

Metagenomic analysis indicates that stressors induce production of herpes-like viruses in the coral *Porites compressa*

Rebecca L. Vega Thurber^{a,b,1}, Katie L. Barott^a, Dana Hall^a, Hong Liu^a, Beltran Rodriguez-Mueller^a, Christelle Desnues^{a,c}, Robert A. Edwards^{a,d,e,f}, Matthew Haynes^a, Florent E. Angly^a, Linda Wegley^a, and Forest L. Rohwer^{a,e}

^aDepartment of Biology, ^dComputational Sciences Research Center, and ^eCenter for Microbial Sciences, San Diego State University, San Diego, CA 92182;

^bDepartment of Biological Sciences, Florida International University, 3000 North East 151st, North Miami, FL 33181; ^cUnité des Rickettsies, Unité Mixte de Recherche, Centre National de la Recherche Scientifique 6020, Faculté de Médecine de la Timone, 13385 Marseille, France; and ^fMathematics and Computer Science Division, Argonne National Laboratory, Argonne, IL 60439

Communicated by Baruch S. Blumberg, Fox Chase Cancer Center, Philadelphia, PA, September 11, 2008 (received for review April 25, 2008)

During the last several decades corals have been in decline and at least one-third of all coral species are now threatened with extinction. Coral disease has been a major contributor to this threat, but little is known about the responsible pathogens. To date most research has focused on bacterial and fungal diseases; however, viruses may also be important for coral health. Using a combination of empirical viral metagenomics and real-time PCR, we show that *Porites compressa* corals contain a suite of eukaryotic viruses, many related to the Herpesviridae. This coral-associated viral consortium was found to shift in response to abiotic stressors. In particular, when exposed to reduced pH, elevated nutrients, and thermal stress, the abundance of herpes-like viral sequences rapidly increased in 2 separate experiments. Herpes-like viral sequences were rarely detected in apparently healthy corals, but were abundant in a majority of stressed samples. In addition, surveys of the *Nematostella* and *Hydra* genomic projects demonstrate that even distantly related Cnidarians contain numerous herpes-like viral genes, likely as a result of latent or endogenous viral infection. These data support the hypotheses that corals experience viral infections, which are exacerbated by stress, and that herpes-like viruses are common in Cnidarians.

coral reefs | disease | Herpesviridae | viral-like particles | virome

Coral disease is a major cause of the pandemic decline of tropical reefs (1, 2). Increases in coral disease incidence have occurred during the last several decades (3, 4), and, although their devastating effects on coral populations are no longer in question (5), in most cases, it is not yet clear whether specific or opportunistic pathogens are involved (6, 7).

A challenge to identifying causative agents of coral disease is that corals naturally harbor a variety of microbiota including symbiotic algae, fungi, Bacteria and Archaea (8–13). Corals demonstrate species-specific and potentially mutualistic relationships with communities of Bacteria (14), and these associated communities are altered during episodes of coral disease (7, 15). To date, no coral viral diseases have been positively identified, but pathologies, such as yellow blotch and white band disease, have been hypothesized to be the results of viral infection (16).

Virus-like particle abundances shift both across reefs and with proximity to coastal areas and corals themselves (17, 18). On tropical reefs viral numbers range from 0.3 to 1.25×10^7 particles per milliliter in reef water (19) to $0.8\text{--}1 \times 10^9$ particles per cubic centimeter within the sediment (20). Small-scale variations in viral numbers occur with changing proximity to coral colonies, with viral particles peaking $\approx 2\text{--}4$ cm above the coral surface. These abundances are elevated in waters surrounding diseased coral colonies from 1.0×10^7 to 1.5×10^7 particles per milliliter (21). Virus-like particles (VLPs) also occur within corals and their relatives. For example, VLPs have been found within the tissues of anemones and within the symbiotic zoochlorellae and zooxanthellae of various Cnidarians (22–24). While the identity of these VLPs has yet to be

established, an increase in viral particles within dinoflagellates has been hypothesized to be responsible for symbiont loss during bleaching (25–27). VLPs also have been identified visually on several species of scleractinian corals, specifically: *Acropora muricata*, *Porites lobata*, *Porites lutea*, and *Porites australiensis* (28). Based on morphological characteristics, these VLPs belong to several viral families including: tailed phages, large filamentous, and small (30–80 nm) to large (>100 nm) polyhedral viruses (29). Metagenomic data have also shown that both phages and eukaryotic viruses are associated with *Porites astreoides* (30) and healthy and bleaching *Diploria strigosa* corals (31).

Environmental stress often results in coral bleaching, disease, and death. Increased temperature, nutrient loading, dissolved organic carbon pollution, and reductions in ambient seawater pH are of particular concern due to their effects on the coral-symbiont relationship, host homeostasis, microbial overgrowth, and skeletal deposition (32–34). To determine whether environmental perturbations shift the eukaryotic viral assemblage present in corals, these 4 parameters were manipulated, and the resulting viral consortia characterized through the generation of 6 metagenomes. We show here that changes in the viral repertoire varied across each treatment, but all treatments contained genomic sequence similarities to specific eukaryotic viral families, most notably the Herpesviridae. Furthermore, a unique herpes-like viral sequence (HLVs) increased up to 6 orders of magnitude in coral specimens exposed to increased temperature, nutrients, and pH stress indicating viral production within the host. From these and other data, we hypothesize that a distantly related herpes-like virus or group of viruses is commonly associated with corals and Cnidarians in general and are produced as a result of stressor events.

Results and Discussion

Experimental Design and Rationale. To characterize coral-associated viruses, specimens of the finger coral, *Porites compressa*, were collected in the Hawai'i Marine Laboratory Refuge. This species of coral was chosen because it is a common and endemic species of coral in the Hawaiian archipelago and is the

Author contributions: R.L.V.T. and F.L.R. designed research; R.L.V.T. and K.L.B. performed research; R.L.V.T., H.L., B.R.-M., C.D., R.A.E., M.H., F.E.A., and L.K. contributed new reagents/analytic tools; R.L.V.T., D.H., L.W., H.L., B.R.-M., C.D., R.A.E., and F.E.A. analyzed data; and R.L.V.T. wrote the paper.

