Discovery of Symbiotic Nitrogen-Fixing Cyanobacteria in Corals

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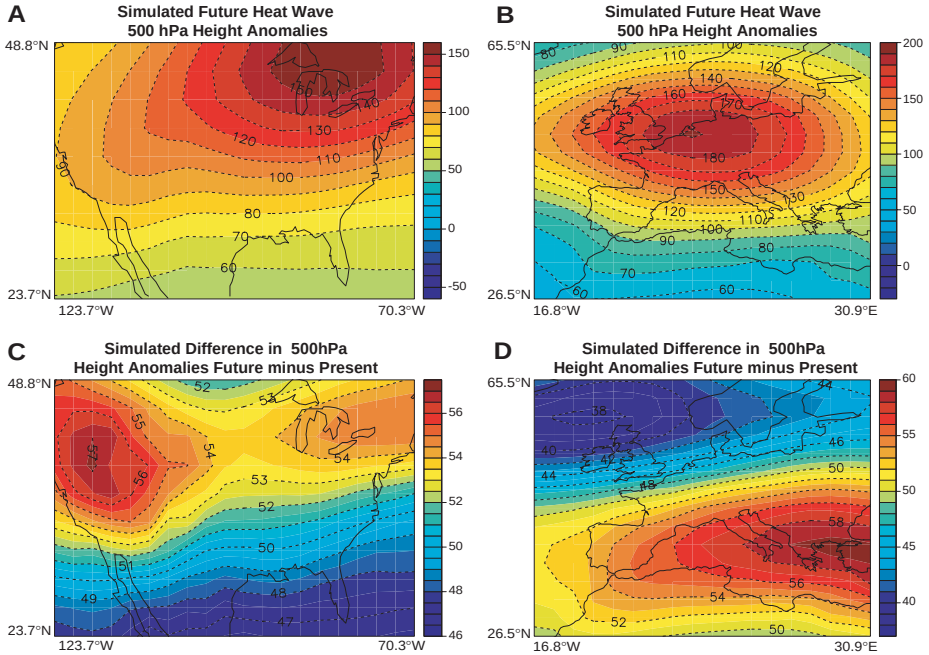

Fig. 4. Height anomalies at 500 hPa (gpm) for events that satisfy the heat wave criteria in the model in future climate (2080 to 2099) for grid points near Chicago (**A**) and Paris (**B**), using the same base period as in Fig. 3, C and D. Also shown are changes (future minus present) in the model's 500-hPa height mean base state, for North America (**C**) and Europe (**D**).

maximum temperatures (fig. S2). This is consistent with a widening of the distribution of temperatures in addition to a shift in the mean (*5*), and suggests that there is an increase in heat wave occurrence beyond that driven by changes in the mean circulation.

The 500-hPa height anomalies are most strongly related to positive warm season precipitation anomalies over the Indian monsoon region and associated positive convective heating anomalies that drive mid-latitude teleconnection patterns (such as those in Fig. 4, C and D) in response to anomalous tropical convective heating in future climate (figs. S3 to S5). Thus, areas already experiencing strong heat waves (e.g., southwest, midwest, and southeast United States and the Mediterranean region) could experience even more intense heat waves in the future. But other areas (e.g., northwest United States, France, Germany, and the Balkans) could see increases of heat wave intensity that could have more serious impacts because these areas are not currently as well adapted to heat waves.

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Figs. S1 to S5
References

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Discovery of Symbiotic Nitrogen-Fixing Cyanobacteria in Corals

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Colonies of the Caribbean coral *Montastraea cavernosa* exhibit a solar-stimulated orange-red fluorescence that is spectrally similar to a variety of fluorescent proteins expressed by corals. The source of this fluorescence is phycoerythrin in unicellular, nonheterocystis, symbiotic cyanobacteria within the host cells of the coral. The cyanobacteria coexist with the symbiotic dinoflagellates (zooxanthellae) of the coral and express the nitrogen-fixing enzyme nitrogenase. The presence of this prokaryotic symbiont in a nitrogen-limited zooxanthellate coral suggests that nitrogen fixation may be an important source of this limiting element for the symbiotic association.

