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A Profile of an Endosymbiont-enriched Fraction of the Coral *Stylophora pistillata* Reveals Proteins Relevant to Microbial-Host Interactions*

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This study examines the response of *Symbiodinium* sp. endosymbionts from the coral *Stylophora pistillata* to moderate levels of thermal "bleaching" stress, with and without trace metal limitation. Using quantitative high throughput proteomics, we identified 8098 MS/MS events relating to individual peptides from the endosymbiont-enriched fraction, including 109 peptides meeting stringent criteria for quantification, of which only 26 showed significant change in our experimental treatments; 12 of 26 increased expression in response to thermal stress with little difference affected by iron limitation. Surprisingly, there were no significant increases in antioxidant or heat stress proteins; those induced to higher expression were generally involved in protein biosynthesis. An outstanding exception was a massive 114-fold increase of a viral replication protein indicating that thermal stress may substantially increase viral load and thereby contribute to the etiology of coral bleaching and disease. In the absence of a sequenced genome for *Symbiodinium* or other photosymbiotic dinoflagellate, this proteome reveals a plethora of proteins potentially involved in microbial-host interactions. This includes photosystem proteins, DNA repair enzymes, antioxidant enzymes, metabolic redox enzymes, heat shock proteins, globin hemoproteins, proteins of nitrogen metabolism, and a wide range of viral proteins associated with these endosymbiont-enriched samples. Also present were 21 unusual peptide/protein toxins thought to originate from either microbial consorts or from contamination by coral nematocysts. Of particular interest are the proteins of apoptosis, vesicular transport, and endo/exocytosis, which are discussed in context of the cellular processes of coral bleaching. Notably, the protein complement provides evidence that, rather than being expelled by the host, stressed endosymbionts may mediate their own departure. ***Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.015487, 1–19, 2012.***

Key to the evolutionary success of reef-building corals that inhabit the often nutrient-poor waters of tropical oceans is the metabolic cooperation between the host coral and unicellular algae residing within its cells. These endosymbiotic algae typically are dinoflagellates of the genus *Symbiodinium*, which are often referred to as "zooxanthellae," although this catch-all name established by Brandt in 1883 does include other resident taxa of microbial endosymbionts (1). In this photoautotrophic symbiosis, fixed organic carbon produced by *Symbiodinium* is released to the animal host for its nutrition, whereas the dinoflagellates benefit by acquiring inorganic waste from the host (2–4).

Breakdown of the coral symbiosis is manifested as bleaching, made apparent by the loss of photobionts (5) or destruction of their photosynthetic pigments (6). Because heterotrophic feeding by polyps does not provide sufficient nutrition for corals to survive (7), death of colonies may result from severe bleaching if the obligate association is not re-established, thereby threatening coral reef ecosystems (8). The symbiotic association typically fails when corals experience temperature anomalies as little as 2–3 °C above mean summer seawater temperatures (9–11), which in combination with high solar irradiance leads to the production of excessive

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Received October 28, 2011, and in revised form, January 28, 2012
Published, MCP Papers in Press, February 20, 2012, DOI 10.1074/mcp.M111.015487

levels of reactive oxygen species (ROS)[1] from over-reduced photosystems of *Symbiodinium* that overwhelm the antioxidant defenses of the holobiont (12–14). In 1998, worldwide sea surface temperature anomalies associated with a strong El Niño Southern Oscillation resulted in a major global bleaching event, causing coral mortality on 16% of the world's coral reefs (15), which demonstrates the critical importance of symbiosis to coral reef health.

A consensus has emerged as to the biochemical mechanisms underlying ROS production in early stages of cnidarian bleaching (reviewed in Refs. 16 and 17). Briefly, under conditions of ambient light exposure, endosymbiotic algae liberate oxygen as a by-product of photosynthesis, causing host tissues to be hyperoxic on a diel cycle (18). In addition, bright sunlight and thermal stress can damage the photosynthetic reaction centers of *Symbiodinium*, leading to excessive levels of ROS production by generating the highly reactive hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) that by diffusion can overwhelm the antioxidant capacity of both the algal symbiont and host (19), thereby causing further cellular damage. Direct photoexcitation, particularly in the presence of solar ultraviolet radiation, may produce ROS in both the endosymbionts and the host cells (20). A major mechanism for detoxification of ROS is the production of the antioxidant enzymes superoxide dismutase (SOD) and catalase, which are found in both partners (21), and ascorbate peroxidase in the algal symbiont (22). Our understanding of the contribution that each partner makes to the physiological response of the holobiont to oxidative stress, however, remains limited (23), including the involvement of ROS sensing and oxidative signaling of apoptotic pathways in symbiotic cnidarians (14).

Ample evidence for stress-induced progression of programmed cell death and necrosis in partners of the coral symbiome has been reported (24–25), which corresponds with activation of apoptotic genes of the coral host (26–29) and in aposymbiotic coral larvae and embryos (30, 31). Apoptotic elements are similarly induced by hyperthermal stress in the host tissues of the symbiotic sea anemones *Anemonia virdis* (32), *Aiptasia pallida* (33, 34), and *Anthopelura elegantissima* (35). Elements of the NF-κB signaling pathway of apoptosis in cnidarians are highly conserved traits (36), including genes of the caspase cascade and the pro-apoptotic and anti-apoptotic B-cell lymphoma 2 (Bcl-2) family of proteins (33, 29) that likely predate the evolution of animal multicellularity via endosymbiotic incorporation of aerobic bacteria as the ancestral proto-mitochondria of unicellular eukaryotes (37).

Careful examination of the coral *Agaricia agaricities* at above normal temperatures under daylight exposure has revealed accumulation of H_2O_2 at a rate that exceeds its removal by cellular protection or loss by diffusion, thereby causing oxidative stress, damage to the calcium exclusion system, and exocytotic liberation of *Symbiodinium* into the coelenteron with subsequent fragmentation of the gastrodermis (38). Furthermore, the thermal tolerance of *Symbiodinium* spp. is demonstrably constrained by the production and accumulation of H_2O_2 (39) that would account for variation in the thermal sensitivity of *Symbiodinium* phylotypes (40). It has been suggested that excess H_2O_2 generated by endosymbiotic algae may provide the signal to initiate the expulsion of these photobionts from corals (41) and/or trigger apoptosis in cnidarian host tissues by activation of the transcription factor NF-κB that leads directly to host cell apoptosis and autophagy (42).

Alternatively, NF-κB can induce the expression of nitric-oxide synthase (NOS) to produce NO, a well known promoter of the pro-apoptotic transcription factor p53 that is the cell cycle gatekeeper of the caspase cascade. NOS activity in a sea anemone was first reported by Trapido-Rosenthal *et al.* (43), and NO production has since been linked to cnidarian bleaching (44, 45), presumably via up-regulation of NF-κB (46). Perez and Weis (47) found that the major source of stress-induced NO production was in the host cells of the symbiotic anemone *A. pallida*. Consistent with this finding, NOS activity was absent in symbiotic dinoflagellates isolated with rigorous cleansing from three phylogenetically distinct cnidarians and from their cultured algal symbionts, whereas significant activities were found in the host cytosol (48). Furthermore, cytosolic NO might combine with superoxide produced by heat-stressed *Symbiodinium* to afford the powerful oxidant peroxynitrite (47) known to damage host mitochondrial membranes and cause the release of cytochrome *c* to activate the intrinsic apoptotic pathway of caspase-induced cell death (49). Although expressed markers of apoptosis, including the Bcl-2 family of pro- and anti-apoptotic proteins, are observed in the response of freshwater *Hydra* to stress (50), a direct link between molecular regulation of the Bcl-2 family of apoptotic mediators to activate cell death in cnidarian bleaching has yet to be demonstrated (29).

Other cellular mechanisms leading to bleaching that have been described include *in situ* symbiont degradation and autophagy (42), host cell detachment (51), host cell necrosis (24), and exocytosis (5, 52, 53). It is generally agreed that cnidarian bleaching involves multiple complex processes that depend on the type, synergy, and duration of the bleaching stress. For instance, in a recent publication, we have shown that thermal stress contributing to bleaching of the coral *Stylophora pistillata* is exacerbated by a limitation in the bioavailability of trace metals (specifically, iron) essential as cofactors to major metabolic processes, perhaps including antioxidant enzymes, in both the host and dinoflagellate partners

[1] The abbreviations used are: ROS, reactive oxygen species; Bcl-2, B-cell lymphoma 2; NF-κB, nuclear factor-κB; NOS, nitric-oxide synthase; MES, 2-(N-morpliolino) ethanesufonic acid; PAR, photosynthetically active radiation (400–700 nm); SOD, superoxide dismutase; SNARE, soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment receptor proteins; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TEAB, triethylammonium bicarbonate; TMT, tandem mass tag; KEGG, Kyoto Encyclopedia of Genes and Genomes.

(54). Conversely, iron hyperenrichment can also be detrimental to coral reef health arising from increased algal abundance and enhanced microbial activity as found in shipwreck-affected coral atoll mesocosms (55).

Although genomic techniques are yielding valuable insights into how the partners of coral symbiosis respond to environmental stress at transcription, the use of mRNA abundance as a correlation proxy to protein abundance and its functional activity has limitations in assessing phenotypic adaptation to abiotic stress (56). Accordingly, inroads to stress assessment at the proteome level of cnidarian symbioses have been progressing (57–62), as has understanding of the proteomic response of other marine organisms to environmental stress (63). In an attempt to understand the physiological response of symbiotic corals to the stresses of high irradiance, temperature, and trace metal limitation, we construct for the first time, using a quantitative high throughput platform, a comprehensive profile of the major proteins and peptides in a *Symbiodinium*-enriched fraction obtained from a reef-building coral under experimental conditions of environmental stress. Herein, we report the results of trace metal restriction at different temperatures in *S. pistillata* leading to a bleaching response in this coral.

