assay, with bovine serum albumin as a standard. To measure phenol oxidase activity, 10 μl of the protein extract was diluted in 50 μl sterile DNA-grade water (Fisher Scientific, Atlanta, GA, USA), and the oxidation of 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA; Sigma-Aldrich, St. Louis, MO, USA) to dopachrome was measured. Twenty-five microliters of 10 mM stock L-DOPA was added and the reaction was initiated with the addition of trypsin (50 μg ml^{-1}) by activating prophenoloxidase to phenoloxidase, and the absorbance was monitored at 490 nm for 80 min. Prophenoloxidase (PPO) activity was calculated with the formula: ΔA490/80 min/mg protein.

Results and Discussion

A. pallida Infections with *V. coralliilyticus* and *V. shiloi*

Six polyps were infected with either *V. coralliilyticus* or *V. shiloi* AK1 at each infectious dose (10^6, 10^7, and 10^8 cfu of pathogen ml^{-1}) with uninfected animals as negative controls. Anemones responded to *Vibrio* infection with darkening of the tissue, retraction of tentacles, and mortality (defined as polyp tissue degradation by the pathogen, Fig. 1, and data not shown for *V. shiloi*). Although both *V. coralliilyticus* and *V. shiloi* AK1 are associated with bacterial bleaching in corals, no bleaching signs were observed in the *A. pallida* polyps post-infection at any of the tested concentrations (Fig. 1a). When cells of *Symbiodinium* within the diseased tissues were counted by flow cytometry, no differences between the treatments were observed (Supplemental Figure S1), indicating that *V. coralliilyticus* does not cause the loss/expulsion of zooxanthellae in the *Aiptasia* model. *V. shiloi* infections were only carried out for 3 days due to complete mortality of the polyps infected at the highest concentration (data not shown). In all cases, *Vibrio* spp. were recovered from the diseased animals when slurries of ground tissues were plated onto TCBS medium.

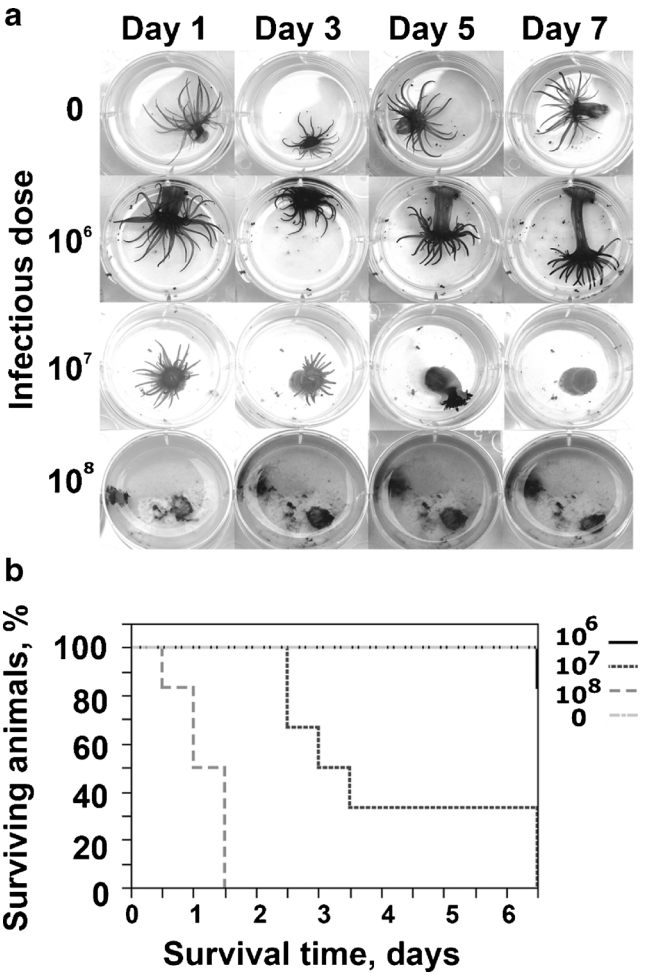

Fig. 1 Infections of *A. pallida* with *V. coralliilyticus*. Following 24-h acclimation in wells of a six-well plate, polyps were infected with serially diluted suspensions of *V. coralliilyticus* in ASW (10^6–10^8 cfu ml^{-1}). Experiments were conducted with 15 polyps, with at least six animals per dose. Infections were carried out for 7 days at room temperature, and polyps were monitored and photographed daily. **a** Appearance of representative animals. **b** Kaplan–Meier analysis of animal mortality in the presence of the pathogen (χ^2=37.951; *df*=3; p<0.0001)

