MARINE BIOLOGY

Heat-evolved microalgal symbionts increase coral bleaching tolerance

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Coral reefs worldwide are suffering mass mortalities from marine heat waves. With the aim of enhancing coral bleaching tolerance, we evolved 10 clonal strains of a common coral microalgal endosymbiont at elevated temperatures (31°C) for 4 years in the laboratory. All 10 heat-evolved strains had expanded their thermal tolerance in vitro following laboratory evolution. After reintroduction into coral host larvae, 3 of the 10 heat-evolved endosymbionts also increased the holobionts' bleaching tolerance. Although lower levels of secreted reactive oxygen species (ROS) accompanied thermal tolerance of the heat-evolved algae, reduced ROS secretion alone did not predict thermal tolerance in symbiosis. The more tolerant symbiosis exhibited additional higher constitutive expression of algal carbon fixation genes and coral heat tolerance genes. These findings demonstrate that coral stock with enhanced climate resilience can be developed through ex hospite laboratory evolution of their microalgal endosymbionts.

INTRODUCTION

Coral populations globally have deteriorated over the past two to three decades due to climate change–related marine heat waves (*1*). For example, coral cover on the Great Barrier Reef (GBR) decreased by about half due to summer heat waves in 2016 and 2017 (*2*, *3*), followed by an 89% drop in coral larval recruitment in 2018 (*4*). Although corals are adapting to changing environments (*5*), the rapid coral loss indicates that the speed of climate change outpaces the natural rate of coral adaptation (*4*). This realization has spurred the exploration of assisted evolution approaches targeted at increasing coral thermal tolerance and reducing the incidence of coral bleaching (*6*). Coral bleaching is the loss of the algal endosymbionts in the family Symbiodiniaceae from the coral tissues. The algae provide the coral with most of their nutrition via translocation of photosynthate, and the loss of algal symbionts—coral bleaching—leads to starvation and coral mortality if the symbiosis is not reestablished. Assisted evolution approaches aimed at increasing coral thermal tolerance may contribute to the persistence of coral reefs until global warming is halted (*6*, *7*).

It is widely documented that coral thermal tolerance is largely dependent on the physiological tolerance of their associated Symbiodiniaceae (*8*). The alga's thermal tolerance in vitro has been shown to increase over as few as ~40 asexual generations through exposure to elevated temperatures, also known as directed, laboratory, or experimental evolution (*9*, *10*). However, when one of the heat-evolved algal strains was reintroduced into coral recruits, no significant benefits were observed for the thermal tolerance of the holobiont (i.e., the coral host and its associated microbiota) (*9*). Here, we show that other heat-evolved strains derived from the same wild-type clone can increase heat-induced bleaching tolerance of coral larvae, and we identify some of the adaptations that underpin enhanced thermal tolerance.

To improve algal thermal tolerance, we exposed clonal strains of a common pan-tropical algal symbiont of reef-building corals to elevated temperatures (31°C) for 4 years in the laboratory (~120 asexual generations). After subsequent acclimation of the cell cultures to 27°C for 6 weeks, we compared the thermal tolerance of the 10 heat-selected strains (SS1 to SS10) to 2 wild-type strains (WT1 and WT2), which were derived from the same clonal culture but maintained at 27°C for 4 years (schematic of experimental design in fig. S1 and table S1). Performance during acute heat exposure of 31°C for 3 weeks in vitro and 1 week in hospite was assessed by examining (i) temporal changes in algal cell densities, to assess growth, and (ii) photosynthetic health using a number of parameters measured with pulse-amplitude modulation chlorophyll fluorometry (*11*); (iii) the amount of reactive oxygen species (ROS) that leaked out of the algal cells; and (iv) the algal and coral transcriptomes of larvae inoculated with a subset of the wild-type or heat-evolved strains at ambient temperature.

RESULTS AND DISCUSSION

All 10 heat-evolved strains were more tolerant to elevated temperature in vitro than the 2 wild-type strains, as indicated by an increase in cell densities of ~66% over the three weeks at 31°C, whereas the wild-type (WT) strains showed a ~79% decrease in cell densities (Fig. 1A and fig. S2; generalized linear model $P_{GLM} < 1 \times 10^{-6}$ for SS versus WT strain, raw data and database S1). Photochemical stress was minimal during exposure to elevated temperatures in the heat-evolved strains, as shown by stable and relatively high F_v/F_m values in 8 of the 10 heat-evolved algae (strains SS2 to SS9, with only a slight decrease for SS1 and SS10; Fig. 1B). Conversely, the two wild-type strains showed signs of photoinhibition manifested by significantly decreased F_v/F_m values (~42%; Fig. 1B and fig. S3).

To further examine the physiological responses to acute heat exposure, we measured the effective quantum yield of photosystem II ($\Delta F/F_m'$) and calculated the relative electron transport rate (ETR) and proportion of open photosystem II reaction centers [photochemical quenching (qP)], measurements related to the overall photosynthesis rate, and efficiency of downstream dark reactions, such as carbon fixation (*11*). In addition, we calculated a coefficient