EXPERIMENTAL PROCEDURES

Collection and Maintenance of Corals—Colonies of the reef-building coral *S. pistillata* (Esper, 1797) were collected at Pith Reef (18°13.434′S 147°01.052′E), Great Barrier Reef, Australia in January 2010 (Australian Institute of Marine Science Trip 4912). Collection depth ranged from 7 to 9 m at an average water temperature of 28 °C. Following collection, the corals were kept in shaded plastic tanks (64 liters) with flow-through seawater until transferred to the Australian Institute of Marine Science. On arrival at the Australian Institute of Marine Science, the corals were moved to a shaded 1000-liter tank with aeration and flow-through seawater (~2 liters min^{-1}, 28 °C ± 0.5 °C, salinity 30–32). Incoming seawater was filtered to 5 μm, and the corals received intensities of photosynthetically available radiation (PAR) ranging between 60 and 100 μmol photons m^{-2} s^{-1}. Coral colonies were cut into 3–5-cm pieces and prepared for experimental procedures as described by Shick *et al.* (54). Only corals without obvious endolithic organisms were chosen for experimentation. Coral fragments were then transferred to indoor holding tanks for recovery and acclimation (28 °C ± 0.5 °C, PAR 200 μmol photons m^{-2} s^{-1}, salinity 30–32) for 4 weeks prior to their exposure to experimental treatments.

Manipulative Trace Metal Experiment—Experimental treatments were conducted in an indoor aquarium room where water parameters, air temperature, and irradiance were controlled. Four 32-liter tanks were set in line, serving as temperature-controlled water baths. Each tank was fitted with a submersible pump to generate constant water circulation (1000 liters h^{-1}) and its own seawater inlet from a temperature-controlled reservoir set to a flow rate of 400 ± 25 ml min^{-1}. Incident PAR was subsaturating, 200 μmol photons m^{-2} s^{-1} (400 W Metal Halide Fixture Complete; Lamp Technologies International Pty. Ltd., Nunawading, Australia) at the water's surface, and illumination was provided for 12 h/day for the first 6 days. On day 6, the irradiance was increased to 425 ± 75 μmol photons m^{-2} s^{-1} (sufficient to saturate photosynthesis in this coral) for the remaining 7 days of the experiment. The irradiance inside the treatment chambers (see below) was ~87% of incident irradiance. There was no detectable UVB radiation beneath the chamber lids, and UVA was only 25 μW cm^{-2} (54). Water temperature was regulated using 2-kW heating bars controlled by a CR1000 measurement and control datalogger (Campbell Scientific, Hyde Park, Australia) regulated to within ± 0.1 °C by a computer controlled system. The room's air temperature was set to 25 °C and circulated across the exposed tops of the experimental treatment chambers to minimize radiative heating.

The experiments were conducted in low density polyethylene containers (3 liters; Sistema, Dandenong, Australia) including 2 liters of treatment media and sealed by lids with silicone gaskets. Prior to use, treatment containers and fittings were acid-cleaned thoroughly following the protocol described by Shick *et al.* (54). Coral fragments were suspended in the experimental media using a nylon monofilament line secured to polyethylene bolts and wing nuts attached to the container lid. Seawater inside the experimental chambers was mixed and aerated by a flow of air through a 0.45-μm filter delivered from an aquarium pump; air exited the sealed chamber via a 0.40-μm filter pressure-fitted into the lid. The chambers were externally ballasted and immersed in the temperature-regulated water baths to a level just below the sealing gasket. The experimental medium (treated seawater) in each chamber was changed daily in a room clean of trace metals as described by Shick *et al.* (54).

Trace metal-free seawater was prepared by oxidizing seawater sent through a 0.5-μm filter by exposure to UV radiation in a purpose-built, flow-through UV reactor using a medium pressure mercury vapor lamp similar in design to that described by Achterberg and van den Berg (64) to release trace metals bound in dissolved organic complexes. The UV-treated seawater was then passed through a metal chelating resin (Chelex® 100; Bio-Rad) at a flow rate of 8 ml min^{-1} to remove total trace metals (65). A nutritional suite of essential trace metals and vitamins was added back to the UV-Chelex-treated seawater at concentrations used to obtain optimal growth for most phytoplankton (66) (supplemental information Data Set 1). Trace metal and vitamin amendments were added to 45 ml of UV-Chelex-treated seawater passed through a 0.5-μm filter prior to daily medium changes. Amendments sat for a maximum of 3 h prior to addition to experimental chambers, where they were brought up to 2 liters with UV-Chelex-treated seawater passed through a 0.5-μm filter.

The trace metal-replete medium was prepared by adding all metals and vitamins listed in supplemental information Data Set 1, whereas the effects of iron- and manganese-limiting conditions were tested by individually omitting their additions. The effects of trace metal limitation and temperature on *S. pistillata* were examined by exposing coral fragments to five treatments: 1) low temperature (28 °C) plus all metals (LT +M), 2) low temperature minus iron (LT −Fe), 3) low temperature minus manganese (LT −Mn), 4) high temperature (31 °C) plus all metals (HT +M), and 5) high temperature minus iron (HT −Fe). An additional treatment using high temperature minus manganese was also included in the experimental design, but coral fragments in this treatment died suddenly during the experiment, before endosymbiotic algae could be harvested. Three fragments from each of two coral colonies were placed in each treatment chamber. Water temperature in high temperature treatments was increased gradually (0.75 °C/h) from 28 to 31 °C on the 10th day of the experiment.

Isolation of Endosymbiotic Dinoflagellates—Endosymbiotic dinoflagellates were isolated on day 0 and following termination of the experiment at day 13. The day 0 sample for one of the coral colonies was designated replicate A, whereas the day 0 samples from the second colony was divided to become replicates B and C. Replicates A, B, and C at $t = 0$ represented the base-line proteome prior to stress against which the effects of the treatments were compared. Isolation was carried out under low light conditions and on ice. Coral tissue was removed from the skeleton of *S. pistillata* using an airgun and filtered (0.2 μm, Absolute D, Cuno Pacific Pty. Ltd., NSW, Australia)

seawater. The resulting slurry was homogenized in a hand-held homogenizer (PRO 250; PRO Scientific, Oxford, CT) for 45 s and passed through a 25-μm Nitex mesh. The filtered slurry was then centrifuged for 20 min at 500 $\times$ *g* and 4 °C to sediment the algae. The supernatant containing the host tissue was discarded, and the pellet containing the freshly isolated endosymbiotic dinoflagellates was resuspended in Moore's calcium-free artificial seawater (www.mbl.edu/Biological-Bulletin/COMPENDIUM/CompTab2.html) containing 0.02% (w/v) SDS to remove any adhering host phagosomal membrane and to minimize clumping and again centrifuged for 20 min at 500 $\times$ *g* and 4 °C. To remove SDS, the algal pellet was washed three times with artificial seawater without SDS and centrifuged for 20 min at 500 $\times$ *g* and 4 °C after each wash. Following the final wash, the pellet was resuspended in 1 ml of artificial seawater and transferred to vials of a known weight. A small subsample was microscopically examined to assess and confirm the purity of the algal isolate. The suspension of cleaned endosymbiotic dinoflagellates was again centrifuged for 10 min at 16,000 $\times$ *g* and 4 °C, the supernatant was aspirated, and the wet weight of the pellet was recorded. A minimum of 200 mg of wet algae were freeze-dried. Samples having a dry weight between 3 and 110 mg were subsequently prepared for protein extraction.

Protein Extraction and Tandem Mass Tag Labeling—A volume of 0.5 ml of 50 mM triethylammonium bicarbonate (TEAB) buffer (pH 7.5) was added to each freeze-dried sample in a microcentrifuge tube. Then ~300 μl of a suspension of acid-washed and rinsed glass beads (400–625-μm diameter; Sigma) was added. Each sample was vortexed for 1 min and then placed on ice for 1 min. This procedure was repeated 10 times. Lysis was visually verified by green (chlorophyll) discoloration of the lysis buffer. The beads were allowed to settle, and the supernatant was transferred to a fresh microcentrifuge tube and centrifuged at 12,000 $\times$ *g* for 10 min at 4 °C. The supernatant was then assayed with Bradford reagent (Sigma) to ensure that sufficient protein was available for labeling with amine-reactive tandem mass tag (TMT) reagents to enable the identification and quantification of extracted proteins by mass spectrometry (67). Proteins from our five treatments plus a $t = 0$ sample representing the unstressed proteome were labeled using a TMT sixplex tagging kit (Thermo Scientific, East Riding, UK) comprising an isobaric set of six mass tags with five isotopic substitutions. Each tag produces a unique reporter ion during MS/MS fragmentation analysis enabling concurrent identification and quantification of proteins in different samples by labeling of digested proteins at the peptide level (bottom-up strategy) (68) or by labeling at the protein level followed by to tryptic digestion (top-down strategy) (69). Simultaneous processing of six samples minimizes analytical variation introduced by differences in gel loading or extraction and eliminates the need for six individual analyses that would be affected by LC-MS/MS signal fluctuations between operations. Extracted proteins were labeled with TMT126–TMT130 reagents for each respective treatment, and the process was repeated for the three replicates (A–C) from each experimental treatment. For the unstressed $t = 0$ samples representing the basal proteome, TMT131 reagent was used for labeling. Thus, in total, 18 protein samples were labeled.

Procedures for protein (top-down) TMT labeling were as follows. Briefly, each TMT tag was reconstituted in 24 μl of acetonitrile and equilibrated at room temperature before adding it to each sample. Protein quantities were adjusted to reduce variability between replicates. Appropriate volumes of the protein extract from each treatment sample or pretreatment $t = 0$ sample equivalent to 25 μg were lyophilized and reconstituted in 50 μl of 50 mM TEAB containing 0.04% SDS for resolubilization. The samples were then reduced by adding 5 μl of 9 mM tris(2-carboxyethyl)phosphine hydrochloride in 50 mM TEAB at 55 °C for 1 h and then alkylated with 5 μl of 16 mM iodoacetamide in 50 mM TEAB at room temperature for 30 min in darkness. A volume of 8 μl of 5% hydroxylamine (w/w) was added according to TMT procedures to terminate the reaction. Combined TMT-tagged samples for each treatment replicate A1–5, B1–5, and C1–5 were mixed individually with their corresponding TMT-tagged, pretreatment samples A($t = 0$), B($t = 0$), and C($t = 0$), respectively. The samples were then lyophilized and reconstituted in 20 μl of 50 mM TEAB and 20 μl of Laemmli buffer (70). In these samples, excess TMT reagents were removed during protein SDS-PAGE separation.