In polyps infected with *V. coralliilyticus*, 100 % of polyps infected at 10^8 cfu ml^{-1} succumbed to infection by day 3 and at the 10^7-cfu ml^{-1} concentration (Fig. 1b). Survivorship analysis indicated that while the two higher concentrations resulted in 100 % mortality, the curves were significantly different from each other (χ^2=37.95; *df*=3; p<0.0001; Fig. 1b).

V. coralliilyticus, in addition to infecting corals, also causes disease in *Artemia* nauplii and in many fish species [34]. While infections of corals with *V. coralliilyticus* can lead to bleaching or tissue necrosis, depending on the ambient temperature [12], fish infected with the pathogen developed severe muscle tissue necrosis with hemorrhaging, and infected *Artemia* died [34]. It is clear that the pathogenic vibrios do not elicit the same signs in each host and that no one model organism is able to mimic the response of all hosts absolutely. Therefore, perhaps it is expected that the disease signs elicited by *V. coralliilyticus* in *A. pallida* would differ somewhat from those observed in corals.

A. pallida Infections with the Caribbean Yellow Band Disease Consortium

Environmental samples of the CYBD consortium were collected from infected colonies of *M. faveolata* at Looe Key, FL, USA. The mortality of the infected polyps increased with decreasing concentration of the environmental sample (Fig. 2). After 24–48 h after infection, polyps infected with dose 2 and dose 3 began showing disease signs and mortality after 4 days. There was no mortality of the uninfected polyps; a significant difference in the survivorship trends for the dilutions was observed (χ^2=15.74; *df*=3; p=0.0013; Fig. 2).

Based on the culture limitations of polymicrobial infections, we could not determine the LD_{50} for the environmental samples of CYBD. Therefore, to follow up on this experiment, a re-constituted consortium of the four strains isolated from the CYBD-infected corals [15] was used as inoculum. The polyps were infected with the reconstituted consortium at doses ranging from 3.3×10^5 to 3.3×10^6 cfu ml^{-1}. The infection with the culturable strains resulted in the same disease signs in the infected polyps as the environmental CYBD samples and led to mortality after 4 days of infection (Fig. 2). The highest dose (3.3×10^6 cfu ml^{-1}) resulted in 100 % mortality after 6 days, 1 day sooner than the environmental dilution samples (Fig. 2). The lowest dose tested (3.3×10^5 cfu ml^{-1}) resulted in 50 % mortality after 7 days. LD_{50} for the reconstituted CYBD consortium was calculated to be ~10^5 cfu ml^{-1}.

To ascertain that the infected polyps contained the introduced strains, DGGE analysis was conducted using infected and uninfected polyps, with the DNA extracted from the mix of the strains of the reconstituted CYBD consortium as a control. As shown in Fig. S2, in addition to the strong signature bands associated with the infected and un-infected polyps, bands corresponding to those found within the BBD consortium were present in the infected polyps and