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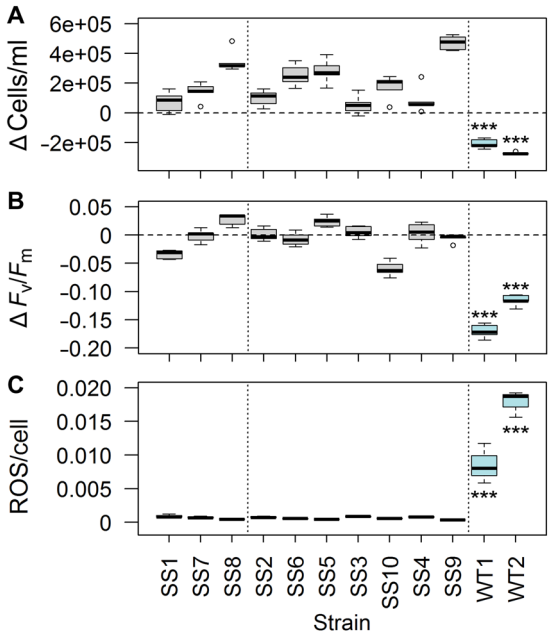

Fig. 1. In vitro thermal tolerance assessment of heat-evolved (SS) and wild-type (WT) algal symbiont strains at 31°C. ΔValue differences (end-beginning, 21 days) in (**A**) cell densities in culture and (**B**) maximum quantum yield of photosystem II. (**C**) ROS secreted into culture medium after 21 days, measured in fluorescence and normalized to cell numbers. Box colors represent (gray) heat-evolved strains and (blue) wild-type strains. Whiskers, maximum and minimum value; boxes, first and third quartile; line, median; $n = 5$ for each group; *Significant difference to the three heat-evolved strains SS1, SS7, and SS8 (at $P_{GLM} < 1 \times 10^{-6}$, generalized linear model, database S2).

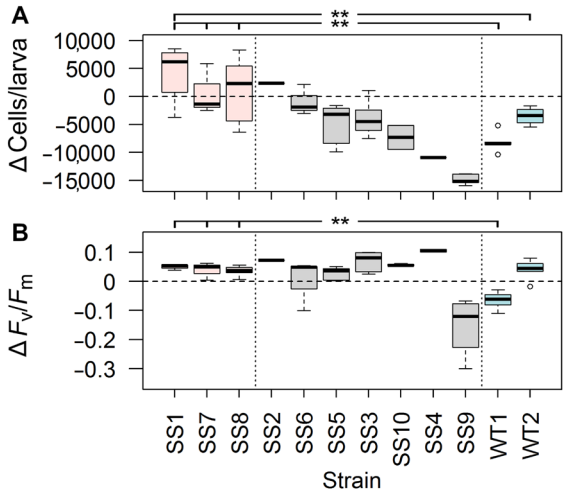

Fig. 2. In hospite comparison of coral larvae bleaching tolerance between heat-evolved (SS) and wild-type (WT) algal symbiont strains at 31°C. ΔValues (end-beginning, 7 days) are displayed for (**A**) cell densities per larva and (**B**) maximum quantum yield of photosystem II (F_v/F_m). Box colors: red, heat-evolved strains that confer their thermal tolerance to the coral holobiont; gray, heat-evolved strains that do not confer thermal tolerance; blue, wild-type strains. Larvae biological replicates at beginning/end; cell densities: SS1, $n = 5/4$; SS2, $n = 4/1$; SS3, $n = 6/6$; SS4, $n = 5/1$; SS5, $n = 5/5$; SS6, $n = 5/3$; SS7, $n = 5/3$; SS8, $n = 5/5$; SS9, $n = 5/5$; SS10, $n = 5/4$; WT1, $n = 6/6$; WT2, $n = 5/5$. F_v/F_m: SS1, $n = 4/4$; SS2, $n = 3/1$; SS3, $n = 5/6$; SS4, $n = 5/2$; SS5, $n = 4/5$; SS6, $n = 4/3$; SS7, $n = 5/3$; SS8, $n = 5/5$; SS9, $n = 4/4$; SS10, $n = 3/3$; WT1, $n = 3/6$; WT2, $n = 4/5$. Because of mortality of SS2 and SS4 holobionts, we had only one biological replicate at the end of the heating period; strains were excluded from statistical analyses. Whiskers, maximum and minimum value; boxes, first and third quartile; line, median. *Significant difference of wild-type strains to the respective heat-adapted strains SS1, SS7, and SS8 (generalized linear model, database S2).

for nonphotochemical quenching [$qN = (F_m - F_m')/(F_m - F_o)$] because this photoprotective mechanism can prevent the formation of excessive levels of damaging free radicals, such as ROS, by dissipating excessive energy (*12*). All heat-evolved strains maintained stable $\Delta F/F_m'$, qP, ETR, and a relatively constant energy dissipation through qN, with all values being significantly higher compared to those of the wild-type strains (figs. S3 to S5, P_{GLM} significant differences for strain comparisons; database S1). In contrast, both wild-type strains decreased $\Delta F/F_m'$ and ETR by 94% and showed a 100% decline to a qP of zero, with WT1 also showing a decline in qN of ~75%. These results suggest that the functionality of photosystems, nonphotochemical quenching, and downstream dark reactions in the selected strains were not affected by the elevated temperatures but were negatively affected in the wild-type strains.

Because coral bleaching can be triggered by excessive amounts of ROS that leak from the symbiont into the host tissue (*13*), we examined the secretion of ROS from the algal cells into the culture medium with a CellROX fluorogenic probe. The secretion of ROS into the culture media by the heat-evolved strains was minimal, whereas the wild-type strains exhibited ~13- to 28-fold higher levels of extracellular ROS at the end of the heating period (Fig. 1C and fig. S7, A and B). Thus, reduced ROS secretion was a common phenotypic trait of the heat-evolved strains and response to long-term exposure to elevated temperatures in vitro. We therefore hypothesized that the heat-evolved strains would also increase the bleaching tolerance of the coral holobiont once they reestablished as coral symbionts.