One-dimensional Gel SDS-PAGE and In-gel Digestion—The sixplex TMT-labeled samples (A–C) were boiled for 5 min before loading across a 4–12% NuPAGE Novex Bis-Tris gel (Invitrogen) with MES (2-(*N*-morpholino)ethanesulfonic acid) buffer alongside Novex® SeeBlue® Plus2 prestained standards (Invitrogen). Electrophoresis was carried out for 100 min at 150 V. The gel was fixed, Coomassie Blue-stained, destained, and visualized with an ImageQuant (GE Healthcare) biomolecular imager. The bands were then selected for excision and in-gel digestion (supplemental information Data Set 2). The three gel lanes were sectioned into 15 portions of ~2 mm^2 in area. In-gel reduction, alkylation, and proteolytic digestion with trypsin were performed prior to subsequent analysis by mass spectrometry (71) as follows. Briefly, cysteine residues were reduced with 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide in 100 mM ammonium bicarbonate to form stable carbamidomethyl derivatives. Trypsin digestion of each section was carried out overnight at 37 °C in 50 mM ammonium carbonate buffer, and the supernatant was retained. The peptides were extracted from the excised gel portions by two washes with 50 mM ammonium bicarbonate and acetonitrile. Each wash involved shaking gel sections for 15 min before collecting the peptide-containing extract. The extract was pooled with the initial digestion supernatant and then lyophilized. The samples were resuspended in 46 μl of 50 mM ammonium bicarbonate per band prior to LC-MS/MS analysis on injection of half the sample volume.

LC-MS/MS—Chromatographic separations of each band were performed using an Ultimate LC system (Dionex Ltd., East Riding of Yorkshire, UK). The peptides were resolved by reversed phase chromatography on an Acclaim® PrepMap100 C18, 3-μm particle, 100 Å (75 μm $\times$ 150 mm) column (Thermo Scientific/Dionex). Depending on the intensity of the gel band, gradients of 30–180 min of acetonitrile in 0.05% formic acid were delivered to elute the peptides at a flow rate of 200 nl/min. Peptides were ionized by electrospray ionization using a Z-spray source fitted to a QToF-micro quadrupole orthogonal acceleration time-of-flight tandem mass spectrometer (Waters Corp., Milford, MA). The instrument was set to operate in automated switching mode, selecting the two most intense precursor ions for sequencing by collision-induced fragmentation. Precursor ions were surveyed across a range of 400–1800 *m/z*. The MS/MS analyses were conducted across a range of 100–2000 *m/z* using collision energy profiles that were chosen based on the mass-to-charge ratio and the charge state of the peptide. Each precursor mass was excluded from further selection from the mass list for 90–120 s following its fragmentation depending on the length of the gradient. Quality control tests with 200 fmol of BSA were analyzed between each set of 15 gel sections to ensure that coverage of the protein was ≥30%.

Data Analysis—Tandem mass spectra were extracted into peak lists by Masslynx version 4.0 and Protein Lynx Global Server 2.2.5 (Waters Corporation, Milford, MA). Charge state deconvolution and deisotoping were not performed. All of the MS/MS spectra were analyzed using Mascot software (version 2.2.03; Matrix Science, London, UK). Mascot was developed to search entries of the uniprot_sprot_100713 database (selected for eukaryotes, 518,415 entries), assuming use of the digestion enzyme trypsin and up to three missed cleavages. The data were searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 1.2 Da. The following were stated as variable modifications: N-terminal acetylation, deamidation of asparagine and glutamine, N-terminal glutamine

to pyroglutamine, oxidation of methionine, iodoacetamide derivative of cysteine, and phosphorylation of serine, threonine, and tyrosine. Mascot Daemon was used to perform merged searches for the 15 peak list files per replicate set. The raw data were recalibrated against internal tryptic peptides where necessary to produce a mass accuracy of less than 50 ppm for most peptides.

A complete sequence for the genome of *Symbiodinium* is unavailable, and only incomplete and fragmentary information for this genus exists in the Uniprot protein database. Therefore, an all-protein search was chosen to match orthologs from other species. To maximize the sweep of coverage of the *Symbiodinium*-enriched protein extract, all of the labeled peptides marked as bold (most likely assignment) by Mascot were used regardless of the probability-based expected value (supplemental information Data Set 4), and data assigned to peptides but not to Mascot protein hits were individually annotated by a manual search of the Uniprot database (supplemental information Data Set 5). All of the Mascot hits of any peptide score and probability-based expected value were accepted meeting the criteria: all peptides rank 1, lysine-containing peptides flagged as bold with one or more tag(s) plus TMT131 detected. Grouping of peptides matched to multiple database entries was not reported. Instead, the assignment declared first in the Mascot peptide summary report was used. Also, using rank 1, peptides marked as bold red ensured that peptides reported here were based on the highest scoring assignments. The Mascot peptide summary report resulting from the merged data was exported as a comma-separated values (.csv) file. An in-house PerlScript program was then used to merge the .csv file with a tab-separated values (.tsv) file resulting from the search that contains the TMT tag intensities. The resulting .csv file was then used for sorting and processing data in Microsoft Excel. False discovery rates were estimated by again doing the merged searches against a Mascot decoy database. The decoy database contains randomized sequences, and therefore any matching peptides would be highlighted as a false positive.

Peptide data for each replicate set were annotated with the sample representing the $t = 0$ base-line proteome prior to stress category (A–C) and combined into one file. The data were then sorted according to protein accession number and replicate category. Proteins present in two or three replicates were then carried forward for further analysis. Data assigned to peptides but not to Mascot protein hits were then individually annotated by Mascot's peptide view (therefore including all eukaryotic, prokaryotic, Achaean, and viral proteins). Quantification was determined for peptides that were present in at least two of the three replicates. TMT tag intensities giving *x*-fold changes of 0 or negative values between treatment and the unstressed $t = 0$ proteome taken prior to treatment were discarded. Peptides without detectable tags do not allow relative quantification and were also eliminated. TMT tag intensities for each assigned peptide present in at least two of the replicates were used to calculate the analysis of variance between treatments relative to the pretreatment $t = 0$ proteome. Experimental condition had a statistically significant effect on the average TMT tag intensity when $p < 0.05$. In these cases, a Student's *t* test was also calculated between individual pairs of samples (*i.e.* between each individual treatment and its $t = 0$ base line and between individual sample/treatments) to confirm that the experimental condition produced a significant effect (72, 73).

RESULTS

Experiments were performed wherein the coral *S. pistillata* in seawater either replete or lacking in iron or manganese was exposed to photosynthetically saturating levels (>400 μmol of photons m^{-2} s^{-1}) of PAR at nonstressful seasonal and at elevated water temperatures. Under these conditions, the dark-adapted photosynthetic efficiency of the symbiotic algae in all treatments declined significantly (paired *t* tests, $p < 0.05$) from day 1 to the end of the experiment, as evinced by reduced maximum quantum fluorescence yields (ratio of variable to maximal chlorophyll fluorescence in photosystem II, F_v:F_m) of the photosynthetic pigments of photosystem II (supplemental information Data Set 3), which is consistent with the symbiotic photophysical dysfunction that occurs during coral bleaching (74).

The MS/MS spectra for single-peptide-based protein identifications are shown in supplemental information Data Set 6. The average *x*-fold change of the TMT tag intensities for each treatment relative to the unstressed $t = 0$ proteome was calculated from all of the peptides assigned to the same protein across two, or better three, replicates (supplemental information Data Set 7). Values <1 or >1 indicate either a reduction or an increase in tag intensity as compared with the pretreatment $t = 0$ proteome, whereas a value equal to 1 indicates no change in protein abundance. Of the 109 peptides meeting our stringent criteria for quantification, 83 (76%) did not show any significant change in TMT tag intensity compared with the unstressed $t = 0$ proteome. Fig. 1 shows the remaining 26 assigned peptides where differences in TMT tag intensities between treatments relative to the pretreatment $t = 0$ proteome were found to be statistically significant. Of these, 20 of 26 peptides have a significant reduction in TMT tag intensity compared with the unstressed $t = 0$ proteome in at least one of the low temperature treatments. Even in the relatively benign treatment of low temperature (*i.e.* unchanged from the day 0 temperature) in seawater replete with trace metals, 17 of 26 peptides had a significant reduction in tag intensity, perhaps a response to the more than doubling of irradiance on day 6, which was manifested in an immediate decrease in photosynthetic efficiency, such that photosynthetic efficiency in all treatments was lower at the end of the experiment on day 13 than immediately before the start of the experiment on day 1 (supplemental information Data Set 3). Only 12 of 26 assigned peptides showed a statistically significant increase in TMT tag intensity compared with the unstressed $t = 0$ proteome; these 12 peptides were all associated with high temperature treatments and were generally involved in protein biosynthesis (perhaps reflecting a general accelerating effect of the increased temperature on metabolism) with the exception of proteins Q9J4Z9 and P27551, which are of viral origin. The only statistically significant increase in TMT tag intensity observed at low temperature was caused by manganese depletion (LT −Mn) to enhance the abundance of a protein from the TCA cycle (B3EN95, succinyl-CoA ligase) and that of the abundance of a protein of viral origin (P27551, viral replication protein E1). Only 5 of 26 proteins showed varying TMT tag intensities under both temperature conditions that were statistically significant compared with the pretreatment $t = 0$ proteome. No statistically significant decrease in TMT tag intensity was as-