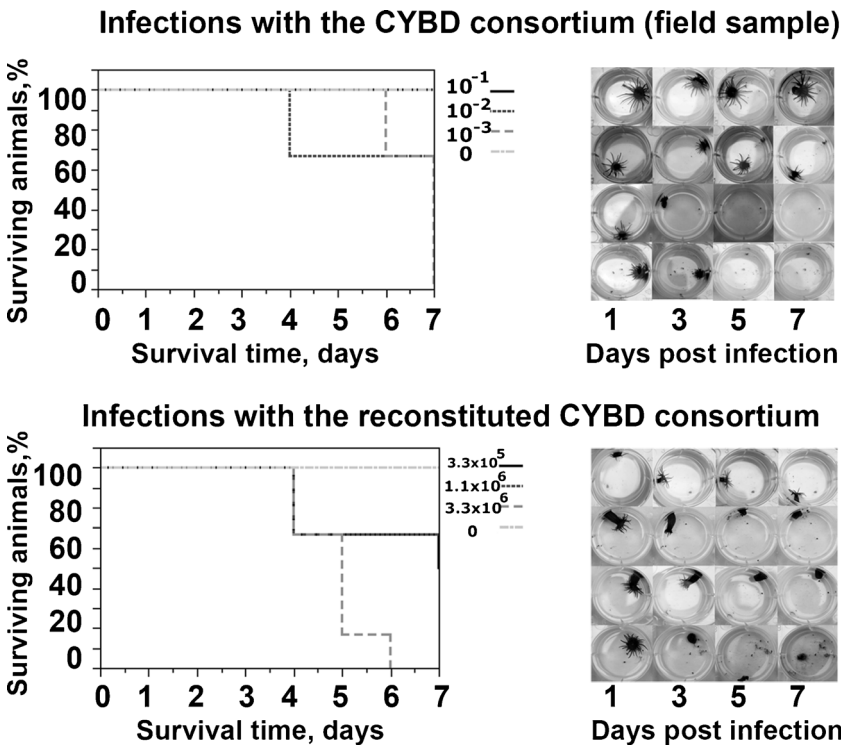

Fig. 2 Survivorship of *A. pallida* polyps infected with the Caribbean Yellow Band Disease (*CYBD*) consortium. Infections were carried out either with the dilutions of the CYBD consortium collected from the symptomatic *Montastraea faveolata* (*top panels*) or the reconstituted consortium carrying culturable vibrios (*bottom panels*). Survivorship, estimated by Kaplan–Meier analysis, decreased significantly with the increasing concentration of the environmental CYBD sample (χ^2=15.738; *df*=3; p=0.0013) and a reconstituted consortium of CYBD isolates (χ^2=9.058; *df*=3; p=0.0285). Appearance of representative polyps is shown in the *panels on the right* (*top row* uninfected polyps, *bottom row* the highest infectious dose)

absent from the un-infected polyps, thus fulfilling Koch's postulates.

Enzymatic Activities Within the CYBD Consortia

Because reconstituted CYBD consortium (containing *Vibrio* spp. 1B4, 3B7, 1H5 and *Photobacterium* sp. 2H12) was more virulent than the consortium collected in situ from a diseased *Montastraea* colony, we tested whether enzymatic profiles could be at least in part responsible for the observed differences in virulence. Previously, the ability to more efficiently utilize mucus and produce a broader range of glycosidases was linked to an increased virulence of another coral pathogens, *S. marcescens* [23, 30]. As shown in Fig. 3, the totality of glycosidase activities in the starved cultures of the reconstituted consortium was significantly broader than in the environmental sample, with strong activities of β-D-*N*-acetyl-galactosaminidase, β-D-*N*-acetyl-glucosaminidase, and β-D-galactopyranosidase. It is of note that strong activities associated with the degradation of acetylated aminosugars were present in other coral pathogens, but are significantly weaker in the members of commensal microbiota [23, 30].