To test this hypothesis, we inoculated coral larvae with 1 of the 12 algal strains and exposed them to 31°C for 7 days, following an acclimation period of the holobiont for 10 weeks at 27°C. The acclimation period started with the algal inoculation of the larvae and ended when algal cell densities in the larvae were similar in all treatments ($1.0 \pm 0.5 \times 10^4$ cells/larva; fig. S7C). Our criteria for enhanced bleaching tolerance of the holobiont at 31°C were (i) stable or increasing algal cell densities and (ii) maintenance of F_v/F_m (absence of photochemical stress). Algal cell densities increased by ~26% on average in three heat-sensitive holobionts only over the one week at 31°C ($P_{GLM} < 0.01$ for SS1, SS7, and SS8 versus WT1; database S1). All other holobionts decreased in algal cell densities. Various levels of bleaching were observed for larvae harboring each of the other heat-evolved strains, whereas the two wild-type strains lost ~61 and ~30% of their algal symbionts, respectively (Fig. 2A and fig. S7C). F_v/F_m values were maintained in the heat-evolved holobionts showing significantly higher values compared to the decreased F_v/F_m values of WT1, except SS9 (Fig. 2B and fig. S7D; significant comparisons: $P_{GLM} < 0.01$ for SS1, SS3, SS5, SS7, SS8, and SS10 versus WT1; $P_{GLM} < 0.05$ for SS6 and SS9 versus WT1; database S1). Comparison of the algal cell densities and F_v/F_m values showed no significant differences between larvae inoculated with any of the SS strains and those inoculated with WT2. However, the cell density data for larvae inoculated with SS1, SS7, and SS8 were stable or increased during heat stress, whereas cell densities decreased in WT2. These findings demonstrate that a considerable level of physiological variability arises among biological replicates after long-term culturing, but it also shows that larvae in symbiosis with SS1, SS7, and SS8 had a clear trend consistent

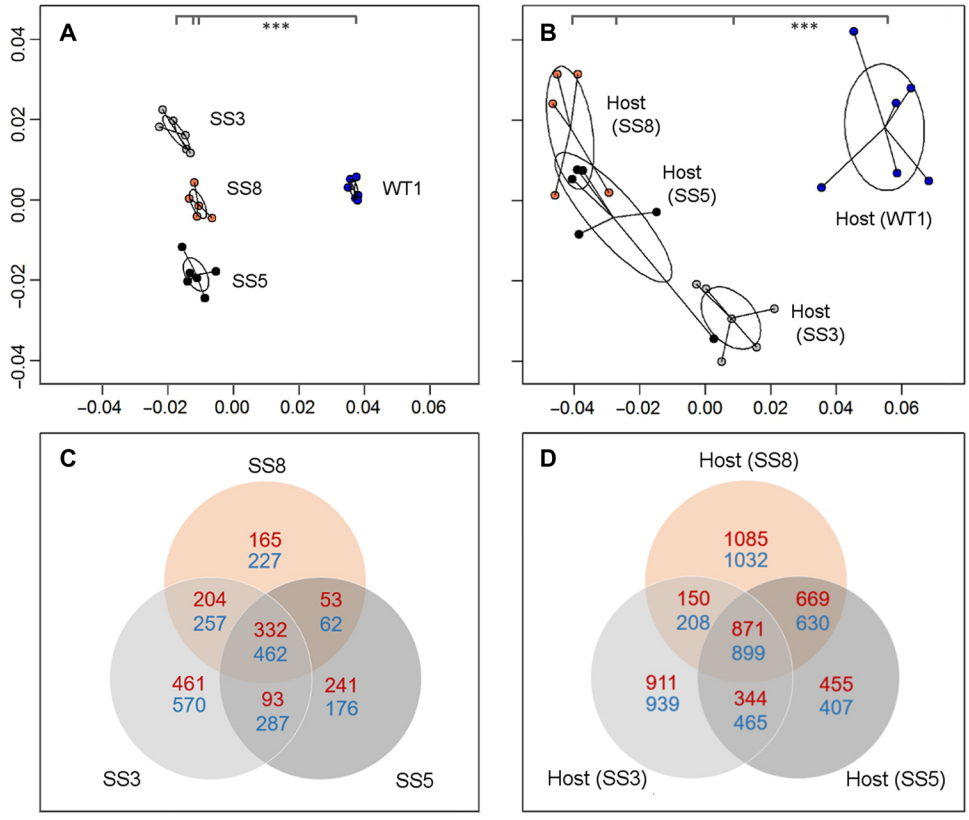

Fig. 3. Gene expression patterns of coral holobionts. Multidimensional scaling plots illustrate the general transcriptomic responses of (**A**) the algal symbionts and (**B**) their respective coral hosts at rest temperatures of 27°C. *Significant differences at $P < 0.001$ between holobionts with heat-evolved and wild-type endosymbionts based on a permutational multivariate analysis of variance using distance matrices (adonis) with Tukey post hoc comparisons (database S3). Venn diagrams show the shared and unique DEGs for both algal symbiont (**C**) and host (**D**) in symbiosis compared to WT1. Red numbers indicate up-regulated genes, and blue numbers indicate down-regulated genes, both at <0.05 FDR, $n = 6$ per strain, SS8 $n = 5$, each replicate with 10 pooled larvae.

with enhanced bleaching tolerance. Our results confirm previous findings that strain SS4 is unable to confer bleaching tolerance to the coral holobiont (*9*), because larvae inoculated with this strain showed high levels of mortality in our experiments.