The authors declare no conflict of interest.

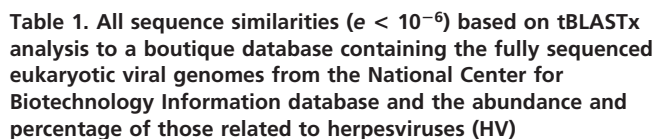
Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (genome project and accession nos. 28415, 28417, 28419, 28421, 28423, and 28425 and EU660955).

¹To whom correspondence should be addressed. E-mail: rvegathurber@gmail.com.

This article contains supporting information online at www.pnas.org/cgi/content/full/0808985105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA



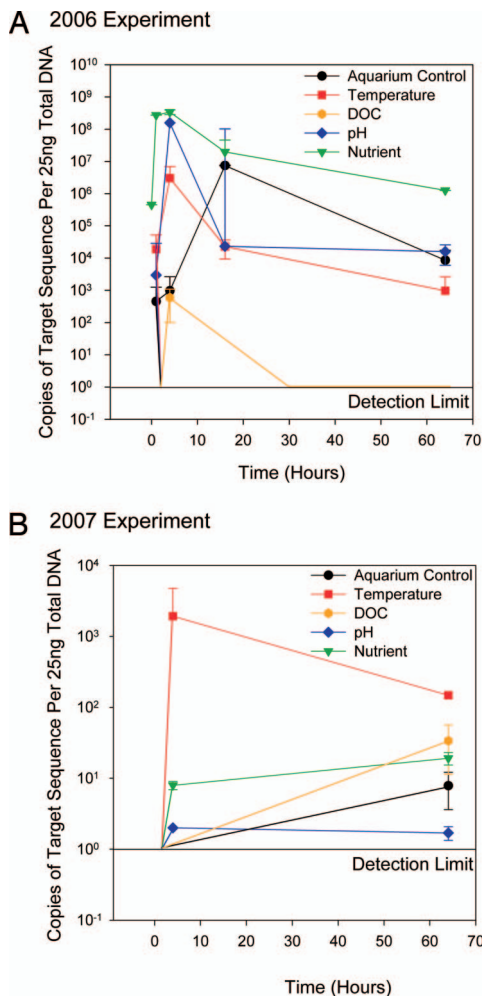


Fig. 3. Herpes-like viral sequence abundance across two 64-h experiments. (A and B) Real-time PCR on a putative herpes-like virus thymidylate synthetase gene was conducted on all temporal samples collected during the original metagenomic experiment (A) and samples from a second experiment conducted 1 year later (B). In the original experiment, coral specimens were exposed to thermal (5°C above ambient), dissolved organic carbon (25 mg·mL⁻¹ glucose), nutrient (10 μM nitrate, nitrite, phosphate, and ammonium), and pH (1.7 units below ambient) stress for 64 hours. (B) In the second 2007 experiment, corals were acclimated to the tanks for 2 weeks before treatment with stressor, thermal stress was an elevation of seawater temperature 3°C, acidity stress was a 0.2 unit reduction of ambient pH, and samples were collected at 0, 4, and 64 h.

exception of the Nutrient (green triangles). The target sequence was also elevated in all of the 1-, 4-, 16-, and 64-h treated corals. The abundance of this target sequence typically peaked at 4 h after treatment exposure, and the maximum increase was ≈6 orders of magnitude. For example, the pH (blue diamonds) and Temperature (red triangles) stressors increased the abundance of this viral sequence from below the detection limit (≈10 copies) to >10⁵ and 10⁷ copies in only 4 h. Viral abundance was then reduced to 10³ for the remainder of those experiments.

In the Aquarium Control sample (Fig. 3, black circles) large numbers (≈10⁷) of this HLV specific target gene were detected after 16 h and demonstrated that even in the absence of applied exogenous abiotic stress, physical manipulation of the corals led to viral production. The viral sequence was detected in only 1 of the 5 DOC (orange hexagons) time points, corroborating the low abundance of sequences in the DOC metagenome. Both this low copy number in the DOC samples and the large copy number in the nutrient zero time point indicate that external herpesvirus contamination was not a confounding factor.

Second Experiment Conducted One Year Later Confirms that HLVs Are Commonly Associated with Stressed Corals. The possibility that the viruses discovered in the corals were unique to the original metagenomic experiment and/or the result of unnatural stress conditions was tested by conducting a second experiment 1 year later. This second repeat experiment used the same sampling scheme (Fig. S1) with minor modifications to reflect more likely environmental conditions: (i) corals were acclimated to the tanks for 2 weeks before treatment with stressor, (ii) thermal stress was an elevation of seawater temperature 3°C, (iii) acidity stress was a 0.2 unit reduction of ambient pH, and 4) nubbins were only collected at 0, 4, and 64 h. Identical primer sets were used to amplify the HLV thymidylate synthase sequence.

These analyses showed an increase in the herpes-like virus target sequence when stressors were applied to the corals (Fig. 3B). The target sequence was not detected at the zero hour time point in any of the tissue samples, but was found in the temperature (red squares), pH (blue diamonds), and nutrient (green triangles) samples at 4 and 64 h. The DOC exposed and aquarium control corals contained no detectable target sequence until 64 h.

Together, these results show that, although the temporal changes in HLV abundance were analogous in both experiments, the magnitude of this change was different. Compared with the 2006 metagenomic experiment, the total abundance of HLVs was lower in the second 2007 experiment. This may have resulted from the more environmentally relevant levels of stressors applied in the repeat experiment. However, environmental conditions were also milder during the second 2007 experiment with no extended rainy or windy periods before or during the experiment. In contrast, during the original 2006 experiment, precipitation along with significant runoff occurred. As previously mentioned, the abundance of the HLVs was found to be high in the original experiment's nutrient zero hour sample. This indicates that production of virus had already begun on the reef, perhaps as a result of degraded environmental conditions.