Protein Accession Number	Protein Description	Average x-fold change of TMT tag intensities				
		LT+M	LT -Fe	LT -Mn	HT+M	HT -Fe
Q9Z7A5	UvrABC system protein B involved in DNA repair SOS response	0.2	0.1	NTD	0.3	0.8
Q9VU84*	Drebrin-like protein involved in actin binding	0.2	0.8	0.7	9.0	3.9
Q9V557*	Probable cytochrome P450 4p2, iron co-factor	0.3	0.9	1.0	2.3	2.4
Q9J4Z9	Putative ankyrin repeat protein FPV241 – viral origin	0.0	0.2	4.0	35.4	43.4
Q9HIS2	30S ribosomal protein	1.1	1.2	3.4	3.5	2.1
Q9FDN7	Nitric oxide reductase, iron co-factor	0.1	0.2	0.4	1.2	1.4
Q7NNH7	30S ribosomal protein S6	0.4	0.3	0.4	2.3	2.2
Q6XZ78	Fructokinase-2, starch metabolism	0.5	0.8	1.5	2.1	1.3
Q6P2L6*	Histone-lysine N-methyltransferase, zinc cofactor	0.3	0.5	1.1	4.8	5.6
Q6NY15*	Testis-specific gene 10 protein involved in flagella assembly	0.0	2.0	2.7	5.0	1.5
Q6F260	Translocase involved in protein transfer across membranes	0.3	1.3	6.3	6.6	4.1
Q3UYK3*	GTPase-activating protein for Rab family proteins, calcium ion binding	0.4	0.2	0.5	1.2	1.1
Q30S82	3-phosphoshikimate 1-carboxyvinyltransferase – shikimate pathway	0.0	1.2	0.9	21.6	3.1
Q1RML7*	Protein MAK16 homolog required for maturation of 25S and 5.8S rRNAs	0.1	0.6	0.5	1.1	1.3
Q0VNJ7	Probable translational activator of cytochrome c oxidase 1	0.1	0.1	0.3	0.5	1.1
P76369	Uncharacterized HTH-type transcriptional regulator yeeY	NTD	0.1	0.8	4.4	6.0
P54122	Ribonuclease J, involved in maturation and stability of mRNAs, metal ion binding	0.9	0.6	0.8	4.5	1.6
P32330	Protein involved in increasing cellular tolerance to 2-deoxy-glucose	0.3	0.0	0.9	2.5	3.2
P27754	Ribosomal protein S3, mitochondrial	0.2	1.0	1.6	2.0	1.1
P27551	Replication protein E1, viral origin	0.0	1.7	7.6	114.5	10.3
O08451*	Aconitate hydratase, TCA cycle enzyme, iron co-factor	NTD	0.1	NTD	0.3	0.4
B8J1N0	L-seryl-tRNA(Sec) selenium transferase	0.5	1.4	0.5	7.5	6.3
B5EAX0	Lipoyl synthase, incorporates sulphur into proteins, iron co-factor	0.1	0.1	0.0	1.3	0.9
B3EN95*	Succinyl-CoA ligase, TCA cycle enzyme, magnesium or manganese co-factor	0.3	0.2	1.1	7.5	6.8
A8GN90	Prolyl-tRNA synthetase	0.0	0.2	0.0	2.0	2.1
A8EZ44	DNA helicase ruvB involved in recombination & DNA repair	0.1	0.6	1.6	3.0	2.3

FIG. 1. **Statistically significant average fold change of the TMT tag intensities for each treatment relative to control $t = 0$.** The treatments are low temperature (28 °C) plus all metals (*LT +M*), low temperature minus iron (*LT −Fe*), low temperature minus manganese (*LT −Mn*), high temperature (31 °C) plus all metals (*HT +M*), and high temperature minus iron (*HT −Fe*). Corals in the treatment high temperature minus manganese (*HT −Mn*) became necrotic before the endosymbiotic algae could be harvested. Values of <1 or >1 would indicate either a reduction or increase in tag intensity compared with the control, whereas a value of 1 indicates no change. Statistically significant changes are indicated by shading *red* for fold decrease and *green* for fold increase. *NTD*, no tag detected; *, proteins also detected in coral genome sequences.

sociated with treatments at the higher temperature treatment. Overall, it appeared not to be metal limitation *per se* that influenced TMT tag intensity but rather the maintenance temperature of the coral, regardless of the presence or absence of iron. That is, at the higher temperature, significant increases in TMT tag intensities occurred in five proteins in both HT −Fe and HT +M conditions, an additional four proteins showed significant increases only in HT +M, and a further three proteins only in HT −Fe. Corals in the HT −Mn treatment perished before their symbiotic algae could be harvested.

Protein analysis (supplemental information Data Set 7) gave the presence of antioxidant, metabolic redox, and cytoprotective enzymes (Table I), which were expected to change in response to stress treatments. Surprisingly, only changes in nitric-oxide reductase (Q9FDN7) and a probable thiol peroxidase (Q8NRG3) (supplemental information Data Set 7) could be quantified (Fig. 1), with the TMT tag intensity of the nitric-oxide reductase being significantly repressed in the low temperature treatments. The intensity of the thiol peroxidase TMT tag did not change significantly compared with the pretreatment $t = 0$ proteome in any experimental condition (supplemental information Data Set 7). Interestingly, this protein requires an iron cofactor, as does nitric-oxide reductase, but the TMT tag intensity in the high temperature treatment with iron limitation did not change significantly compared with the unstressed $t = 0$ proteome. Thermal stress enhances the effects of high irradiance on photosystems I and II of the algal symbiont, leading to increased production of ROS (40) having a variety of cytotoxic effects, including DNA damage. Although a variety of DNA repair enzymes were detected in the data set (Table II and supplemental information Data Set 7), surprisingly, only the two proteins Q9Z7A5 and A8EZ44 that belong to the Uvr and Ruv DNA repair systems, respectively, were detected in the quantified data set (Fig. 1) that is potentially linked to damage caused by oxidative stress. The TMT tag intensity of neither protein increased under the experimental stress conditions compared with the unstressed $t = 0$ proteome. Indeed, the tag intensities were significantly reduced under low temperature conditions compared with the unstressed $t = 0$ proteome.

The most extreme increase in protein expression was observed for the viral replication protein E1 (P27551), with a massive 114-fold increase in TMT tag intensity under high temperature, metal-replete conditions (Fig. 1 and supplemental information Data Set 7). The only protein associated with apoptosis that could be quantifiably measured (supplemental information Data Set 7) was the Tax1-binding pro-

TABLE I

Antioxidants, metabolic redox enzymes, and cytoprotective heat shock proteins detected in the Symbiodinium-enriched fraction of Stylophora pistillata

Protein accession number	Protein description
B2SAW2	Alkyl hydroperoxide reductase
P21688	β-Carotene hydroxylase
P29422[a], P12365[a], B1W5Q9, B1W5Q9, B1W5Q9, A1SWM1, B3EBB7, B4SAT7, C0ZSW0, P0C8F0, Q0U324, Q1M498, Q3KE62, Q87WL6, A0K4Q2, O08404, Q8EIV5	Catalase/Catalase-peroxidase
P52649[a]	Cytochrome b_{245} heavy chain
Q5N5B0[a]	Cytochrome b_6-*f* complex iron-sulfur subunit
P50686[a], P07255	Cytochrome *c* oxidase
Q6C0Z6, Q6URB0	Cytochrome *c* peroxidase
O09164, P81527, P09213	Superoxide dismutase
Q3TQB2[a]	FAD-dependent oxidoreductase
Q2GCZ2, A4YER6, Q55318[a], B2GEF7, B2U9D2	Ferredoxin-NADP reductase
P00390[a], P0AC65	Glutathione reductase
Q7N809	Hydroxyacylglutathione hydrolase
Q03751	Cysteine-string protein (HSP-binding)
Q54J68	Heat shock protein, 10 kDa
P22774[a], Q01889, Q05746[a], P12078[a], P08418[a]	Heat shock protein, 70 kDa
P33125[a]	Heat shock protein, 82 kDa
P40292[a]	Heat shock protein, 90 kDa
P41887[a]	Heat shock protein, 90 homolog
Q06068[a]	Heat shock protein, 97 kDa
O94641[a], P31539	Heat shock protein, 104 kDa
P04792[a]	Heat shock protein, β1
Q10284[a]	Heat shock protein, Sks2
Q96VB8	Heat shock protein, Sse1
P38530[a]	Heat shock factor, protein 2
Q42592	L-Ascorbate peroxidase S
A2BQ24, A3PBR8, Q85A72	Light-independent protochlorophyllide reductase
C0IW58	Low redox potential peroxidase
Q84TF4	Monothiol glutaredoxin-S13
Q0MQI7[a], Q08822[a], Q2KIG0[a]	NADH dehydrogenase (ubiquinone) flavoprotein
A1ALP0[a], A1BF33[a], A8ERL5[a], B2V988[a], B3QP57[a], Q0BSK3[a], Q89AU5[a], Q1IPE7[a], Q1IS59[a], P56912[a], A1SE34[a], A0QJV5[a], A4SLN5, Q68WJ7[a]	NADH-quinone oxidoreductase
A6MMH7[a], Q09FR3[a], Q32126[a], Q6EW03[a], Q7YJS8, Q8DLN5	NAD(P)H-quinone oxidoreductase
Q43644[a], Q07500[a], Q2I3H4[a], Q70Y29[a], Q9B8C8[a], P05510[a], P12776[a]	NADH-ubiquinone oxidoreductase
Q9FDN7	Nitric oxide reductase
O48677	Peroxidase
Q5HNJ2, Q8NRG3	Probable thiol peroxidase
B2SK49	Putative NADH dehydrogenase/NAD(P)H nitroreductase
Q58065	Putative NADH oxidase
Q7Q161	Putative oxidoreductase
Q11W68, Q488Q1	Putative reductase
Q9FJD6, Q9LZU9	Putative respiratory burst oxidase
Q82ZW4	Redox-sensing transcriptional repressor rex 2
O81209, Q5ZAJ0, O81210, O81211	Respiratory burst oxidase
Q87L91	Sulfite reductase (NADPH) hemoprotein
Q86GL4[a]	Superoxide-generating NADPH oxidase
Q6P902, O48737, Q655X0, P0AA25[a], Q8CDN6[a]	Thioredoxin
Q69PS6, P52215, Q9ZL18	Thioredoxin reductase
Q10216[a], P0AG84[a]	Uncharacterized oxidoreductase
Q58698	Uncharacterized polyferredoxin-like protein
P81701	Vanadium-dependent bromoperoxidase

[a] Proteins also detected in coral genome sequences.

tein (Q86VP1), which actually inhibits TNF-induced apoptosis (75) and is a known transactivator of viral replication and transformation (76). However, the TMT tag intensity of this protein did not change significantly compared with the pretreatment $t = 0$ proteome under any condition of experimental treatment. Likewise, the only protein associated with exocytosis was a Ras GTPase-activating protein (4Q6PFQ7), the TMT tag intensity of which also did not change significantly compared with the unstressed $t = 0$ proteome (supplemental information Data Set 7).