We explored the mechanisms behind the differential bleaching tolerances in four coral-algal combinations by examining the gene expression profiles of the four holobionts before heat exposure to assess normal functioning (RNA-seq raw data and bioinformatics in databases S2 and S3). One was a heat-tolerant holobiont with the heat-evolved SS8 algae, two were heat-sensitive holobionts with the heat-evolved SS3 and SS5 algae, and the fourth was the heat-sensitive holobiont with WT1 algae. Despite the relatively short evolutionary time separating the algal strains (~8 years), we found considerable differences in their gene expression profiles in hospite (Fig. 3 and fig. S8A). We subsequently examined patterns among the commonly up- and down-regulated genes in the SS strains compared to WT1. Ranked-based gene ontology (GO) analysis with adaptive clustering (*12*) showed that the genes that were commonly down-regulated in all three heat-evolved strains were enriched for GO terms implicated in photosynthesis, such as chlorophyll binding, the thylakoid membrane, and proton transmembrane transport (no clear overall pattern was observed for the commonly up-regulated algal genes). This down-regulation of photosynthesis in hospite complements the active photoprotective mechanisms observed in vitro and likely contributes to the enhanced thermal tolerance of the heat-evolved strains.

The differentially expressed genes (DEGs) and enriched GO terms in SS8 highlight the adaptive changes of the holobiont with enhanced bleaching tolerance. In symbiosis, SS8 had 165 uniquely up-regulated genes and 227 uniquely down-regulated genes at <0.05 false discovery rate (FDR) compared to WT1, SS3, and SS5 (Fig. 3). Among the up-regulated genes were significantly enriched GO categories implicated in carbon fixation and glyoxylate metabolic processes (photorespiration) (fig. S9A, table S2, and database S4), both linked to the dark reaction of photosynthesis through the Calvin-Benson cycle and the synthesis of high-energy glucose molecules (*14*). Two specific genes involved in this association encoded ribulose-bisphosphate carboxylase (RuBisCO) and phosphoglycolate phosphatase (photorespiration) (Fig. 4A). They were only expressed up to 2.4-fold higher in SS8 compared to WT1, but this is consistent with the low-fold gene expression differences observed among in hospite Symbiodiniaceae (*15*, *16*). In cyanobacteria, an increased expression of RuBisCO can compensate for reduced photosynthesis rate (*17*). Further, heat stress has been shown to decrease CO_2 fixation (*18*) and RuBisCO activity in Symbiodiniaceae (*19*, *20*), and in some coral species, the inhibition of the Calvin-Benson cycle can contribute to coral bleaching (*21*, *22*). We therefore hypothesize that the increased expression of genes encoding enzymes associated with the Calvin-Benson cycle, in combination with reduced photosynthesis and active photoprotection mechanisms, contributed to the enhanced bleaching tolerance of the larvae-SS8 symbiosis. Because the progenitor strain was a monoclonal

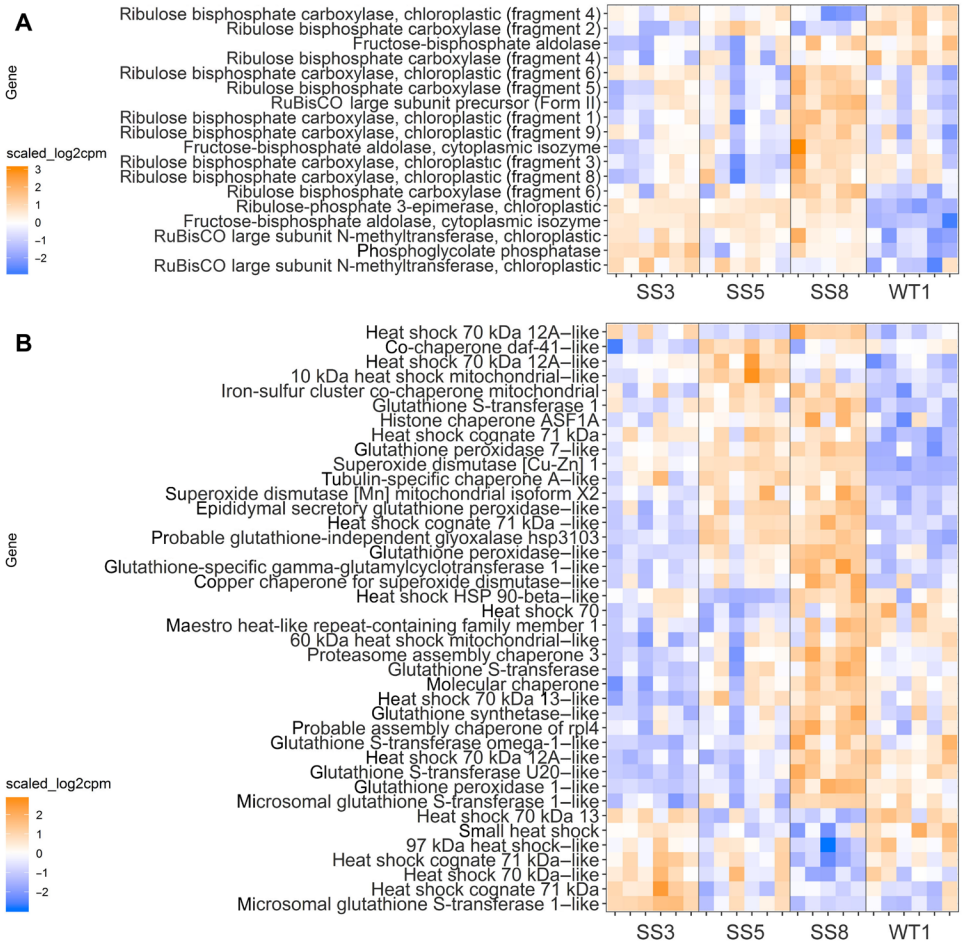

Fig. 4. Relative expression of genes involved in specific physiological functions. Genes differentially expressed (FDR < 0.05) in holobionts containing laboratory-evolved symbionts versus WT1 symbionts. (**A**) Symbiont genes coding for parts of Calvin-Benson pathway (RuBisCO, phosphoglycolate phosphatase, fructose-bisphosphate aldolase). (**B**) Host genes coding for stress-related proteins (such as glutathione *S*-transferases, heat shock proteins, and superoxide dismutases).