A two-way ANOVA conducted on the real-time data indicated significant effects of both treatment ($F = 9.80$, $P < 0.01$) and time ($F = 4.82$, $P < 0.01$), and a significant interaction effect ($F = 4.54$, $P < 0.01$). The combination of the two main effects and the interaction term explains >70% of the variance. These results indicated that the different treatments applied to corals stimulated herpes-like viral production and that the level of production varied significantly over the time course of the experiment. A significant interaction term suggested that the effects of both treatment and time on viral production were amplified multiplicatively. This implies that just studying each treatment individually over time or all treatments simultaneously over 1 time point would not have shown the same differences in production.

Additional Evidence for Herpes-Like Viruses in Corals and All Cnidarians. In addition to our datasets, transmission electron micrographs (TEM) and sequences from the *Nematostella* genome and hydra EST project provide further evidence that Cnidarians, not just corals, are hosts for herpes-like viruses. Corals contain large enveloped icosahedral virions morphologically similar to the *Herpesviridae* (28, 29). Herpesvirus essential genes are also present in Cnidarian genomes (28, 51, 52). Within the *Nematostella* draft genome, alkaline exonucleases, herpes major outer envelope proteins, herpes DNA primases, herpes ubiquitin-specific proteases as well as a herpes surface glycoprotein and transcription activation factor were identified (Table S4). Also, several copies of a latent membrane protein were found suggesting the presence of endogenous or latent herpes-like viruses in *Nematostella*. Additionally, expressed sequence tags from *Hydra magnipapillata* contain herpes specific tegument proteins and herpes-associated proteases, suggesting that herpes-like viral transcripts were produced.

Herpes-like viral sequences were also recently identified in metagenomic libraries from *P. astreoides* and *D. strigosa* corals. In

the *P. astreoides* metagenome, most of the sequences with similarity to eukaryotic viruses were related to fish iridoviruses, but the second most abundant viral similarities were to *Herpesviridae* (30). In addition, >50% of the all viral sequence similarities (including phages and Archaeal viruses) from linker amplified shotgun libraries of healthy and bleached *D. strigosa* were to *Herpesviridae*, indicating that herpesviruses are commonly associated with corals and not a result of our methodology.

Caveats. The initial metagenomic experiment was designed both to address questions about the viral consortia present in corals and to determine whether this high-throughput metagenomic approach is amenable for empirical experiments. Therefore, treatments were designed to be harsh and as a result did not reflect realistic stressor levels. However, the second 2007 experiment was redesigned to represent more environmentally relevant levels of stress and used to confirm trends from the metagenomes.

In addition, equal amounts of DNA were combined from each replicate coral at each time point to generate individual stressor libraries that contained between 36 and 45 separately extracted viromes. This pooling approach provided a reduction in the variance, or normalizing shifts in the viral consortia over-time. The metagenomes therefore represent an underestimation of the true changes in the viral consortia. The individual temporal samples were then used for real-time PCR to verify identified trends.

The method used to collect the coral subsamples or nubbins required removal of the corals from the aquaria and repeated tissue damage to the coral finger. Corals from the no treatment aquarium control had elevated numbers of herpes-like viral sequences in both experiments. It was apparent from the real-time PCR that this method was disruptive enough to induce herpes-like viral particle production albeit at a reduced level compared with the other stressors. However, naturally occurring damage to corals results from predation, weathering events like hurricanes and earthquakes, and human activities, such as the aquarium coral and ornamental fish trade, recreational diving, and research (53). Our experimental design may have inadvertently mimicked fragmentation and small scale predation on corals. It is therefore likely that these activities and events are stressful enough to induce production of herpes-like viruses on the reef and may result in short or long term reductions in coral health that have until now gone unnoticed.

Last, the metagenomic DNA was amplified using multiple displacement amplification with Phi29 polymerase before sequencing, which could artificially inflate the occurrence of rare sequences, ssDNA, and small circular genomes, thus biasing the analysis (54, 55). However, multiple displacement amplification generally provides an even representation of genomes except at the ends (56) and, as evidenced by the elevation of ssDNA viruses in only the DOC sample, did not appear to bias these data.

Conclusions

This experiment used 6 empirically derived metagenomes to investigate shifts in previously unexplored viral consortia. The coral holobiont is a symbiotic community of animal host, zooxanthellar algae, and various other microbiota (57). This diversity of organisms is reflected in the viral consortia present within the tissues of corals, which included representatives of viruses that infect many protist and metazoan phyla (Fig. 1B), and viruses that infect Bacteria and Archaea (data not shown). Due to the predominance of herpes-like viral sequences in both our samples and *Nematostella*, an aposymbiotic anemone, we hypothesize that these distantly related herpesviruses infects the coral host.

Environmental and Physical Stressors Are Sources of Viral Production.

The metagenomic and temporal experiments presented here demonstrate that exposure to stressors results in the production of a herpes-like virus or a consortium of herpes-like viruses in *P. compressa* corals. Thermal stress, eutrophication, and decreasing seawater pH have each

been shown to disrupt coral health. Increases in sea surface temperature causes coral bleaching and increased coral disease incidence (58, 59). Nutrient addition exacerbates coral diseases, and reduced pH results in loss of corallite deposition (60, 61). This study demonstrates that, in addition to symbiont loss and bacterial and fungal disease (62), temperature and nutrient elevation and pH reduction result in increased HLV production.