Despite not finding a greater range of proteins differentially expressed in our experimental treatments in response to ex-

TABLE II
DNA repair enzymes detected in the Symbiodinium-enriched fraction of Stylophora pistillata
None of the proteins were detected in coral genome sequences.

Protein accession number	Protein descriptor
Q180Q8, Q730N8	(6–4)DNA photolyase
C1E3X9	A/G-specific adenine DNA glycosylase repair
Q3MDP1, Q602J1, Q7ZYL5, Q2J2I0, B5ZAV3	AP-base excision repair system
Q9KGL3, Q4JB80	DNA polymerase ϵ
Q92QX2, B3CRA8, B4S2C6, Q3YRC1, O50248, Q8UFV3, Q11NR3, O32158, C4K4K4	Double-strand break repair system
Q9A5I1, A6VXD2, A6VXD2, A7GM37, A7GXS4, A7HVV8, C0QKP5, O33600, P10862, P54560, Q2J6D7, Q3A3F2,Q8R5G2, Q8WZS3, Q91G76, Q96UP6,Q97LQ2	Formamidopyrimidine-DNA glycosylase base excision repair system
Q9ZJ57, Q8K9A9, Q92889	MutS mismatch repair system
Q18BJ4, Q0H8X2, Q0E2Y1, P97313, C0ZZ46, B6EGQ6, B0UWW2, B0KLZ2, P75437, Q4JAM1, Q6HBC2, Q54UC0, Q0SM15, C4LIX8, A9ADC9, A8EZ44, Q4JVD9, P58965, B1IBR6, Q6J6J0	Rec recombination repair system
Q89U80, Q8DML0, Q8J2R3, Q864U1,Q5JGV6, Q5A0W7, Q2NE95, Q2G9X2, B3EG36, B3DRV5, A8AY33, Q8EPI1, Q89B02, Q87Y35, Q6AFJ3, B7HHV4,B2THC7, B0BXK8, B2G8T9, P36592, Q3KKQ1, Q5P3R8	RuvABC recombination repair system
B2UX57, B6JB45, C3LQ59, O09053, O73810, P22706, Q10445, Q5KVB5, Q5L5Q2, Q5QUB8, Q5R4N7, A9KPL0, B4RJQ9, B6YRR8, C5BKM1, O35923, P59971, Q18AN9, Q68XI5, Q6BSB8, Q6L8L5, Q6LMU2, Q6R2P8	UvrABC base excision repair system

perimental stress, our analysis gave an exceptional depth of proteome coverage to reveal a comprehensive profile of proteins relevant to the metabolic interactions of coral symbiosis. Quantification of proteins can be achieved by several nonradioactive isotope labeling approaches, notably by stable isotope labeling and by stable isotope tagging. The former method affords protein labeling by the incorporation of isotopically enriched constituent amino acids in culture (77) or by *in vivo* metabolic labeling in whole organisms (78). Both metabolic labeling and isobaric chemical tagging are generally capable of accurate, precise, and reproducible quantification capable of deep proteome coverage (79). Tagging includes the use of TMTs and isobaric tags for relative and absolute quantitation (iTRAQ). Both approaches involve differential labeling of protein mixtures with isobaric (equal mass) reagents that bond to primary amine groups that fragment during tandem MS into unique reporter ions corresponding to the original labeled samples. The use of labeling with stable isotope-substituted metabolites, such as the approach of stable isotope labeling with amino acids in cell culture (SILAC), is limited in application because not all model biological systems are amenable to these methods. Furthermore, isobaric tagging enables a higher throughput than a stable isotope labeling approach with multiplexing of up to six and eight protein mixtures by TMT and iTRAQ reagents, respectively. Our rationale in choosing TMT labeling at the protein level was to reduce the complexity of the samples to maximize the number of digested peptides that could be resolved in sufficient detail to be identified and quantified on a high throughput analytical platform. However, there was trade-off between gaining the resolution required to separate peptides in sufficient detail to be identified and to achieve quantitative precision. This limitation arises because only lysine and protein N-terminal residues are labeled by TMT reagents. Also, fewer peptides are produced by tryptic digestion because most lysine residues are modified by TMT reagents, resulting in missed cleavages at TMT-labeled lysine sites, thus reducing the efficiency of detection.

Nevertheless, high resolution TMT-labeled protein analysis of the symbiont-enriched fraction of *S. pistillata* has yielded valuable information on the protein structure of coral symbiosis. In addition to the quantified data set, there were a total of 8098 MS/MS events relating to individual peptides that could be separated to allow sufficient amino acid sequence to be used to search Uniprot. Only ~1% of peptides were labeled in this experiment, although this is not uncommon for an experiment of this nature but adequate for peptide detection by the model instrument of LC-MS/MS used. Of the 8098 MS/MS spectra acquired, 7790 peptides could be further assigned a biochemical function based on KEGG gene ontology descriptors (supplemental information Data Set 8, with the peptide sequences for the remaining 308 unassigned proteins listed in supplemental information Data Set 9). The qualitative data set (supplemental information Data Set 8) was

searched for antioxidant enzymes using the KEGG biological process descriptors "oxidation-reduction process" and "cell redox homeostasis," as well as the KEGG molecular function descriptors "oxidoreductase activity" and "peroxidase activity" (Table I). To this table we have also added the cytoprotective heat shock proteins extracted from supplemental information Data Set 8. Proteins associated with repair to DNA following ROS damage were searched in the qualitative supplemental information Data Set 8 using the descriptor "DNA" (Table II). Both the qualitative and quantitative data sets were searched also for effectors of bleaching using the KEGG biological process descriptors "vesicle-mediated transport," "exocytosis," "apoptosis," "autophagy," and the molecular function descriptor "NF-κB" (Table III). While going through the qualitative data set, we noticed a striking number of proteins that are not usually associated with algal metabolism. These included oxygen-binding globins (Table IV), nitrogen fixation and metabolizing enzymes (Table V), and an astonishing complement of biotoxins (Table VI); the last we thought might arise from bacterial consorts or contamination by coral nematocysts (stinging capsules of cnidarians). We were intrigued by the massive increase of protein expression in the viral replication protein E1 (P7551) and viral ankyrin repeat protein (Q9J4Z9) in high temperature treatments (Fig. 1), and a greater complement of viral proteins were uncovered from supplemental information Data Set 8 that are involved in viral replication and protein biosynthesis (Table VII). Photosystem proteins were present as expected for *Symbiodinium*, and the additional presence of phycoerythrin and phycocyanin (Table VIII) gives evidence that our corals harbored cyanobacteria as either consorts (80) or pathogens (81). Interestingly, proteins analogous to photo-system proteins could be found in the coral genome sequences which warrants further investigation. The presence of surface-associated unicellular cyanobacteria also is consistent with a strong indication of nitrogen-fixing enzyme systems (82).

DISCUSSION

Study of the molecular basis for the physiological response of coral-algal symbiosis to environmental stress has centered on transcriptomics using various methods of differential gene expression to assess the genetic response of corals to stress (Ref. 83 and references therein). Those studies used high throughput sequencing to obtain large libraries of expressed sequence tags representing host, symbiont, or holobiont transcripts; smaller scale differential gene expression libraries originating from isolated algal endosymbionts; or cDNA microarray analysis of endosymbiont gene expression *in hospite*. Although these studies have enhanced considerably our understanding of host, symbiont, and holobiont molecular responses during environmental stress, transcriptome analyses are limited in that they do not represent the true phenotype of the symbiosis at the protein level of microbial-host interaction. This is because the relative abundance of transcripts is low and attempts to amplify the sequences by PCR can introduce bias. Furthermore, as in transcription, the translation of mRNA can be controlled by a number of processes, mostly at the level of initiation, either by induction or repression. In marine biology, transcriptomics is further hampered by poor sequence annotation because many organisms of marine origin are under-represented in databases, and it appears from comparative analysis that DNA sequences from marine and terrestrial organisms are highly divergent (84).

A more accurate reflection of biological function may be gained from the proteome because proteins generally are of higher abundance and have a longer half-life within cells than do transcripts. Also, many proteins are post-translationally modified, which cannot be detected in the transcriptome. It has been estimated that 80% of a cell's phenotype can be described by the proteome compared with only 30% by the transcriptome (56). A very limited number of studies have examined the proteome of both symbiotic and aposymbiotic cnidarians using two-dimensional SDS-PAGE analysis (57–62). The success of such attempts was restricted by the applicability of low throughput protein analyses. Herein we report the use of high resolution, quantitative high throughput protein analysis to obtain the first comprehensive proteome expression profile of symbiont-enriched fractions taken from an intact coral holobiont to examine the response of the endosymbiont to varied conditions of environmental stress. Although differential expression of proteins proved low in our experiments, relative yet quantifiable measurements of expressed proteins were attained by use of TMT reagents for protein level amino group labeling to provide an isotopic and isobaric tag enabling coelution of peptides from multiplexed samples during liquid chromatography with sample differentiation provided by MS/MS fragmentation within the mass reporter region. Protein quantities used for TMT labeling were standardized, and all three symbiont replicates obtained from individual coral treatments were analyzed in parallel. The loading of proteins from all treatments and the unstressed $t = 0$ proteome in a single sample eliminated problems arising from variation in the consistency of sample loading, the in-gel digestion process, and LC-MS/MS performance between sample runs, to afford protein expression profiles with a high degree of accuracy.