culture, it is unlikely that selection on preexisting genetic diversity contributed to the enhanced thermal tolerance. Therefore, we conclude that somatic mutations and/or epigenetic changes over the 4 years at 31°C have led to different levels of thermal resistance.

Gene expression patterns of the coral larval host were also distinct from one another and correlated by the type of algal strain they harbored (Fig. 3 and fig. S8B). Coral larvae in symbiosis with strain SS8 had 1085 and 1032 up- and down-regulated genes, respectively (Fig. 3), with 16 significantly enriched GO categories all involving down-regulated genes (fig. S9B). These GO categories included G protein–coupled receptor (GPRC) signaling pathways, potassium transmembrane transport, and proteins related to dendrites, integral components of membranes, and protein homooligomerization. The relationships of these enrichments to the phenotype are unclear, but we note that some functionally similar GPCRs can be related to metamorphosis and settlement in a distant coral relative, *Leptastrea purpurea* (*23*). In addition to these global analyses, we interrogated the symbiont and host data for specific genes known to be involved in the heat stress response and bleaching tolerance, such as glutathione *S*-transferases, superoxide dismutases, peroxidases, heat shock proteins, and other chaperones (*24*). Each of the four sorts of holobionts showed a unique expression profile of heat stress genes. Although the heat-evolved algal symbionts did not show increased expression of known heat stress response genes, the coral host in symbiosis with SS8 showed up-regulation of such genes compared to larvae harboring SS3, SS5, and WT1 (Fig. 4B and fig. S10). Because the transcriptomes were obtained from holobionts before heat stress, these data suggest that SS8 elicits a potential "front-loading" response in the host, which may be beneficial for coping with elevated temperatures. Front-loading responses have been reported previously for related coral species, including heat shock and antioxidant genes (*25*, *26*).

In conclusion, 4 years of experimental evolution resulted in an expansion of in vitro algal cell growth and photochemical efficiency capacities toward 31°C, whereas the optimum growth and photosynthesis capacity remained close to ambient temperatures. These findings suggest that the use of heat-evolved strains can be suitable for coral reef restoration because marine heat waves are summer phenomena with ambient temperatures occurring for the remainder of the time. However, further research is required to examine whether the enhanced heat tolerance of the laboratory-evolved strains is maintained in the field under long-term exposure to ambient temperature (i.e., outside the summer season). The comparison of closely related strains of algal endosymbionts demonstrates that, in addition to reduced algal secretion of ROS, specific adaptations are

required to enhance the bleaching tolerance of the coral-algal symbiosis. These adaptations include a higher expression of algal symbiont genes involved in carbon fixation and higher levels of constitutive expression of coral host heat-stress genes. Our findings provide compelling evidence that heat-induced bleaching tolerance of coral can be increased through long-term heat exposure of cultured algal endosymbionts, followed by reintroduction into coral. Coral stock with enhanced climate resilience provides an opportunity to assist corals in coping with climate change until global warming is halted.

MATERIALS AND METHODS

Experimental design and heat selection

Dinoflagellates of the species *Cladocopium goreaui*, a haploid single-celled microalga and endosymbiont of corals, were isolated as described by Chakravarti *et al.* (*9*). In brief, a heterogeneous *C. goreaui* cell mixture was isolated from an *Acropora tenuis* coral colony collected at Magnetic Island, central GBR in 2010. Subsequently, a monoclonal subculture was established in the laboratory, kept at 27°C in 0.2-µm filter-sterilized seawater with added IMK nutrients (Nihon Pharmaceutical Co.) and at a light intensity of 65 ± 10 µmol photons m/s with a 14:10 light:dark cycle (Sylvania FHO24W/T5/865 light tubes) at the Australian Institute of Marine Science. Twelve replicate subcultures were established from the *C. goreaui* monoclonal progenitor culture, each with a starting cell density of 300,000 cells/ml. Ten strain replicates were then subjected to a thermal selection experiment in the form of a stepwise temperature increase from 27° to 31°C, a so-called ratchet protocol. The technique applies a selection pressure, here increased temperature, to maximize mutations and thermal adaptation of the microalgae to the new culturing conditions (*27*). Each temperature step at 28° and 30°C lasted 1 month. The four strains with the highest growth rates were subcultured and subjected to the next temperature step, resulting in 10 thermally selected strains (SS) according to the experimental details of Chakravarti *et al.* (*9*) (table S1). The selected strains were then maintained continuously at 31°C, whereas the wild-type (WT) strains were always kept at 27°C. For general maintenance and subculturing once a month, 1 ml of a culture with about 1×10^6 cells/ml was transferred into 24 ml of fresh medium. The current study was conducted ~4 years after the start of the first stepwise temperature increase (table S1A). Because of the different growth rates, the number of generations for SS and WT strains was ~120 and ~225, respectively. Because mutations occur randomly in each algal strain (*27*), we considered each strain (table S1) as a biological replicate that is independent from the other strains (*9*). The treatment temperature levels of 27° and 31°C are comparable to environmental seawater temperatures on the GBR. The symbiotic algae have been isolated from around Magnetic Island in the central GBR, where ~26°C has been the average seawater temperature over the last 8 years (data center from the Australian Institute of Marine Science; data.aims.gov.au/aimsrtds/yearlytrends.xhtml). In comparison, according to a previous study, the temperature 32°C was the upper thermal limit of the *C. goreaui* strains that exhibited no net growth (*9*).