A hypothesis that was not directly tested is that the duration of each stressor maybe critical to the overall effect on viral production. Treatments were only 64 h in length, but stress events may last significantly longer on the reef. A reduction in the viral abundance after 4 h in both experiments may suggest that corals can withstand these short stressors, but prolonged stress, such as sustained seawater temperature elevation, may overwhelm a coral's ability to fight off infection.

It is currently unclear how these viruses affect coral physiology or ecology. We hypothesize that viral production can (i) directly cause down stream changes in physiology such as a reduced innate immunity response, secondary opportunist infection by microbes, symbiont expulsion, cell detachment, and apoptosis, or (ii) negatively combine with other stressor induced changes.

Herpesviruses, Human Disease, and the Evolution of Metazoans.

Herpesviruses are characterized by several morphological and genomic commonalities, including large enveloped icosahedral virions, long linear double stranded DNA genomes, and similar life cycle pathways (63). Although herpesviruses were once considered exclusively vertebrate diseases, members of the ostreid *Herpesviridae* are known to infect and kill invertebrates, specifically industrially cultured bivalves (64). The genomes of these shellfish-infecting herpesviruses contain few gene sequences in common with their vertebrate counterparts. Genome examination has demonstrated that herpesviruses can contain upwards of 70% unique genes (65). Across all of the genomes of *Herpesviridae* there is only a single subunit of a terminase shared in common (66). These data suggest that *Herpesviridae* is not a phylogenetically related group of viruses, but perhaps consists of 2 phenetic Families, one related to vertebrate disease and the other to invertebrate disease.

Our coral metagenomes contain more sequences similar to the α -herpesviruses (including several human associated herpesviruses), which infect predominantly mammals and often exhibit broad host range (37). The data presented here support the hypothesis that herpes-like viruses are common in Cnidarians and could perhaps represent distant relatives of the vertebrate lineage of *Herpesviridae*. Our studies may also provide insight into the evolution of these viruses. Herpesviruses typically infect nervous tissue, and it is tempting to suggest that the herpes-like viruses in Cnidarians may have ancient origins, because Cnidarians are the first metazoans to develop rudimentary nervous systems (52).

This was the first experiment to use massively parallel pyrosequencing for empirical experiments, demonstrating that this high-throughput approach can be used as a first step to identify novel viruses in virtually any system. Yet caution should be taken when designing empirical metagenomics. Our statistical analysis on the real-time PCR suggests static analysis of metagenomes may fail to identify critical changes between and among viral consortia.

Overall, we have demonstrated that herpes-like viruses are commonly associated with corals and perhaps all Cnidarians and are produced as a result of changes in ambient water conditions and physical disruption. It is not yet clear how these viruses may affect the physiology or resilience of their hosts, but these data provide a critical foundation for future studies on eukaryotic viral disease ecology of corals.

Materials and Methods

Virome Generation. Virome DNA from each time point and treatment was isolated and extracted to generate twenty-five temporal samples that were

subsampled, amplified, and then pooled by treatment for metagenomic library construction (see Fig. S1).

Virome Purity Verifications. To verify that the viral libraries contained no human contaminating DNA, sequences were compared with the human genome using BLASTn and a minimum 200 score as measured by the formula $(0.267 \times \text{raw score} - \ln(0.041)) / \ln(2)$. Raw scores were the sums of the alignment pairwise scores. Across all 6 viromes low numbers of sequences (<0.8%) were assigned "human" indicating that human contamination was not an issue.

Real-Time PCR. Real-time PCR on the putative herpes-virus target sequence was conducted using SYBR GreenER qPCR SuperMix from Invitrogen according to the manufacturer's recommendations with exactly 25 ng of total viral DNA. Primers used were: forward, 5'-AAAATAAGATTGGGAGATCTAGGC-3' and reverse, 5'-TGCCATTTTAGGTAAATCAGAAAC-3'. Standards were made

from purified target and standard curves were generated using 0.5, 0.05, 0.005, and 0.0005 ng of the target (GenBank accession no. EU660955). A two-way ANOVA (time and treatment) was conducted using Minitab Version 15 (State College, PA). Both parametric and non-parametric tests provided similar results, and the residuals were randomly distributed with a mean of 0, justifying the assumptions.

ACKNOWLEDGMENTS. We thank the Rappé laboratory and all those at Hawai'i Institute of Marine Biology who sponsored R.V.T. in Hawaii. E. Brown, A. Thurber, D. Drumm, and M. Hatay were also instrumental in the collections of the corals and viral particles. R. Schmieder also provided bioinformatic assistance. This work was sponsored by National Science Foundation Postdoctoral Fellowship DBI-0511948 (to R.V.T.) and a Marine Microbial Initiative grant from the Gordon and Betty Moore Foundation (to F.L.R.). Corals were sampled under the auspices of an Hawai'i Institute of Marine Biology collection permit and on Department of Land and Natural Resources Special Activity Permit SAP 2007-72 (to R.V.T.).