Many clades of *Symbiodinium* can be grown in pure culture, although often with considerable difficulty. However, exposing these pure cultures to conditions of environmental stress would not necessarily provide a true picture of the response of the endosymbiont *in hospite.* To achieve this, we extracted and rapidly cleaned the *Symbiodinium* fraction obtained from coral that had been exposed to conditions designed to elicit a bleaching response. However, absolute removal of host tissue contamination by repeated washing and centrifugation is nearly impossible given the abundance and robust nature of host nematocysts and phagosomal membranes that typically persist as contaminants. Differentially expressed proteins of this fraction from experimental treatments are presented un-

TABLE III

Major proteins detected in the Symbiodinium-enriched fraction of Stylophora pistillata related to coral bleaching: apoptosis, vesicle transport, and endo/exocytosis

Protein accession numbers	Protein function	Protein description
O04093	Apoptosis	Putative inactive disease susceptibility protein LOV1
O42354[a]	Negative regulation of apoptosis	E3 ubiquitin-protein ligase Mdm2
O60125	Apoptosis	BAG family molecular chaperone regulator 1A
O64789	Apoptosis	Probable disease resistance protein
O92529	Apoptosis	Genome polyprotein
P58801[a]	Positive regulation of apoptosis	Receptor-interacting serine/threonine-protein kinase 2
Q12933[a]	Regulation of apoptosis	TNF receptor-associated factor 2
Q32NG6	Induction of apoptosis	Tumor necrosis factor receptor type 1-associated DEATH domain protein
Q6UXS9[a]	Apoptosis	Inactive caspase-12
Q6ZNE5	Positive regulation of autophagy	Beclin 1-associated autophagy-related key regulator
Q8RXS5	Apoptosis	Probable disease resistance protein At5g63020
Q8W3K3	Apoptosis	Putative disease resistance protein At1g58400
Q8YTC2[a]	Apoptosis	Uncharacterized WD repeat-containing protein Alr2800
Q95KV0[a]	IκB kinase activity	Inhibitor of nuclear factor κB kinase subunit β
Q99KF0	Regulation of apoptosis	Caspase recruitment domain-containing protein 14
Q9BZR8	Regulation of apoptosis	Apoptosis facilitator Bcl-2-like protein 14
Q9FL92	Apoptosis	Probable WRKY transcription factor 16
Q9LVT1	Apoptosis	Putative disease resistance protein At5g47280
Q9QAX1	Apoptosis	Genome polyprotein
Q9SZA7	Apoptosis	Probable disease resistance protein At4g33300
Q86VP1[a]	Inhibits TNF-induced apoptosis	Tax1-binding protein 1
Q3UHC7[a]	Inhibits CHUK-NF-κB signaling	Disabled homolog 2-interacting protein
Q54DK4[a]	ARF GTPase activator activity	α-Protein kinase 1
Q8H100[a]	ARF GTPase activator activity	Probable ADP-ribosylation factor GTPase-activating protein AGD8
O14234[a]	Calcium ion transport	Calcium-channel protein Cch1
Q0VD05	Calcium ion transport	Voltage-dependent calcium channel γ-3 subunit
Q8NC96[a]	Endocytosis	Adaptin ear-binding coat-associated protein 1
Q9UU81[a]	Endocytosis	AP-1 complex subunit γ-1
Q2KJ81[a]	Endocytosis	AP-1 complex subunit μ-1
Q9Y6Q5[a]	Endocytosis	AP-1 complex subunit μ-2
O00203[a]	Endocytosis	AP-3 complex subunit β-1
A2RV61[a]	Endocytosis	GTPase-activating protein and VPS9 domain-containing protein 1
P90761[a]	Endocytosis	Putative stoned B-like protein
Q681Q7	Endocytosis	Uncharacterized protein At1g03900
Q9Z1C7[a]	Guanine nucleotide exchange factor	Rap guanine nucleotide exchange factor 4
Q8C0Q9[a]	Guanine nucleotide exchange factor	Rap guanine nucleotide exchange factor 5
Q06836	Nucleotide exchange from the GDP- to the GTP-bound form	Arf guanine nucleotide exchange factor SYT1
P47102	Nucleotide exchange from the GDP- to the GTP-bound form	ARF guanine-nucleotide exchange factor 1
Q8NEV8	Rab effector protein in vesicle trafficking	Exophilin-5
Q8R3D1[a]	Rab GTPase activator activity	TBC1 domain family member 13
Q8BYJ6[a]	Rab GTPase activator activity	TBC1 domain family member 4
O95759[a]	Rab GTPase activator activity	TBC1 domain family member 8
Q0IIM8[a]	Rab GTPase activator activity	TBC1 domain family member 8B
Q3UYK3[a]	Rab GTPase activator activity	TBC1 domain family member 9
P47709[a]	Rab GTPase binding	Rabphilin-3A
Q9EQZ7[a]	Rab GTPase binding	Regulating synaptic membrane exocytosis protein 2
Q9QXG2[a]	Rab-protein activation	Rab proteins geranylgeranyltransferase component A1
Q8MLZ5	Regulation of small GTPase mediated signal transduction	Ras GTPase-activating protein Gap-2
Q9UJF2[a]	Regulation of small GTPase mediated signal transduction	Ras GTPase-activating protein nGAP

TABLE III—*continued*

Protein accession numbers	Protein function	Protein description
Q3T000[a]	v-SNARE	Synaptobrevin homolog YKT6
Q9R0N5[a]	v-SNARE	Synaptotagmin-5
Q8TDW5[a]	v-SNARE	Synaptotagmin-like protein 5
Q8N4C7	t-SNARE	Syntaxin-19
Q39233	t-SNARE	Syntaxin-21
Q94KK7	t-SNARE	Syntaxin-52
Q9Y2D4[a]	Vesicle docking involved in exocytosis	Exocyst complex component 6B
Q9LXX6	Vesicle docking involved in exocytosis	Probable exocyst complex component 6

[a] Proteins also detected in coral genome sequences.

TABLE IV
Globin hemoproteins in the Symbiodinium-enriched fraction of Stylophora pistillata

Protein accession number	Protein description
P0ABD3[a], O68926	Bacterioferritin
P13578	Extracellular globin-2B
P12549	Globin CTT-VIIB-6
P19645	Hemoglobin subunit α
Q9PVU6	Hemoglobin embryonic subunit α
Q1AGS4	Hemoglobin subunit α-2
P04444	Hemoglobin subunit β-H1
Q94FT8[a]	Nonsymbiotic hemoglobin 3
Q47952[a]	Hemoglobin-haptoglobin binding protein
Q9DGJ0, P02193	Myoglobin
P42578[a]	Yolk ferritin (snail)

[a] Proteins also detected in coral genome sequences.

der "Results"; thus, significant elements of the qualitative expression profile remain to be discussed.

Protein and Peptide Toxins—Despite direct microscopic examination of our cleaned algal isolates to ensure that contamination was removed as far as possible, we acknowledge that this host-derived contamination was evident particularly by the presence of non-dinoflagellate toxins (Table VI) that appear in the qualitative protein data set (supplemental information Data Set 8), which are thought to be derived either from microbial consorts or from the venome of coral nematocysts. To identify contaminating host toxins in our analysis data sets, protein sequences were compared against the very recently available genome sequences for the corals *Acropora millipora* (http://coralbase.org/) and *Acropora digitifera* (85), mindful that these corals are of a different genus and harbor different clades of *Symbiodinium*. To our surprise, we were unable to find homolog toxin sequences encoded in either of the published coral genomes, although a sequences homologous to botulinum toxin substrate was found in the *A. digitifera* genome (supplemental information Data Set 10). It is intriguing that so many in our data set are related to toxins from such dissimilar organisms (*e.g.* bacteria, fungi, invertebrates, and vertebrates). The venoms of many species of cnidarians contain a highly complex mixture of peptides, proteins, phospholipids, phospholipases, glycoproteins, bioactive amines, and carbohydrates that, with the exception of the sea anemones and several dangerous species of jellyfish, are largely uncharacterized (86).

The sequence of one toxin (G9TWG1) from our *Symbiodinium*-enriched coral fraction was similar to the K^+ channel peptide toxin kaliseptine (36 amino acids) obtained from the sea anemone *Anemonia sulcata* (87). Isolated also from this anemone are the kalicludines (58–60-amino acid peptides) that are structurally related to the neurotoxins produced by mamba snakes (88). Further studies on the provenance, biological properties, and evolutionary significance of early metazoan toxins are clearly warranted, especially the botulinum-like zinc-metalloprotease toxins (Table VI) that may serve as virulence agents of coral disease (89). Additionally, marine venoms are a rich source of neuroactive agents; sea anemone toxins are being applied to the treatment of multiple sclerosis (90), whereas analog toxins of a marine cone snail are in clinical use for the suppression of neuropathic pain (91).

Antioxidant and Cellular Redox Enzymes and Globin Hemoproteins—A major mechanism for detoxification of ROS is the production of the antioxidant enzymes SOD and catalase, which have previously been reported in both partners (21, 22), and ascorbate peroxidase, reported only in the algal symbiont (22). All three detoxification enzymes were detected in our data set (Table I), as were other peroxidases and thiol reductases. Not surprisingly, mining of coral sequences for the first three enzymes revealed only SOD and catalase encoded in these genomes, confirming previous experimental findings that ascorbate peroxidase is of algal origin, although a plant-like ascorbate peroxidase gene has been reported in host tissue of the symbiotic cnidarian *Hydra viridis* (92). The only quantifiable proteins that had antioxidant functions were a thiol peroxidase and surprisingly nitric-oxide reductase (Fig. 1), the latter serving as a redox enzyme for NO detoxification. NOS and its encoding gene have previously been detected in only host cnidarians (44, 48) but not their endosymbionts.