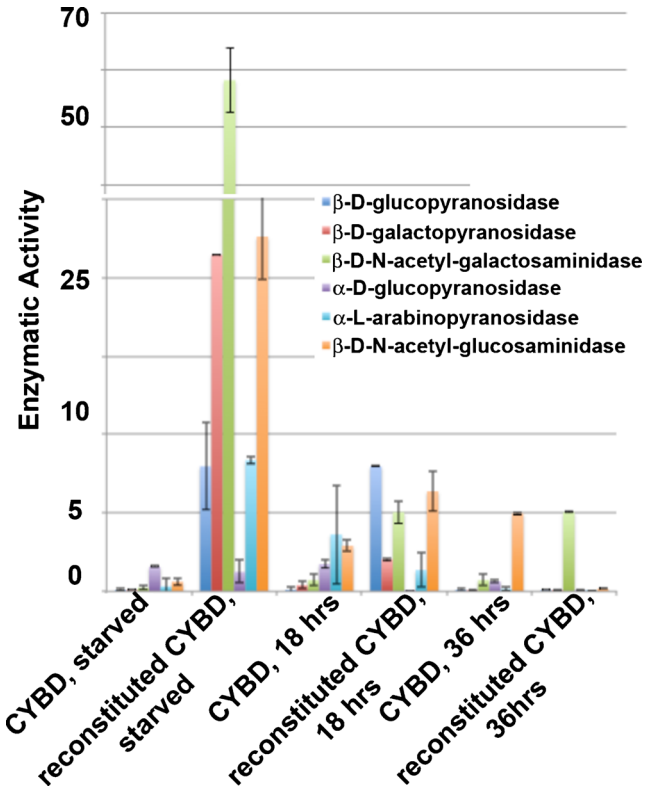

Fig. 3 Glycosidase activities induced by growth on *Montastraea faveolata* mucus. Environmental sample of the CYBD consortium or the reconstituted CYBD consortium was first starved in 10 mM HEPES-buffered artificial seawater and then mixed with filter-sterilized mucus from the host coral *M. faveolata*. Induction of the glycosidase activities was assessed with the appropriate *p*-nitro-phenyl substrates following 18 and 36 h of incubation with *M. faveolata* mucus. Data from a representative experiment are shown. All enzymatic assays were carried out for the exact same length of time. Enzymatic activity is presented as modified Miller units (A_{405}/OD_{590})

Melanin Production Within Infected Tissues

Darkening of the polyp tissue, retraction of the tentacles, and polyp mortality at higher concentrations were the main observable signs of the infection. To test whether darkening of the tissues is associated with melanin production (a well-documented defense response in cnidarians [35]), PPO activity was measured. The basal levels of PPO activity/mg protein averaged 0.0189±0.0008 (mean±SD). The PPO activity in polyps infected at 10^5 cfu ml^{-1} was 8.5 times greater than that of healthy polyps at 0.160±0.006, and the activity in polyps infected at 10^6 cfu ml^{-1} was nearly 10 times greater than healthy polyps at 0.184±0.008. Though the sample size was limited, these results indicate that melanin production (measured indirectly as PPO activity) in *A. pallida* polyps increases in response to bacterial infection, similarly to the responses of other cnidarians to various stressors [36].

The Effect of the Temperature Stress on Susceptibility of *A. pallida* to Infections with *Vibrio* spp.

Because relatively modest increases in seawater temperature have been linked with an increase in susceptibility of corals to opportunistic pathogens (and to CYBD specifically [37]), we also tested how transient exposure of *A. pallida* to elevated temperatures will affect its susceptibility to CYBD. While exposure of *A. pallida* to elevated temperature for 24 did not affect mortality of the polyps in the absence of the pathogen, this treatment significantly increased their susceptibility to the pathogens present in the reconstituted CYBD consortium (Fig. 4).

Conclusions

In this study, we tested the suitability of *A. pallida* as a surrogate host for studying virulence mechanisms in opportunistic coral pathogens. *A. pallida* appears to be susceptible to infection by coral opportunistic pathogens, although none of the tested pathogens elicited observed bleaching signs in the anemones, nor was associated with the loss of *Symbiodinium* spp. A short-term exposure of *A. pallida* to elevated temperatures increased its sensitivity to pathogens. Furthermore, the ability to more efficiently utilize mucus of the host coral *M. faveolata* resulted in the increased virulence in *A. pallida* of the consortium of pathogenic vibrios. While it is almost certain that the entire complex multi-level interplay between