- Harvell CD, et al. (1999) Review: Marine ecology—Emerging marine diseases—Climate links and anthropogenic factors. *Science* 285:1505–1510.
- Carpenter KE, et al. (2008) One-third of reef-building corals face elevated extinction risk from climate change and local impacts. *Science* 321:560–563.
- Remily ER, Richardson LL (2006) Ecological physiology of a coral pathogen and the coral reef environment. *Microbial Ecol* 51:345–352.
- Ward JR, Lafferty KD (2004) The elusive baseline of marine disease: Are diseases in ocean ecosystems increasing? *PLoS Biol* 2:542–547.
- Rosenberg E, Ben-Haim Y (2002) Microbial diseases of corals and global warming. *Environ Microbiol* 4:318–326.
- Cooney RP, et al. (2002) Characterization of the bacterial consortium associated with black band disease in coral using molecular microbiological techniques. *Environ Microbiol* 4:401–413.
- Pantos O, et al. (2003) The bacterial ecology of a plague-like disease affecting the Caribbean coral *Montastrea annularis*. *Environ Microbiol* 5:370–382.
- Ritchie KB (2006) Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. *Mar Ecol Prog Ser* 322:1–14.
- Rohwer F, Breitbart M, Jara J, Azam F, Knowlton N (2001) Diversity of bacteria associated with the Caribbean coral *Montastrea franksi*. *Coral Reefs* 20:85–91.
- Rohwer F, Seguritan V, Azam F, Knowlton N (2002) Diversity and distribution of coral-associated bacteria. *Mar Ecol Prog Ser* 243:1–10.
- Kramarsky-Winter E, et al. (2006) Identification of a protist-coral association and its possible ecological role. *Mar Ecol Prog Ser* 317:67–73.
- Wegley L, et al. (2004) Coral-associated archaea. *Mar Ecol Prog Ser* 273:89–96.
- Kellogg CA (2004) Tropical Archaea: Diversity associated with the surface microlayer of corals. *Mar Ecol Prog Ser* 273:81–88.
- Lesser MP, Mazel CH, Gorbunov MY, Falkowski PG (2004) Discovery of symbiotic nitrogen-fixing cyanobacteria in corals. *Science* 305:997–1000.
- Kushmaro A, Rosenberg E, Fine M, Loya Y (2004) Bleaching of the coral *Oculina patagonica* by *Vibrio* AK-1. *Mar Ecol Prog Ser* 273:159–165, 1997.
- Cervino JM, Hayes R, Goreau TJ, Smith GW (2004) Zooxanthellae regulation in yellow blotch/and other coral diseases contrasted with temperature related bleaching: In situ destruction vs expulsion. *Symbiosis* 37:63–85.
- Wetz JJ, et al. (2004) Presence, infectivity, and stability of enteric viruses in seawater: Relationship to marine water quality in the Florida Keys. *Mar Pollut Bull* 48:698–704.
- Griffin DW, et al. (1999) Detection of viral pathogens by reverse transcriptase PCR and of microbial indicators by standard methods in the canals of the Florida Keys. *Appl Environ Microb* 65:4118–4125.
- Seymour JR, Patten N, Bourne DG, Mitchell JG (2005) Spatial dynamics of virus-like particles and heterotrophic bacteria within a shallow coral reef system. *Mar Ecol Prog Ser* 288:1–8.
- Hewson I, Vargo GA, Fuhrman JA (2003) Bacterial diversity in shallow oligotrophic marine benthos and overlying waters: Effects of virus infection, containment, and nutrient enrichment. *Microbial Ecol* 46:322–336.
- Patten NL, Seymour JR, Mitchell AG (2006) Flow cytometric analysis of virus-like particles and heterotrophic bacteria within coral-associated reef water. *J Mar Biol Assoc UK* 86:563–566.
- Wilson WH, Chapman DM (2001) Observation of virus-like particles in thin sections of the plumose anemone, *Metridium senile*. *J Mar Biol Assoc UK* 81:879–880.
- Meints RH, Vanetten JL, Kuczmarski D, Lee K, Ang B (1981) Viral-infection of the symbiotic chlorella-like alga present in *Hydra viridis*. *Virology* 113:698–703.
- Van Etten JL, Meints RH (1999) Giant viruses infecting algae. *Annu Rev Microbiol* 53:447–494.
- Davy SK, et al. (2006) Viruses: Agents of coral disease? *Dis Aquat Organ* 69:101–110.
- Lohr J, Munn CB, Wilson WH (2007) Characterization of a latent virus-like infection of symbiotic zooxanthellae. *Appl Environ Microb* 73:2976–2981.
- Frias-Lopez J, Zerkle AL, Bonheyo GT, Fouke BW (2002) Partitioning of bacterial communities between seawater and healthy, black band diseased, and dead coral surfaces. *Appl Environ Microb* 68:2214–2228.
- Davy JE, Patten NL (2007) Morphological diversity of virus-like particles within the surface microlayer of scleractinian corals. *Aquatic Microb Ecol* 47:37–44.
- Patten NL, Harrison PL, Mitchell JG (2008) Prevalence of virus-like particles within a staghorn scleractinian coral (*Acropora muricata*) from the Great Barrier Reef. *Coral Reefs*, 10.1007/s00338-008-0356-9.
- Wegley L, Edwards R, Rodriguez-Brito B, Liu H, Rohwer F (2007) Metagenomic analysis of the microbial community associated with the coral *Porites astreoides*. *Environ Microbiol* 9:2707–2719.
- Marhaver KL, Edwards RA, Rohwer F (2008) Viral communities associated with healthy and bleaching corals. *Environ Microbiol* 10:2277–2286.
- Bruno JF, et al. (2007) Thermal stress and coral cover as drivers of coral disease outbreaks. *PLoS Biol* 5:1220–1227.
- Kuntz NM, Kline DI, Sandin SA, Rohwer F (2005) Pathologies and mortality rates caused by organic carbon and nutrient stressors in three Caribbean coral species. *Mar Ecol Prog Ser* 294:173–180.
- Kleypas JA, et al. (1999) Geochemical consequences of increased atmospheric carbon dioxide on coral reefs. *Science* 284:118–120.
- Shackleton LA, Holmes EC (2004) The evolution of large DNA viruses: Combining genomic information of viruses and their hosts. *Trends Microbiol* 12:458–465.
- Angly FE, et al. (2006) The marine viromes of four oceanic regions. *PLoS Biol* 4:2121–2131.
- Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA eds (2005) *Virus Taxonomy: Classification and Nomenclature of Viruses* (Elsevier, Amsterdam), Vol VIII.
- Lawrence JG, Hatfull GF, Hendrix RW (2002) Imbrogios of viral taxonomy: Genetic exchange and failings of phenetic approaches. *J Bacteriol* 184:4891–4905.
- Gronenborn B (2004) Nanoviruses: Genome organization and protein function. *Vet Microbiol* 98:103–109.
- Herniou EA, Olszewski JA, O'Reilly DR, Cory JS (2004) Ancient coevolution of baculoviruses and their insect hosts. *J Virol* 78:3244–3251.
- Stoltz DB, Krell P, Summers MD, Vinson SB (1984) Polydnariviridae—a proposed Family of insect viruses with segmented, double-stranded, circular DNA genomes. *Intervirology* 21:1–4.
- Webb BA, et al. (2006) Polydnariviruses genomes reflect their dual roles as mutualists and pathogens. *Virology* 347:160–174.
- Wommack KE, Bhavsar J, Ravel J (2008) Metagenomics: Read length matters. *Appl Environ Microb* 74:1453–1463.
- Dolan A, Jamieson FE, Cunningham C, Barnett BC, McGeoch DJ (1998) The genome sequence of herpes simplex virus type 2. *J Virol* 72:2010–2021.
- McGeoch DJ, et al. (1988) The complete DNA-sequence of the long unique region in the genome of Herpes-Simplex Virus Type-1. *J Gen Virol* 69:1531–1574.
- Tyler SD, Severini A (2006) The complete genome sequence of herpesvirus papio 2 (Cercopithecine herpesvirus 16) shows evidence of recombination events among various progenitor herpesviruses. *J Virol* 80:1214–1221.
- Perelygina L, et al. (2003) Complete sequence and comparative analysis of the genome of herpes B virus (Cercopithecine herpesvirus 1) from a rhesus monkey. *J Virol* 77:6167–6177.
- Bahr U, Darai G (2001) Analysis and characterization of the complete genome of tupaia (tree shrew) herpesvirus. *J Virol* 75:4854–4870.
- Song WJ, et al. (2004) Functional genomics analysis of Singapore grouper iridovirus: Complete sequence determination and proteomic analysis. *J Virol* 78:12576–12590.
- Legoff J, et al. (2006) Real-time PCR quantification of genital shedding of herpes simplex virus (HSV) and human immunodeficiency virus (HIV) in women coinfected with HSV and HIV. *J Clin Microbiol* 44:423–432.
- Putnam NH, et al. (2007) Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science* 317:86–94.
- Putnam NH, et al. (2007) Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science* 317:86–94.
- Smith LD, Hughes TP (1999) An experimental assessment of survival, reattachment, and fecundity of coral fragments. *J Exp Mar Biol Ecol* 235:147–164.
- Spits C, et al. (2006) Optimization and evaluation of single-cell whole, genome multiple displacement amplification. *Hum Mutat* 27:496–503.
- Pinard R, et al. (2006) Assessment of whole genome amplification-induced bias through high-throughput, massively parallel whole genome sequencing. *BMC Genomics* 7.
- Dean FB, et al. (2002) Comprehensive human genome amplification using multiple displacement amplification. *Proc Natl Acad Sci USA* 99:5261–5266.
- Knowlton N, Rohwer F (2003) Multispecies microbial mutualisms on coral reefs: The host as a habitat. *Am Nat* 162:551–562.
- Hoegh-Guldberg O (1999) Climate change, coral bleaching and the future of the world's coral reefs. *Mar Freshwater Res* 50:839–866.
- Fitt WK, Brown BE, Warner ME, Dunne RP (2001) Coral bleaching: Interpretation of thermal tolerance limits and thermal thresholds in tropical corals. *Coral Reefs* 20:51–65.
- Jones RJ, Bowyer J, Hoegh-Guldberg O, Blackall LL (2004) Dynamics of a temperature-related coral disease outbreak. *Mar Ecol Prog Ser* 281:63–77.
- Ben-Haim Y, Zicherman-Keren M, Rosenberg E (2003) Temperature-regulated bleaching and lysis of the coral *Pocillopora damicornis* by the novel pathogen *Vibrio coralilyticus*. *Appl Environ Microb* 69:4236–4242.
- Alker AP, Smith GW, Kim K (2001) Characterization of *Aspergillus sydowii*, a fungal pathogen of Caribbean sea fan corals. *Hydrobiologia* 460:105–111.
- McGeoch DJ, Rixon FJ, Davison AJ (2006) Topics in herpesvirus genomics and evolution. *Virus Res* 117:90–104.
- Davison AJ, et al. (2005) A novel class of herpesvirus with bivalve hosts. *J Gen Virol* 86:41–53.
- Alba MM, Das RJ, Orengo CA, Kellam P (2001) Genomewide function conservation and phylogeny in the herpesviridae. *Genome Res* 11:43–54.
- Davison AJ Evolution of the herpesviruses. *Vet Microbiol* 86:69–88, 2002.