In vitro experiment

We grew the 10 heat-evolved and 2 wild-type strains (wild type for thermal tolerance, WT) at their respective temperatures of 27° and 31°C in 250-ml culturing flasks (vent cap, rectangular shape, Corning) about 4 months before commencement of the experiments to obtain enough biological material for the experiment. To ensure that we were assessing stable adaptive changes to the respective temperatures rather than reversible changes, we acclimated the Symbiodiniaceae strains for 6 weeks at 27°C before the start of the experiment. After the acclimation phase, we refreshed the culture medium and created 10 biological replicates for each strain in experimental flasks (tissue collection flask surface area 25 cm^2, Corning) with each 300,000 cells/ml starting material and each 20 ml of IMK medium (Nihon Pharmaceutical Co.) (total number of biological replicates, $n = 120$). We placed half of the flasks, five biological replicate per strains, into each of the two temperature treatments of 27° and 31°C for 3 weeks (fig. S1B) and measured the thermal tolerance of the strains according to the parameters described below. The spatial flask arrangement was randomized within the incubator every other day.

Coral spawning and larval collection

Six coral colonies of the species *A. tenuis* were collected from Magnetic Island, central GBR, in November 2017 (collection permit number G12/35236.1) and taken to the National Sea Simulator at the Australian Institute of Marine Science, Townsville, Australia. On the night of spawning, bundles of coral eggs and sperm from all six colonies were pooled and placed into a 420-liter tank for fertilization and larval rearing at a density of 0.5 larvae/ml of seawater until fully developed and competent to metamorphose, as described in detail by Pollock *et al.* (*28*). About 1200 larvae were taken randomly from the pool for the in hospite experiment.

In hospite experiment

To establish a symbiosis between coral larvae and the 12 *C. goreaui* strains, 100 coral larvae were transferred to each of twelve 200-ml plastic jars filled with filter-sterilized seawater (0.02 µm filtered) and kept at 27°C (0.5 larvae/ml of seawater). We exposed the larvae with WT and SS strains to ambient temperature (27°C) to ensure that any differences between the holobionts were due to stable adaptive changes and not cofounded by reversible acclimation to the elevated temperatures. In addition, the coral larvae only established a symbiosis with the WT and SS strains at ambient temperatures, not at elevated temperatures. The respective *C. goreaui* strains (table S1) were then added to the jars twice, right away with the coral larvae and after 2 weeks, at a final cell density of ~10^5 cells/ml (one strain per jar) and left for 30 days to establish a symbiosis with the coral larvae (fig. S1C). The larvae were subsequently transferred to new vessels (glass scintillation vials) with new filter-sterilized seawater without additional Symbiodiniaceae cells and left for another 45 days to get fully pigmented and build up equal numbers of *C. goreaui* cells in the tissue. Coral larvae with heat-evolved and wild-type strains reached about 10,000 *C. goreaui* cells per larva (10,185 ± 5103 cells/larva on average; database S1). Before starting the thermal stress experiment at 31°C, we collected samples for transcriptomics (fig. S1D), measured the photosynthetic performance and cell densities the of Symbiodiniaceae strains in the coral larvae (t_0 values; fig. S7D), and then distributed the larvae across three to six scintillation vials with four larvae each (the number of scintillation vials depended on the available number of larvae per strain). Scintillation vials for each strain were as follows: WT1, $n = 6$; WT2, $n = 6$; SS1, $n = 4$; SS3, $n = 6$; SS5, $n = 5$; SS6, $n = 3$; SS7, $n = 3$; SS8, $n = 5$; SS9, $n = 5$; SS10, $n = 3$. To avoid a thermal shock of the coral larvae, the holobionts were exposed to 29°C for 1 day before commencing with the

experimental heat stress at 31°C. At t_7 of heat stress at 31°C, we measured again the photosynthetic performance and cell numbers of the *C. goreaui* strains in the coral larvae to assess their bleaching tolerance at 31°C (fig. S1E). Larvae were first used for photosynthetic measurements and then for cell counts, as described below. The criteria for an enhanced bleaching tolerance of the coral larvae at 31°C were (i) stable or increasing algal cell densities and (ii) maintenance of F_v/F_m (absence of photochemical stress).

Photosynthetic performance

In the in vitro experiment, we assessed the photosynthetic performance twice per week with an imaging pulse-amplitude modulation chlorophyll fluorometer (IPAM M-series, Walz, Germany) (*12*, *29*). We measured the maximum quantum yield (F_v/F_m) of photosystem II on 2-hour dark-adapted cultures and the effective quantum yield ($\Delta F/F_m'$) on light-adapted cultures, together with the relative ETR [$\Delta F/F_m' = (F_m' - F)/F_m'$], photochemical quenching [qP], and non-photochemical quenching [$(F_m - F_m')/(F_m - F_o)$]. Individual flasks were considered as biological replicates ($n = 5$ per strain), each with three point locations averaged as technical replicated measurements with the following parameters: measurement intensity, 6; saturation pulse, 10; gain, 2; damping, 2. Because the IPAM measurement was noninvasive, the same flasks were measured throughout the experiment and used for Symbiodiniaceae cell counts (see below). The raw data are available in database S1.