NOS transcription is activated by NF-κB, which itself is up-regulated by H_2O_2 arising from stress-induced overproduction by the algal symbiont. By this process, it is thought that excess H_2O_2 of the endosymbiont is released by diffusion to the host cnidarian where NOS induction affects a NO-mediated response of the host to cause the initiation of cni-

TABLE V
Major proteins of nitrogen and purine metabolism in the Symbiodinium-enriched fraction of Stylophora pistillata

Protein accession number	Protein description
A0RHQ8[a], A9WSA8[a], B1W463[a], C5C687[a], Q2FHN5[a], Q5FJC0[a], Q87WP4[a], Q8FT42[a], Q8Y665[a], Q9K8V7[a]	Carbamoyl-phosphate synthase
O61608[a], Q92037[a], Q9TUX8[a]	Nitric-oxide synthase
Q8H157[a], Q9M173[a]	Nitrate transporter
P36859[a] Q7VJT5[a], P43101[a], P49050[a], P22945[a], A8LLY9[a]	Nitrate reductase (NADH)
P32712[a]	Formate-dependent nitrite reductase NrfG
P30667[a]	Nif-specific regulatory protein NifA
B2J5Z9	Nitrogenase-stabilizing/protective protein NifW
P10996[a]	Nitrogen cofactor biosynthetic protein NifE
P26248[a], B7KG76[a] P25768[a]	Nitrogenase iron protein NifH
P00468, P10996, P25313, B7KG76, P26248, P16267[a]	Nitrogenase molybdenum-iron protein NifD
Q9X192[a]	NifU-like protein
P10577, P17429, O13415, P30667, P28349[a]	Nitrogen assimilation regulatory protein
A3LYV8[a], Q0CQ46[a]	Nitrogen permease regulator
P17429[a], O13415[a]	Nitrogen regulatory protein
P26050[a]	Nod factor export ATP-binding protein I
P32288[a], Q42689[a], Q9HU65	Glutamine synthetase
Q0DG35[a]	Glutamate synthase
P46011	Bifunctional nitrilase/nitrile hydratase NIT4
B0TT70[a], Q3IRZ6[a], Q2FEK3[a], Q79VJ3[a], Q8XXT1[a]	Urease
A1U501, Q144D8, Q4KJ05, A6X1Q4, Q1MCW3, Q826R6	Urease accessory proteins
B2G592[a]; Q2FEZ3[a]; Q5DYV8[a]	Purine nucleoside phosphorylase
A7GCI1[a]	Xanthine phosphoribosyltransferase
P77165[a]	Xanthine dehydrogenase
Q8MKJ[a]	Uricase (urate oxidase)

[a] Proteins also detected in coral genome sequences.

TABLE VI
Proteins and peptide toxins detected in the Symbiodinium-enriched fraction of Stylophora pistillata

None of the proteins were detected in coral genome sequences.

Protein accession number	Protein description
Q45894	Botulinum neurotoxin type A (bacterium)
P46081	Botulinum neurotoxin type C1 (bacterium)
P19321	Botulinum neurotoxin type D (bacterium)
Q00496	Botulinum neurotoxin type E (bacterium)
P81242	Nonhemolytic enterotoxin (bacterium)
P55981	Vacuolating cytotoxin autotransporter (bacterium)
Q01886	HC-toxin synthetise (fungal plant pathogen)
Q9TWG1	Potassium channel toxin kaliseptin (sea anemone)
Q9BPG0	Conotoxin Pn-B0151 (marine cone snail)
P0CH23	Conotoxin Pu3.5 (marine cone snail)
Q9XZK2	ω-Conotoxin SO-3 (marine cone snail)
P58917	ω-Conotoxin CVIA (marine cone snail)
P0C8U3	μ-Conotoxin-like SxIIB (marine cone snail)
D5GSJ8	Toxin CpTx1 (spider)
D2Y284	Hainantoxin-XVI-14 (tarantula)
D2Y2G1	Hainantoxin-XI-10 (tarantula)
P0C8D5	Scolopendra toxin (centipede)
Q86QU3	Potassium channel toxin γ-KTx 4.10 (scorpion)
P60209	Potassium channel toxin α-KTx 9.4 (scorpion)
A0ZSK4	Neoverrucotoxin subunit β (reef stonefish)
P0CAR3	Short neurotoxin E1 (coral snake)
A2CKF6	Neurotoxin 3FTx-8a (banded krait)
Q9WVC2	Ly-6/neurotoxin-like protein (mouse)

darian bleaching (44, 47). Although NOS was detected in our data set (Table V), likely as a contaminant, the gene encoding this enzyme was found in the published coral genome sequences, whereas nitric-oxide reductase was not. That TMT tagging is proportional to protein concentration and that nitric-oxide reductase was tagged at high yield suggest this protein is produced by the algal partner to protect against the damaging effects of the nitric oxide generated by the host. Nitric oxide can be produced independently in the symbiont by nitrite reductase (P32712) but intriguingly also via the redox-regulated nitrite reductase activity of the highly conserved heme superfamily of proteins (93, 94) that are abundant in coral endosymbionts (Table IV). These hemoproteins may function as post-translationally activated redox-regulated nitrite reductases that could mediate the NO response to cause cnidarian bleaching. Separate from the hemoproteins of metabolism and photosynthesis, these oxygen-binding globins may serve additionally as O_2 sensors to activate cellular defense pathways by regulating the transcription of protective oxygen-responsive genes (95), particularly those factors activated in response to hypoxia (96), an usual condition of coral tissues during dark respiration (18). Expressed also are the ubiquitous ferritin intracellular iron storage proteins providing iron homeostasis in heme metabolism (Table IV). These proteins are reported to be transcriptionally up-regulated by corals during thermal bleaching, possibly in response to iron release caused by ROS disruption to heme-ferritin binding (97).

TABLE VII

Viral proteins detected in the Symbiodinium-enriched fraction of Stylophora pistillata

None of the proteins were detected in coral genome sequences.

Protein accession number	Protein description
Q8V2M9	A-type inclusion protein, viral reproduction
O93182, P03344, Q00071	Gag polyprotein, viral reproduction
O89940, P0C211, P18042	Gag-Pol polyprotein, viral reproduction
Q6QLN1, Q8JJX1	Nonstructural polyprotein pORF1, viral genome reproduction
P06163, Q66T64, P32530	Phosphoprotein, viral genome replication
P36309, O73556, Q6XW15, Q99D35, P33515, Q85197, Q85197, Q9QEJ5, Q9QAX1, P29152, O92529, O92529, Q66474, P03302, A0AUJ5, Q1X881, P89509, P19724, Q01901, Q83883, P07564, Q04544, Q5UCB8, Q6J3P1, P03303	Polyprotein, viral genome reproduction
Q5NDM9	Protein P2-P3, viral reproductive process
P03168	Protein X, viral genome replication
A7IXI8	Regulation of viral transcription
P36796	Regulatory protein E2, viral reproduction
P0C6U3, Q68772, P19811, P0C6Y5, Q88920, P0C6W4, P0C6V9, P0C6Y4, P0C6Y0, P0C6W0, P0C6V8, O36966	Replicase polyprotein, viral genome replication
P17779, Q8UZB6, P17965	RNA replication protein, viral
Q1HVH8	Protein BOLF1, virion assembly
Q64746	Protein involved in viral genome replication
Q9H4T2, A6NJL1, Q5RJ54, A6QNZ0, Q9Z2K3	Zinc finger proteins involved in viral reproduction
Q9J566	Late transcription factor VLTF-3, viral protein
P29044	Putative RNA-directed RNA polymerase, viral
Q85431, P03594, A8C8X1, Q05318, Q6WB93, Q9IWW8, P31332, Q8JPX5, Q88434, P35341, Q8B0H5, P41357, O91940, P12577, Q6UY63, Q91DR9, A0PJ24, Q6UY61, P13615, Q6XQH7, P27316, P22956	RNA-dependent RNA polymerase, viral
P68966	Late 100-kDa protein, intracellular transport of viral proteins
Q9DUD9	Fusion glycoprotein F0, viral penetration
O10685	Major surface glycoprotein G, viral entry
P17287	Protein Vpr, viral infection
P36278, P07234, Q9QEE7, P33427, P15100	Capsid protein, viral envelope
Q9WC60, P12430, P03388, Q9QBZ8, P05878	Envelope glycoprotein gp160
P36711	Fiber protein, viral envelope
P28882	Hemagglutinin glycoprotein, viral envelope
P03465	Hemagglutinin-esterase-fusion glycoprotein, viral envelope
Q05138, P09510, P06794,	Major capsid protein L1
P26536, P16715	Major core protein P4a, viral polyprotein
Q5VKP0	Matrix protein, viral envelope
P28959	Membrane protein UL43 homolog, viral tegument
P41483, Q83953	Occlusion-derived virus envelope protein
Q9E779, P12436	Outer capsid glycoprotein, viral envelope
P33422	Protein VP6, viral capsid
Q02385, Q0Q466	Viral envelope spike glycoprotein
P06490	Viral capsid assembly protein
Q91HK5, P13561	Viral capsid RNA2 polyprotein
Q02385, Q0Q466	Viral envelope spike glycoprotein
P21945, Q82680, Q7T6S8, P27318, Q27YE3, Q9DK04, Q06927	Viral nucleocapsid

TABLE VIII
Major photosystem proteins detected in the Symbiodinium-enriched fraction of Stylophora pistillata
None of the proteins were detected in coral genome sequences.

Protein accession number	Protein description
Q9MSC2, A2C057	Photosystem Q(B) protein
A6MMC3	Photosystem I assembly protein ycf3
Q3AZ40	Photosystem I assembly protein ycf4
O04006	Photosystem I reaction center subunit VI
P17229	Photosystem I reaction center subunit IX
Q6EW23	Photosystem II reaction center subunit H
P51874	Peridinin-chlorophyll a-binding protein
P51873	Peridinin-chlorophyll a-binding protein 2
Q01922	R-phycoerythrin β chain
P28557, P0032	C-phycocyanin α chain

DNA Repair Enzymes—Although exposure to sunlight is an absolute requirement for maintaining phototrophic symbiosis in reef-building corals, excessive exposure can lead not only to direct damage of UV-sensitive cellular components, but in combination with thermal stress can lead to damaging or catastrophic effects characteristic of bleaching from excessive production of ROS. The damaging effects of UV are especially pronounced at the structural level of DNA. All of the main elements ascribed for DNA repair from damage caused by UV exposure, including that indirectly from ROS production, were detected in this study, including photolyase, base excision repair, and recombination mechanisms (Table II). Although we note that UV radiation was not a factor in our experiments, these findings suggest either that elements of the cellular UV damage control metabolism (stochastic) remain active weeks (>4) after exposure or that their presence is mainly attributable to the ROS generated from excess electron flow in photosynthesis under the elevated level of PAR used during the experiment. It is noteworthy that we found significant repression of the UvrABC system excinuclease B protein (Q9Z75A) under low temperature, metal-free conditions (Fig. 1), such being a highly conserved DNA repair enzyme that requires manganese ions.