Supporting Information

Vega Thurber *et al.* 10.1073/pnas.0808985105

SI Methods

Virome Generation. Metagenomic viral DNA extractions: Viral particles were isolated using cesium chloride (CsCl) gradient centrifugation. Coral slurries were centrifuged at 3,000 rpm for 15 min to remove coral debris, and the supernatant was placed in a new 50 mL conical tube. CsCl gradients were made with FASW, and loaded in the following densities: 1.7, 1.5, and 1.35 mg·mL⁻¹, respectively. To each gradient, ≈9 mL of sample supernatant was added. Gradients were then centrifuged at 82,000 × *g* for 2 hours in a Beckman Ultracentrifuge at 4°C. The 1.5–1.35 mg·mL⁻¹ fraction was removed with an 18-gauge needle on a sterile syringe and run through an additional gradient to ensure that all bacteria and debris were removed. A subsample of the resulting fraction was analyzed for the presence of contaminating eukaryotic and microbial cells using Sybr Gold (Invitrogen) staining and epifluorescence microscopy as described in ref. 1. The viral fraction was then DNase I treated to remove any residual free DNA. Viral DNA was extracted using formamide and CTAB, as described in ref. 1. To verify the absence of contaminating eukaryotic and microbial DNA 16S and 18S PCR was conducted on all samples before sequencing as described in ref. 2. No bands were detected.