In the in hospite experiment, we used an IPAM microscopy version to measure the maximum quantum yield (F_v/F_m) of photosystem II of the Symbiodiniaceae strains in single coral larvae that were 2-hour dark-adapted at the beginning and end of the experiment (t_0 and t_7) (*12*, *29*). For the measurements, one larva was taken out of each scintillation vial, and the values of five point locations of about 10 µm in size were averaged as technical replicates with the following parameters: measurement intensity, 5; saturation pulse, 4; gain, 2; damping, 2. Larval biological replicate numbers at the beginning/end of the experiment were as follows: SS1, $n = 4/4$; SS3, $n = 5/6$; SS5, $n = 4/5$; SS6, $n = 4/3$; SS7, $n = 5/3$; SS8, $n = 5/5$; SS9, $n = 4/4$; SS10, $n = 3/3$; WT1, $n = 3/6$; WT2, $n = 4/5$. Because of mortality during the 7 days of heat stress, there were only one to two biological replicates available at the end of the experiment for holobionts with SS2 and SS4, which were therefore excluded from the statistical analysis (SS2, $n = 3/1$; SS4, $n = 5/2$). Because the IPAM measurement was noninvasive, larvae were subsequently used for Symbiodiniaceae cell counts (see below). The raw data are available in database S1.

Symbiodiniaceae cell counts

Symbiodiniaceae cells were counted by taking a 200-µl aliquot of the resuspended cell suspension from in vitro replicates ($n = 5$ flask biological replicates per strain). Cell densities per flask were measured twice per week in technical replicates of two on an automatic cell counter (Countess II FL Automated Cell Counter, Life Technologies) and then averaged to the final count. Cell densities below 100,000 cells/ml per flask were counted manually under an inverted microscope (low number counts are more accurately counted manually compared to cell counter) with a hemocytometer, a ×20 ocular magnification, and fluorescence light to distinguish cell debris from live cells.

In hospite, Symbiodiniaceae cell densities of single larvae were counted before and at the end of the experiment (t_0 and t_7). Each coral larva was washed three times in filter-sterilized seawater (0.02 µm filtered) to get rid of any potential algal symbiont cells potentially attached to the outer mucus layer of the larvae. Individual coral larvae were sonicated in 100-µl filter-sterilized seawater for 3 s to break up the larvae tissue, which does not affect the Symbiodiniaceae cell integrity. A 10-µl aliquot of the tissue homogenate was manually analyzed on a hemocytometer with the aforementioned microscope conditions. Larvae biological replicate numbers at the beginning/end of the experiment were the following: SS1, $n = 5/4$; SS3, $n = 6/6$; SS5, $n = 5/5$; SS6, $n = 5/3$; SS7, $n = 5/3$; SS8, $n = 5/5$; SS9, $n = 5/5$; SS10, $n = 5/4$; WT1, $n = 6/6$; WT2, $n = 5/5$. Because of mortality during the 7 days of heat stress, we had only one biological replicate available at the end of the experiment for holobionts with SS2 and SS4, which were therefore excluded from the statistical analysis (SS2, $n = 4/1$; SS4, $n = 5/1$). The raw data are available in database S1.

Reactive oxygen species

For the in vitro experiment, we measured extracellular ROS that has leaked from the cells into the media (*9*, *30*). Subsamples of 1-ml culture media were transferred into 1.5-ml tubes ($n = 3$ flasks per strain). The *C. goreaui* cells were pelleted by centrifugation at 3000*g* for 5 min, and three 250-µl aliquots from the supernatant were transferred as technical replicates for ROS measurements into a 96-well plate (black, clear bottom, CLS3603-48EA Corning). To stain ROS in the media, 0.5 µl of CellROX orange liquid (C10443, Thermo Fisher Scientific) was pipetted into each well at 5 µM final concentration at 2.5 mM dye stock concentration. The mix was incubated at 37°C for 30 min and measured in a 96-well plate reader with 545-nm absorption and 565-nm emission (Synergy H4, BioTek). Measurements across plates were normalized by calculating the dispersion of empty and blank measurements per plate from the overall mean and subtracting the difference from the respective fluorescence measurements and then normalized to the cell numbers per milliliter (see normalization calculations and raw data in database S1).

Transcriptomics

For the in hospite experiment, we collected coral larvae samples to analyze gene expression profiles of a heat-tolerant (SS8) and three heat-sensitive holobionts (larvae with strains SS3, SS5, or WT1). The samples were taken to assess normal functioning of the holobionts after 2.5 months at 27°C (before the start of the exposure to elevated temperatures). A total of 10 larvae were pooled for each transcriptomics sample replicate [number of replicates per strain: SS3 ($n = 6$), SS5 ($n = 6$), SS8 ($n = 5$), WT1 ($n = 6$); n total = 23 RNA samples]. The samples were snap-frozen in liquid nitrogen and stored at −80°C before RNA extraction. RNA was isolated using the Quick-RNA MiniPrep Kit (Zymo) and modified homogenization protocol. In brief, while samples were placed in tubes on dry ice, we added 120 µl of cold (−80°C) absolute methanol and prechilled (−80°C) bead mix (about 50 mg of 425- to 600-µm acid-washed glass beads and ten 2-mm ceramic beads). Samples were then disrupted in TissueLyser II (Qiagen) in prechilled tube holders (−80°C) at 30 s^{-1} for 3 min. Following disruption, 1200 µl of the Zymo RNA lysis buffer was added (to reduce methanol content to 10%). The samples were allowed to warm to room temperature (with mixing), whereupon they were processed in TissueLyser II again at 20 s^{-1} for 1 min. RNA extraction from the entire volume then continued using the standard Zymo kit protocol, including deoxyribonuclease (DNase) treatment. The resulting RNA was eluted in 30 µl of nuclease-free water. Libraries were prepared at the Ramaciotti Centre for Genomics in Sydney according to the Illumina TruSeq Stranded mRNA v2 Kit with 300 ng of