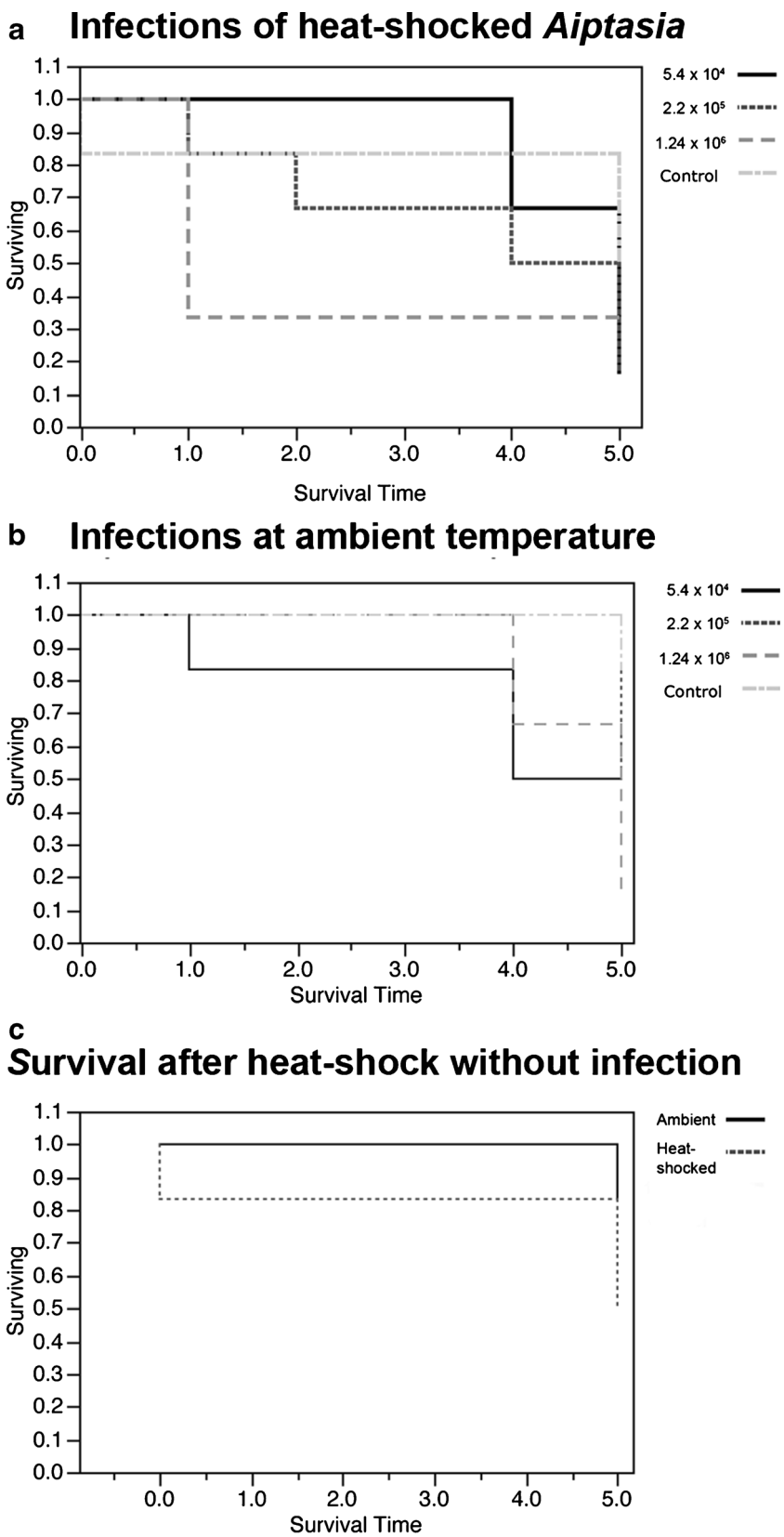

Fig. 4 Heat shock increases susceptibility of *Aiptasia* spp. to CYBD. After acclimation in wells of a six-well plate with fresh artificial seawater, a subset of polyps was kept at 34 °C for 24 h. Heat-treated and un-treated polyps were infected with three dilutions of the reconstituted CYBD consortium (consisting of *Vibrio*-like strains 1B4, 3B7, 1H5, and 2H12, previously isolated from the CYBD-affected *M. faveolata*). *Aiptasia* polyps for these experiments were purchased from Carolina Biological Supply Company. Surviving animals were counted over time

coral hosts, their associated microbiota, and invading pathogens cannot be faithfully replicated in any surrogate host, the use of *A. pallida* can shed light on some of the processes that take place during these interactions.