The success of scleractinian corals since the Triassic (*1*) has been attributed to the establishment of a mutualistic symbiosis between the cnidarian host and a diverse group of endosymbiotic dinoflagellates (zooxanthellae). Zooxanthellae, which are localized within gastrodermal cells of the cnidarian host, can provide more than

100% of the carbon requirements of the animal partner, primarily in the form of carbohydrates and low–molecular-weight lipids. Experimental manipulations of zooxanthellate corals suggest that inorganic nitrogen limits the growth and abundance of zooxanthellae in the coral; indeed, this limitation has been suggested to be essential for the stability of the symbiotic association (*2*, *3*).

In addition to zooxanthellae, a variety of bacteria appear to be associated with scleractinian corals (*4*–*6*), and although these associations also appear to be widely distributed, stable, and nonpathogenic, the function of these bacteria remains largely unknown. However, the presence of cyanobacteria is associated with photosynthesis-dependent nitrogen fixation on coral reefs (*7*) and is suggested to be responsible for nitrogen fixation in living coral tissue (*8*). In this paper, we show that large numbers of endosymbiotic cyanobacteria capable of fixing nitrogen occur in a common scleractinian coral, *Montastraea cavernosa.*

Scleractinian corals, including *M. cavernosa* (*9*–*11*), express a variety of fluorescent proteins, but colonies of *M. cavernosa* have also been observed to fluoresce orange during the daytime (Fig. 1A). This fluorescence is not due to a fluorescent protein but to phycoerythrin. In vivo excitation/emission spectra of these corals showed an emission peak at 580 nm and a shoulder at 630 nm, with excitation bands at 505 and 571 nm (Fig. 1B). Although this spectral signature is similar to those reported for red fluorescent proteins of corals (*12*, *13*), the two excitation peaks also corresponded to absorption by phycoerythrin in marine cyanobacteria that contain both the phycourobilin and phycoerythrobilin chromphores (*14*, *15*). Immunoblots (*16*) of coral homogenates challenged with a polyclonal antibody against phycoerythrin revealed a positive cross-reaction with the 18- to 20-kD β-polypeptide of phycoerythrin (Fig. 1C). These results clearly suggest that intracellular cyanobacteria are associated with the coral.

Fluorescence lifetime analyses (*16*) indicate that the 580-nm excited state is dominated by a single component with a 3.93-ns time constant (Fig. 1D). The slow single-exponential decay of the orange pigment is longer than described for fluorescent proteins (2.6 to 3.7 ns) (*11*, *17*, *18*) and suggests energetic isolation and the absence of excitation energy transfer out of the chromophore. In contrast, phycoerythrin fluorescence normally observed in cyanobacteria exhibits faster kinetics in vivo owing to efficient energy transfer within the phycobilisomes. The lifetime data and the daytime fluorescence indicate that the energy coupling of these pigments to primary photochemistry in the symbiotic cyanobacteria is weak, leading to the relatively high quantum yield of fluorescence for this pigment.

Epifluorescence microscopy (*16*) of host tissue homogenates revealed zooxanthellae exhibiting red chlorophyll fluorescence, as