Virome DNA from each time point and treatment was isolated and extracted to generate twenty-five temporal samples, which were subsampled, amplified, and then pooled by treatment for metagenomic library construction (Fig. S1). Once purified, 1 ng of viral genomic DNA from all samples underwent amplification, using GenomiPhi from GE LifeSciences (Quebec, Canada) and repurification with a DNeasy Blood and Tissue Kit from QIAGEN (Valencia, CA). Each of the metagenomic libraries included equal amounts of the amplified DNA from the 1, 4, 16, and 64 h isolates of each corresponding treatment, except the reference sample time zero, which was a pool of all of the time 0 temporal samples (Fig. S1). Approximately 5 μg of total genomic DNA for each library was sent to 454 Life Sciences (Branford, CT) for pyrosequencing using GS20 technology (Fig. S1).

Each of the reads was parsed, stored, assigned a number, and archived at the San Diego State Center for Universal Microbial

Sequencing (<http://scums.sdsu.edu>). Each viral metagenome can be accessed through this website under the accession nos. 4440374.3, 4440375.3, 4440370.3, 4440371.3, 4440377.3, 4440376.3, but are also found at National Center for Biotechnology Information as genome projects IDs: 28427, 28429, 28431, 28433, 28435, and 28437.

Repeat stressor experiment: All parameters were identical to the initial experiment except that in the Temperature treatment, water was increased to 3°C above ambient instead of 5°C. Also, in the ocean acidity stressor, seawater pH was reduced to 7.8 to reflect a more likely environmental change. Salinity, temperature and pH were measured at every sampling.

Herpes-like thymidylate synthase contig assembly. Sequences from each metagenome that were annotated as herpes-like were assembled using SeqMan from DNASTAR Inc. (Madison, WI) using 99% similarity, 35 base pair overlaps, and a minimum sequence read length of 80 base pairs. Contigs were generated and consensus viral sequences were identified using PSI-BLAST to the non-redundant database at National Center for Biotechnology Information. Primer sets were generated and used first for conventional PCR and then real-time PCR, cloning, and Sanger sequencing.

PCR and putative herpes-like thymidylate synthase gene sequence cloning. For the putative herpes-like virus thymidylate synthase gene, PCR was carried out in 50 μL of standard reactions containing 200 nM primers and 25 ng of total viral DNA. Touch down thermocycling was conducted using a 3 min 95°C hot start and 30 cycles of the following: 95°C for 1 min, 60°C (–0.5°C) for 30 seconds, and 72°C for 1 min. A 10-min extension at 72°C completed the PCR. Samples were run on a 1 or 1.5% agarose gel. PCR reactions were purified using a PCR AccuPrep Kit from BioNeer and cloned using a TopoTA Kit from Invitrogen. Sequencing was conducted at the CSU-PERB Microchemical Core Facility. Sequences were trimmed by hand and homology identified using PSI-BLAST to the nonredundant database at National Center for Biotechnology Information.