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References

1. Bourne DG, Garren M, Work TM, Rosenberg E, Smith GW, Harvell CD (2009) Microbial disease and the coral holobiont. Trends Microbiol 17:554–562. doi:10.1016/j.tim.2009.09.004
2. Rosenberg E, Koren O, Reshef L, Efrony R, Zilber-Rosenberg I (2007) The role of microorganisms in coral health, disease and evolution. Nature Rev Microbiol 5:355–362. doi:10.1038/nrmicro1635
3. Rosenberg E, Kushmaro A, Kramarsky-Winter E, Banin E, Yossi L (2009) The role of microorganisms in coral bleaching. ISME J 3:139–146. doi:10.1038/ismej.2008.104
4. Kushmaro A, Rosenberg E, Fine M, Loya Y (1997) Bleaching of the coral *Oculina patagonica* by *Vibrio* AK-1. Mar Ecol-Prog Ser 147:159–165. doi:10.3354/meps147159
5. Kushmaro A, Banin E, Loya Y, Stackebrandt E, Rosenberg E (2001) *Vibrio shiloi* sp. nov., the causative agent of bleaching of the coral *Oculina patagonica*. Int'l J Sys Evol Microbiol 51:1383–1388
6. Arotsker L, Siboni N, Ben-Dov E, Kramarsky-Winter E, Loya Y, Kushmaro A (2009) *Vibrio* sp. as a potentially important member of the Black Band Disease (BBD) consortium in *Favia* sp. corals. FEMS Microbiol Ecol 70:515–524. doi:10.1111/j.1574-6941.2009.00770.x
7. Ritchie KB (2006) Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. Mar Ecol Prog Ser 322:1–14
8. Sussman M, Loya Y, Fine M, Rosenberg E (2003) The marine fireworm *Hermodice carunculata* is a winter reservoir and spring–summer vector for the coral-bleaching pathogen *Vibrio shiloi*. Environ Microbiol 5:250–255
9. Ben-Haim Y, Thompson FL, Thompson CC, Cnockaert MC, Hoste B, Swings J, Rosenberg E (2003) *Vibrio coralliilyticus* sp. nov., a temperature-dependent pathogen of the coral *Pocillopora damicornis*. Int'l J System Evol Micrbiol 53:309–315. doi:10.1099/ijs.0·02402-0
10. Sussman M, Willis BL, Victor S, Bourne DG (2008) Coral pathogens identified for white syndrome (WS) episootics in the Indo-Pacific. PLoS One 3:e2393. doi:10.1371/journal.pone.0002393
11. Kimes NE, Grim CJ, Johnson WR, Hasan NA, Tall BD, Kothary MH, Kiss H, Munk AC, Tapia R, Green L, Detter C, Bruce DC, Brettin TS, Colwell RR, Morris PJ (2011) Temperature regulation of virulence factors in the pathogen *Vibrio coralliilyticus*. ISME J 6:835–846. doi:10.1038/ismej.2011.154
12. Ben-Haim Y, Zicherman-Keren M, Rosenberg E (2003) Temperature-regulated bleaching and lysis of the coral *Pocillopora damicornis* by the novel pathogen *Vibrio coralliilyticus*. Appl Environ Microbiol 69:4236–4242
13. Weil E, Ritchie KB, Croquer A, Cunning JR, Smith GW (2008) Inoculation of *Vibrio* spp. onto *Montastraea faveolata* fragments to determine potential pathogenicity. Proceedings of the 11th International Coral Reef Symposium, Ft Lauderdale, Florida 1:202–205
14. Cervino JM, Thompson FL, Gomez-Gil B, Lorence EA, Goreau TJ, Hayes RL, Winiarski-Cervino KB, Smith GW, Hughen K, Bartels E (2008) The *Vibrio* core group induces yellow band disease in Caribbean and Indo-Pacific reef-building corals. J Appl Microbiol 105:1658–1671. doi:10.1111/j.1365-2672.2008.03871.x
15. Cunning JR, Thurmond JE, Smith GW, Weil E, Ritchie KB (2008) A survey of vibrios associated with healthy and Yellow Band diseased *Montastraea faveolata*. In: Proceedings of the 11th International Coral Reef Symposium, Ft Lauderdale, Florida, 7–11 July 2008, 1, pp 206–210
16. Ban SS, Graham NA, Connolly SR (2013) Evidence for multiple stressor interactions and effects on coral reefs. Glob Chang Biol. doi:10.1111/gcb.12453
17. Burge CA, Kim CJ, Lyles JM, Harvell CD (2013) Special issue Oceans and Humans Health: the ecology of marine opportunists. Microb Ecol 65:869–879. doi:10.1007/s00248-013-0190-7
18. Weis VM, Davy SK, Hoegh-Guldberg O, Rodriguez-Lanetty M, Pringle JR (2008) Cell biology in model systems as the key to understanding corals. Trends Ecol Evol 23:369–376. doi:10.1016/j.tree.2008.03.004
19. Sunagawa S, Wilson EC, Thaler M, Smith ML, Caruso C, Pringle JR, Weis VM, Medina M, Schwarz JA (2009) Generation and analysis of transcriptomic resources for a model system on the rise: the sea anemone *Aiptasia pallida* and its dinoflagellate endosymbiont. BMC Genomics 10:258. doi:10.1186/1471-2164-10-258
20. Lehnert EM, Burriesci MS, Pringle JR (2012) Developing the anemone *Aiptasia* as a tractable model for cnidarian–dinoflagellate symbiosis: the transcriptome of aposymbiotic *A. pallida*. BMC Genomics 13:271
21. Weis VM (2008) Cellular mechanisms of Cnidarian bleaching: stress causes the collapse of symbiosis. J Exp Biol 211:3059–3066. doi:10.1242/jeb.009597
22. Davy SK, Allemand D, Weis VM (2012) Cell biology of cnidarian–dinoflagellate symbiosis. Microbiol Mol Biol Rev 76:229–261. doi:10.1128/MMBR.05014-11
23. Krediet CJ, Ritchie KB, Cohen M, Lipp EK, Sutherland KP, Teplitski M (2009) Utilization of mucus from the coral *Acropora palmata* by the pathogen *Serratia marcescens* and by environmental and coral commensal bacteria. Appl Environ Microb 75:3851–3858. doi:10.1128/Aem.00457-09
24. Krediet CJ, Carpinone EM, Ritchie KB, Teplitski M (2012) Characterization of the *gacA*-dependent surface and coral mucus colonization by an opportunistic coral pathogen *Serratia marcescens* PDL100. FEMS Microbiol Ecol. doi:10.1111/1574-6941.12064
25. Alagely A, Krediet CJ, Ritchie KB, Teplitski M (2011) Signaling-mediated cross-talk modulates swarming and biofilm formation in a coral pathogen *Serratia marcescens*. ISME J 5:1609–1620. doi:10.1038/ismej.2011.45
26. Kaplan EL, Meier P (1958) Nonparametric estimation from incomplete observations. J Amer Statist Assn 53(282):457–481
27. Hosmer DW, Lemeshow S (1999) Applied survival analysis. Wiley, New York
28. Xiang TT, Hambleton EA, DeNofrio JC, Pringle JR, Grossman AR (2013) Isolation of clonal axenic strains of the symbiotic dinoflagellate *Symbiodinium* and their growth and host specificity. J Phycol 49:447–458. doi:10.1111/Jpy.12055
29. Tolleter D, Seneca FO, DeNofrio JC, Krediet CJ, Palumbi SR, Pringle JR, Grossman AR (2013) Coral bleaching independent of photosynthetic activity. Curr Biol 23:1782–1786. doi:10.1016/J.Cub.2013.07.041
30. Krediet CJ, Ritchie KB, Teplitski M (2009) Catabolite regulation of enzymatic activities in a white pox pathogen and commensal bacteria during growth on mucus polymers from the coral *Acropora palmata*. Dis Aquat Organ 87:57–66. doi:10.3354/Dao02084