Enzymes of Nitrogen Fixation—Evident in our data (Table V) are proteins associated with nitrogen fixation, which is not known in algal endosymbionts such as *Symbiodinium*, and with the exception of NifU, these proteins could not be found encoded in coral genomes, suggesting either that *Symbiodinium* can fix dissolved nitrogen or more likely that these nitrogenases are from coral-associated cyanobacteria (80, 81) or from diazotrophic bacteria (predominantly γ-proteobacteria) that in coral are strongly correlated with dinoflagellate abundance (98), suggesting a tight physiological relationship between these heterotrophic and phototrophic partners. Present also (Table V) is a nodulation factor export ATP-binding protein (P26050), which may provide molecular recognition for this bacteria-dinoflagellate affinity, as is required for sympathogenesis of N_2-fixing rhizobia in plant legumes (99). This bacterial association may be endosymbiotic (as reported in Ref. 80) or instead may include surface-associated cyanobacteria that fix nitrogen at night when photosynthetic production of nitrogenase-destructive O_2 has ceased (100).

Enzymes of Nitrogen Metabolism—Nitrogen assimilation is essential to metabolic interactions between resident algal endosymbionts and their cnidarian hosts (101). Ammonium, nitrate, and free amino acids are taken up by cnidarians, and nitrogen-availability and uptake have been linked to increases in endosymbiont population densities (102–104). Essential amino acid translocation from the symbiotic algae to the animal host is a core element of cnidarian nitrogen-recycling (105). The coral host accumulates waste urea in its tissues as an important store of nitrogen for translocation to its symbiotic partners (106), providing NH_3 as a nitrogen source for photosynthesis-based anabolism on release by urease enzymes, which are plentiful in the *Symbiodinium*-enriched fraction of *S. pistillata* (Table V). Glutamate synthase and glutamine synthetase, both implicated in ammonia assimilation in *Symbiodinium* (see Ref. 107, pages 178–179 for a brief overview), are present in our database (Table V). Clode *et al.* (108) have shown that accretions of uric acid, thought previously to be calcium oxalate (109), are deposited throughout the algal cytosol and within vacuoles of *Symbiodinium* spp. to provide a store of nitrogen when normal routes of cnidarian nitrogen-availability are limited. Additionally, uric acid is a strong reducing agent and potent antioxidant, reacting freely with oxyradicals but not H_2O_2. Uric acid is an end product of purine catabolism by xanthine oxidase from xanthine and hypoxanthine while producing both superoxide anion and hydrogen peroxide. We did find in our data set xanthine dehydrogenase (Table V), which is a xanthine oxidoreductase enzyme that is converted to xanthine oxidase by reversible sulfhydryl oxidation or by irreversible proteolytic modification (110). Present also (Table V) is uricase (urate oxidase) that might be utilized to extract nitrogen stored as uric acid for algal metabolism.

Proteins of Apoptosis, Vesicular Transport, and Endo/exocytosis—There are five separate cellular mechanisms postulated for the loss of algal endosymbionts from cnidarian host tissues in the thermal stress response of coral bleaching: *in situ* symbiont degradation and digestion, symbiont exocytosis, host cell detachment, host cell necrosis, and host cell and symbiont apoptosis (reviewed in Ref. 16). Although no single mechanism of bleaching may be applicable to all conditions of stress causing cnidarians to bleach, it is widely held that cellular changes associated with bleaching are a result of host cell apoptosis. Although ample evidence obtained under controlled experimental conditions supports this view, evidence from the environment suggests that the rates of apoptosis might only reflect that expected from normal cell cycling, as indicated by Starcevic *et al.* (53) and likewise noted by Pernice *et al.* (29) on up-regulation of Bcl-2 as an anti-apoptotic response followed by a decrease in caspase-dependent apoptosis soon after the initial onset of coral bleaching. In con-

trast, algal endosymbionts may exit host cells under moderate stress early in the bleaching process, after which induced apoptosis comes only as a terminal response to prolonged and severe stress for initiation of cell death (13, 14). We found no strong evidence for proteins that mediate apoptosis in this study, and indeed only inactive forms of apoptotic mediators or positive regulators were detected (Table III). Interestingly, none of these proteins were detected as being significantly enhanced by thermal treatment in the quantitative data set (Fig. 1), including Tax1 (Q86VP1.2), which is an inhibitor of NF-κB-induced apoptosis, even though the host corals exhibited signs of early tissue necrosis immediately prior to harvesting. However, the TMT tag intensity of this protein was not significantly different in any of the treatments compared with the $t = 0$ base-line proteome prior to stress (supplemental information Data Set 7). The significant decreases in dark-adapted F_v:F_m that occurred in all treatments in the present study (supplemental information Data Set 3), similar to those seen in our earlier work (54), indicated moderate bleaching under our experimental conditions, but these proteomic data suggest that apoptosis was not a contributing cellular mechanism.

Histological examination establishes that coral host cells are not significantly degraded during bleaching (25), and those intact dinoflagellates *in hospite* are released by exocytosis from the endoderm during coral bleaching (5, 52). Consistent with exocytosis of endosymbionts, we have previously found transcriptomic evidence for differential expression of genes encoding not only Rab-like proteins that are known to regulate vesicle transport in eukaryotes, but also SNARE and calcium transporters that direct exocytosis (53). In this study we provide proteomic evidence for the existence of proteins required for exocytosis, which are expressed during the early stages of coral bleaching. Indeed, the TMT tag intensity of the GTPase activator for calcium binding Rab proteins (Q3UYK3) at the higher temperature is not statistically different from the $t = 0$ base-line proteome prior to stress but is less in the low temperature treatment than at the higher temperature (Fig. 1).

It is becoming increasingly clear from numerous studies on microbial pathogenicity that intracellular pathogens can mediate their own entry into and exit from host cells or lysosomes by encoding their own SNARE proteins (111, 112). These SNAREs can both enhance and inhibit exocytosis (113). Furthermore, an emerging theme (114) provides that SNARE protein function is regulated by complexation with cysteine string protein α (Q03751 in Table I; note that this protein is not encoded in the coral genomes available to search) and Hsc70, a cognate member of the 70-kDa family of stress-induced heat shock proteins, which is a required chaperone of SNARE assembly for exocytosis to occur (115). A range of these proteins (Table III) can be detected when supplemental information Data Set 4 is searched, and opposing vesicular and target SNARE proteins that are necessary for vesicle transport appear to be encoded either by the host (vesicular SNARE, for example, synaptotagmin proteins Q9R0N5 and Q8TDW5) or symbiont (target SNARE, for example, syntaxin proteins Q8N4C7, Q39233, and Q94KK7). We posit that under conditions of stress, heat shock protein chaperones cysteine string protein-Hsc70-SNARE assembly utilizing target SNARE proteins of the endosymbiont, enabling stress-sensitive endosymbionts to mediate their own exit from the host, which departs from customary thinking that the host solely expels the symbiont. This model provides a molecular mechanism for symbiont shuffling (116, 117) whereby thermally sensitive *Symbiodinium* clades are self-displaced during coral bleaching, allowing residual symbionts of a heat-resistant clade to repopulate the symbiosis.

Viral Proteins—A remarkable finding from this quantitative study of the proteome expression profile of the endosymbiont-enriched fraction of coral symbiosis was the massive 114-fold increase in viral replication protein E1 levels that occurred under metal-replete conditions at high temperature (Fig. 1). A review of the qualitative data (supplemental information Data Set 8) for viral proteins reveals a comprehensive complement of proteins required for viral replication, production of new viral particles, and viral-host interactions (Table VII), especially RNA-dependent polymerase indicating the presence of negative strand RNA viruses, which are present in seawater and in marine virus-host assemblages (118). It would seem from the proteomics results that virulence is high during coral stress, which has been reported previously (119–121) when trace metals are replete. Viral replication requires new viral particles to be transmitted to new host cells; these processes include viral budding or host cell lysis in either the infected coral tissue or its endosymbionts. Large scale perturbation of the host cell membrane or host cell lysis has been associated with severe coral bleaching in the past. We suggest that coral recovery post-bleaching not only depends on the retention (or acquisition) of heat tolerant endosymbionts but must also reduce the viral load of the coral to prebleaching levels. Although viruses have been implicated as a cause of coral disease and bleaching (122), the complexity of coral-viral interactions is poorly understood (123), particularly in connection with environmental change (124), which clearly warrants future investigation. Our proteomics data thus provide unique insight to how coral endosymbionts and their microbial consorts respond to conditions of elevated temperature, with and without trace metal limitation, affecting a significant loss of photochemical efficiency in the bleaching response of *S. pistillata*.

In summary, quantitative high resolution TMT-labeled protein analysis of a symbiont-enriched fraction of *S. pistillata* has yielded valuable information on the protein structure of coral symbiosis. Although the sample proteome has revealed a vast complement of proteins involving the metabolic interactions of coral symbiosis, it is the unexpected finding of a massive increase in a key viral replication protein that highlights the po-

tential significance of the coral virome in coral bleaching and disease progression caused by environmental stress. Quantitative high resolution protein analyses in future studies of the intact coral symbiome shall reveal more of the complex and interactive processes that regulate the function of cnidarian symbioses.

Acknowledgments—W. Dunlap gratefully thanks the Institute of Pharmaceutical Science at King's College London for appointment as Honorary Professor. We gratefully thank the master and crew of the RV Cape Ferguson for shipboard field support. Christopher Rigaud, Diving Safety Officer of the University of Maine, provided refresher training for M. Wells and M. Shick. In using the genome data from *A. millipora* (http://coralbase.org/), we acknowledge that these sequence data were produced by the Australian Genome Research Facility and the Australian Research Council Centre of Excellence for Coral Reef Studies in collaboration with the user community.

* This work was supported by National Science Foundation Grant 0648478 (Biological Oceanography), by the National Institutes of Health Research Biomedical Research Centre for Mental Health (King's College London, UK), by Ministry of Science, Education and Sports, Republic of Croatia Grant 058-0000000-3475, and by the Australian Institute of Marine Science. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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