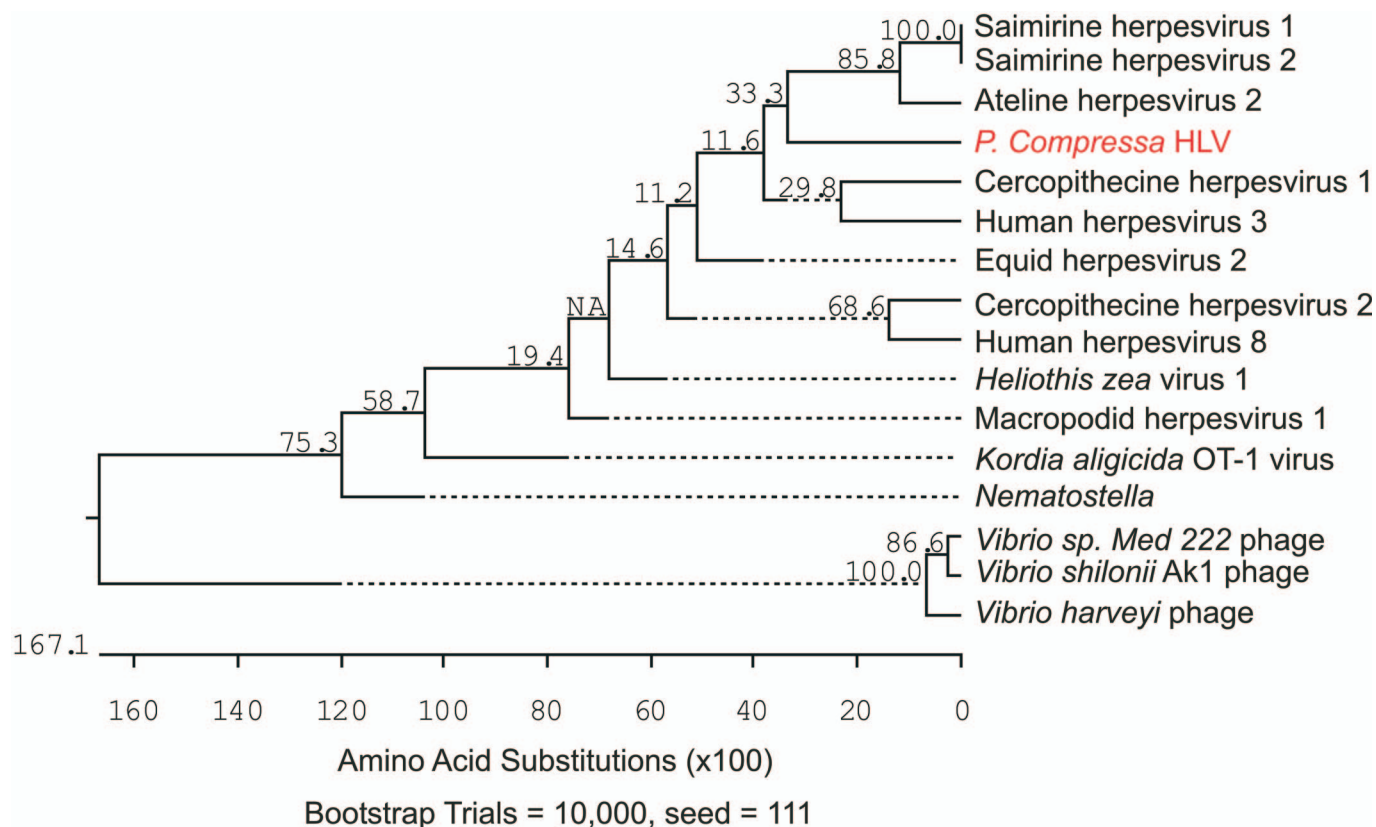


Fig. S2. Coral associated herpes-like viral sequence used for real-time PCR. Sequences from several viral, bacterial, and anemone thymidylate synthase proteins were aligned to the putative Coral HLV sequence using CLUSTAL W and MegAlign from DNASTAR. Blosum 30, PAM 350, and Gonnot 250 matrixes all produced the same tree. Bootstrap analysis was performed using 10,000 iterations.

Table S1. Metagenome characteristics and similarity statistics when compared against the non-redundant ($e < 10^{-4}$) database at NCBI using BLASTn. Each sequence with a similarity in the NR was given a taxonomic assignment (viral, bacterial, eukaryotic) based on its best (smallest e value) similarity

Sample	Reads	Approx. read length	Known, %	GC content, %	Viral, %	Bacterial, %	Eukaryotic, %
Time zero	39,270	101.32	2.12	42.25	1.87	64.14	33.14
Control	39,340	103.7	5.21	46.48	2.89	90.18	6.05
Temperature	39,036	113.38	2.01	48.27	0.98	58.09	40.05
DOC	35,680	102.18	1.88	42.49	8.71	71.24	19.55
pH	50,368	104.73	1.57	43	3.37	68.67	27.44
Nutrient	34,433	107.18	1.83	45.1	6.92	76.82	15.32

Table S2. Coral-associated viral metagenomes were compared for similarity to four previously isolated marine water viromes

Sample	Kingman Reef	Christmas Reef	Palmyra Reef	Tabuaren Reef
Time zero	10.53	10.88	11.10	6.52
Control	2.49	3.65	5.57	1.13
Temperature	29.07	20.12	38.14	25.44
DOC	1.58	2.46	1.74	0.77
pH	11.91	9.63	16.29	9.59
Nutrient	17.41	11.81	23.04	14.27

Percentage similarities between each coral library and reef water viral library were calculated using BLASTn ($e < 10^{-4}$).

Virus	Accession no.	Genome size, kb	Family	Similarities	Coverage
<i>A. polyphaga</i> mimivirus	NC.006450	1181.4	Mimivirus	952	0.09
<i>A. tigrinum</i> virus	NC.005832	106.33	Iridoviridae	64	0.07
<i>A. moorei</i> entomopoxvirus	NC.002520	232.4	Poxviridae	333	0.16
Bovine herpesvirus 1	NC.001847	135.3	Herpesviridae	1071	0.87
Bovine herpesvirus 5	NC.005261	137.82	Herpesviridae	360	0.29
Canarypox virus	NC.005309	359.85	Poxviridae	168	0.05
Cercopithecine herpesvirus 1	NC.004812	156.8	Herpesviridae	666	0.47
Cercopithecine herpesvirus 16	NC.007653	156.5	Herpesviridae	976	0.69
Cercopithecine herpesvirus 2	NC.006560	150.72	Herpesviridae	1282	0.94
<i>E. siliculosus</i> virus	NC.002687	335.59	Phycodnaviridae	174	0.06
<i>E. huxleyi</i> virus 86	NC.007346	407.34	Phycodnaviridae	725	0.20
Equid herpesvirus 2	NC.001650	184.43	Herpesviridae	176	0.10
Human herpesvirus 1	NC.001806	152.26	Herpesviridae	216	0.16
Human herpesvirus 2	NC.001798	154.746	Herpesviridae	283	0.20
<i>M. sanguinipes</i> entomopoxvirus	NC.001993	236.12	Poxviridae	169	0.08
<i>P. bursaria</i> Chlorella virus 1	NC.000852	330.74	Phycodnaviridae	414	0.14
Shrimp spot syndrome virus	NC.003225	305.11	Nimaviridae	125	0.05
Singapore grouper iridovirus	NC.006549	140.13	Iridoviridae	169	0.13
Suid herpesvirus 1	NC.006151	143.46	Herpesviridae	862	0.66
Tupaia herpesvirus	NC.002794	195.86	Herpesviridae	238	0.13

All coral-associated viral metagenomes were combined and the number of sequence similarities ($e < 10^{-6}$) to each genome was calculated. Frequency tables were generated for each 2,500-bp bin across each reference genome. First, the number of synonymous nucleotides between the metagenome and the reference genome was counted. Coverage was then calculated by dividing this number by the total length of reference the genome. Any genome with coverage equal to or greater than 0.05 is listed. Coverage values in bold denotes the 5 highest values.

Table S4. Herpes-like viral sequences in the reference genomes of two Cnidarians

Nematostella Draft	Hydra ESTs
5507928	74131562
5500715	74132680
5503168	68410998
5522046	
5497885	
5517497	
5512689	
5521542	
5498193	
5515317	
5515317	
XP.001626845	
EDO34745	
XP.001641920	
EDO49857	
XP.001636448	
EDO44385	
XP.001629245	
XP.001627817	
XP.001626452	
XP.001624299	
XP.001621368	
XP.001617849	
EDO25749	
EDO29268	
EDO32199	
EDO34352	
EDO35717	
EDO37182	