total RNA input, an adapter dilution of 1:3 to account for the low input, and an amplification of the complementary DNA (cDNA) using 14 cycles of polymerase chain reaction. The libraries were sequenced on NovaSeq 6000 using an S1 flowcell kit at 2 × 100 base pairs (bp) to generate ~87 million reads per sample [1,997,829,399 reads in total; see database S2; raw data available at National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) (repository GSE133082, BioProject PRJNA549921, SRA SRP201999, detailed accession numbers in database S2)]. All reads passed a >36 phred score quality control filter and were analyzed for gene expression profiles. The raw data files containing both algal symbiont and coral host reads were mapped parallel with the software STAR [version 2.5.3 (*31*)] using the default parameters for 2 × 100 bp reads to the published coral and algal symbiont reference genomes obtained from http://refuge2020.reefgenomics.org/ (*A. tenuis* genome and *Symbiodinium goreaui* clade C1 genome) (*32*). On average, 86% (75 million reads out of ~87 million reads per sample) were mapped to the reference genomes: 33% of the reads per sample (29 million) to the algal symbiont genome, and 53% of the reads (47 million) to the coral host genome. Because of the parallel mapping approach, we checked the data for reads that mapped to both genomes. From the ~87 million reads per sample, about 0.0017% (~1500 reads) mapped to both references (see database S2). The double-mapped reads only affected the counts of one differentially expressed exon of the algal symbiont with <0.01% of the counts due to double-mapped reads. For the coral host, 17 differentially expressed exons were affected with on average 0.77% of the reads coming from double-mapped reads (full exon table in database S2). Because these reads were low in numbers and the target exons were not of major importance for the data interpretation, we did not remove these reads from the data.

Both genomes were annotated with a local DIAMOND Blastx scan (*33*) against the nonredundant database (version 10.12.2018) using an *e*-value cutoff of $<10^{-5}$ in combination with an InterProScan in Blast2GO (version 5.0.8) to correct the annotations and to obtain GO categories (annotations in database 3). The gene expression matrix from STAR was then processed with the R package "linear models for microarray data" (limma, version 3.36.5, full R code in database S3). In brief, exon expression was collated to gene expression, and genes with a low count were filtered out of the dataset (minimum count >40 reads). After count normalization, gene expression of the heat-evolved strains was compared to the expression of the wild-type strain. The gene expression matrices for host and symbiont are available through a data repository at NCBI GEO, repository GSE133082. Overall differential gene expression was analyzed for functional enrichment with a ranked-based GO analysis with adaptive clustering using a Mann-Whitney *U* test (MWU) (*34*). The hierarchical clustering trees show significantly enriched GO categories by up-regulated (red) or down-regulated (blue) genes based on gene expression *P* values $\log_{10}$-transformed (clustering trees available in database S4). To assess respective unique adaptations of the strains, significantly DEGs that were unique for the respective sample groups (below the statistical threshold of <0.05 FDR) were used for GO enrichment with the R package GOseq (goseq version 1.34.1) (*35*) (database S4). Enriched GO categories containing less than three DEGs were dismissed. In addition to the functional enrichment, we analyzed the expression of custom gene sets to test for potential front-loading of heat stress–associated gene sets (*25*). For R code and model outputs, see additional databases (table S2).

Statistical analysis

All physiological measurements (cell densities, chlorophyll measurements, and ROS) were tested for significant differences between WT and SS strains with generalized linear models (R package stats, version 3.5.1). To compare strains in vitro and in hospite properly, we calculated the delta values (difference end-beginning of heat exposure) and then the relative percent change (see calculations database S1).

For the in vitro experiment, we used a quasi-binomial distribution with a two-factorial design (TEMPERATURE with the levels 27° and 31°C and STRAIN with the levels SS01 to SS10 and WT1 and WT2). The day 0 in vitro photophysiology measurements had to be removed from the data due to technical issues with the instrument.

For the in hospite experiment, we used a quasi-binomial distribution with a one-factorial design of only STRAIN because we had only one temperature treatment of 31°C (STRAIN levels: SS1, SS3, SS5, SS6, SS7, SS8, SS9, SS10, WT1, and WT2). Because in hospite biological replicates at the beginning and end of the experiment were independent from another (different individual coral larvae), we calculated the average of the beginning values, which was then subtracted from the end value. The two strains SS2 and SS4 were removed from the statistical analysis because we had less than three biological replicates available at the end of the experiment due to mortality.

The ROS data of in vitro were analyzed with a gamma distribution and STRAIN as a factor (levels: SS1 to SS10 and WT1 and WT2) because we only had measurements from the end of the experiment and could not calculate the relative percent change over time. For R code and test outputs, see database S1.

Overall gene expression profiles of larval samples were analyzed for significant differences among the factor STRAIN (levels: SS3, SS5, SS8, and WT1) with a permutational multivariate analysis of variance using distance matrices [adonis, R package vegan version 2.5-4 (*36*)] using 999 permutations, Euclidean distances, and a Tukey's honestly significant difference as a post hoc test. For R code and test outputs, see database S3.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/20/eaba2498/DC1

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Heat-evolved microalgal symbionts increase coral bleaching tolerance

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