31. Yu Z, Morrison M (2004) Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 70:4800–4806. doi:10.1128/AEM.70.8.4800-4806.2004
32. Mshvildadze M, Neu J, Shuster J, Theriaque D, Li N, Mai V (2010) Intestinal microbial ecology in premature infants assessed with non-culture-based techniques. J Pediatrics 156:20–25. doi:10.1016/j.jpeds.2009.06.063
33. Mydlarz LD, Holthouse SF, Peters EC, Harvell CD (2008) Cellular responses in sea fan corals: granular amoebocytes react to pathogen and climate stressors. PLoS One 3:e1811. doi:10.1371/journal.pone.0001811
34. Austin B, Austin D, Sutherland R, Thompson F, Swings J (2005) Pathogenicity of vibrios to rainbow trout (*Oncorhynchus mykiss*, Walbaum) and *Artemia* nauplii. Environ Microbiol 7:1488–1495. doi:10.1111/j.1462-2920.2005.00847.x
35. Mydlarz LD, Harvell CD (2007) Peroxidase activity and inducibility in the sea fan coral exposed to a fungal pathogen. Comp Biochem Physiol A Mol Integr Physiol 146:54–62. doi:10.1016/j.cbpa.2006.09.005
36. Palmer CV, Bythell JC, Willis BL (2011) A comparative study of phenoloxidase activity in diseased and bleached colonies of the coral *Acropora millepora*. Dev Compar Immunol 35:1096–1099. doi:10.1016/J.Dci.2011.04.001
37. Cervino JM, Hayes RL, Polson SW, Polson SC, Goreau TJ, Martinez RJ, Smith GW (2004) Relationship of *Vibrio* species infection and elevated temperatures to yellow blotch/band disease in Caribbean corals. Appl Environ Microbiol 70:6855–6864. doi:10.1128/AEM.70.11.6855-6864.2004