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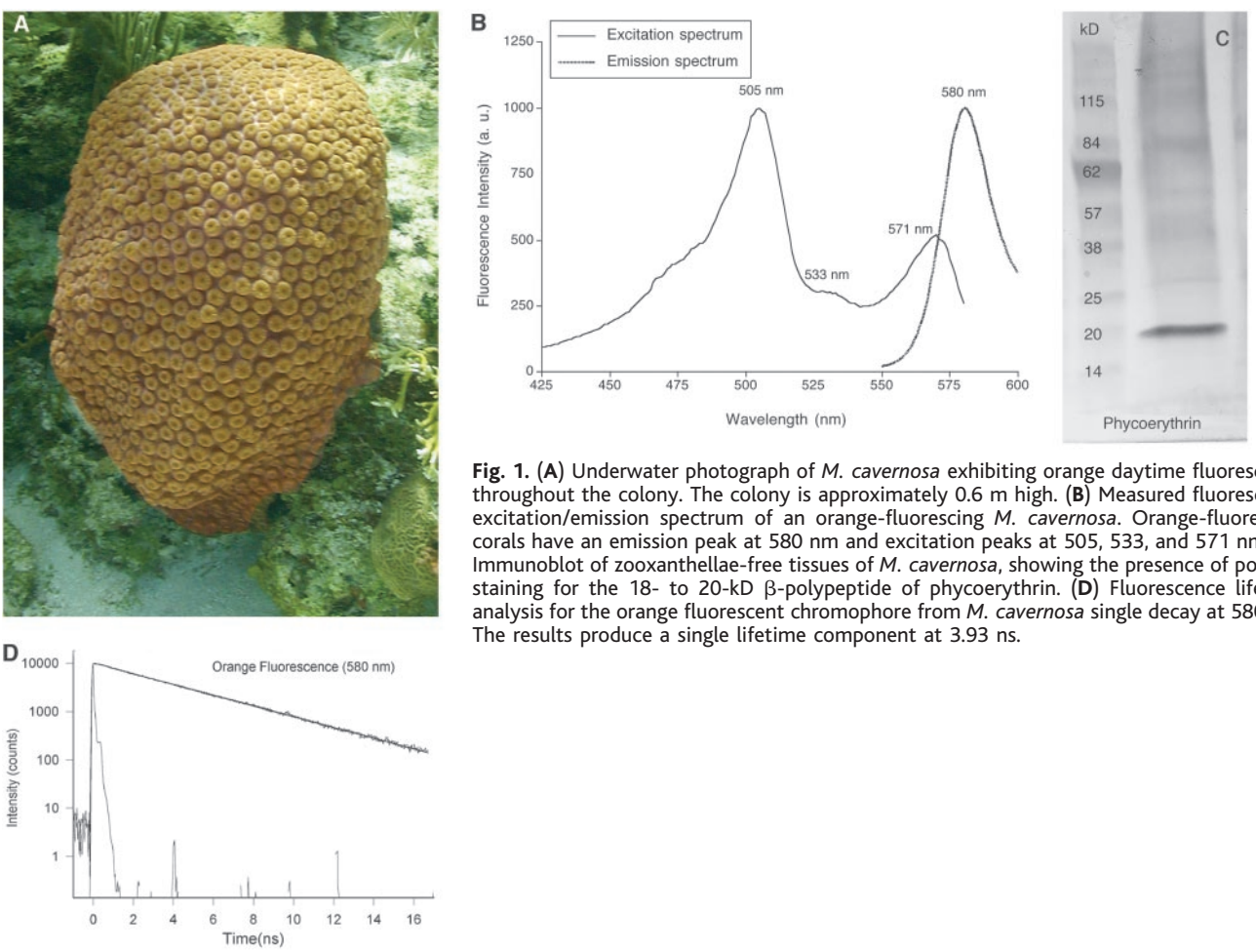

Fig. 1. (**A**) Underwater photograph of *M. cavernosa* exhibiting orange daytime fluorescence throughout the colony. The colony is approximately 0.6 m high. (**B**) Measured fluorescence excitation/emission spectrum of an orange-fluorescing *M. cavernosa*. Orange-fluorescing corals have an emission peak at 580 nm and excitation peaks at 505, 533, and 571 nm. (**C**) Immunoblot of zooxanthellae-free tissues of *M. cavernosa*, showing the presence of positive staining for the 18- to 20-kD β-polypeptide of phycoerythrin. (**D**) Fluorescence lifetime analysis for the orange fluorescent chromophore from *M. cavernosa* single decay at 580 nm. The results produce a single lifetime component at 3.93 ns.

well as many smaller orange-fluorescent cells resembling cyanobacteria (Fig. 2A). An analysis of tissue homogenates using flow cytometry (*16*) showed a distinct phycoerythrin signature and a size range for these cells of 1.0 to 3.0 μm in diameter. The number of phycoerythrin-positive cells from fluorescent samples of *M. cavernosa*, normalized to surface area, ranged from 1.14×10^7 to 2.55×10^7 cells cm^{-2}, whereas nonfluorescent colonies have $\ll 10^2$ phycoerythrin-positive cells per square centimeter. Transmission electron micrographs (*16*) of the coral tissue revealed that the cyanobacteria-like cells are located in the epithelial cells of the animal host and are surrounded by host membrane (Fig. 2B). The cyanobacteria-like cells exhibit an unusual arrangement of their thylakoid membranes that cross randomly throughout the cell and occasionally appear both expanded and appressed. These cells also appear to have fewer electron-dense phycobilisomes associated with the thylakoid membranes than has been reported for other cyanobacteria. Immunogold probing of thin sections (Fig. 2C), using the antibody to phycoerythrin, showed that the antibody binds significantly [analysis of variance (ANOVA), $P < 0.001$] to the cyanobacterial cells [134.9 particles per cell ± 19.4 (SE)] when compared to controls [6.5 particles per cell ± 2.1 (SE)] without the primary antibody. These cells also bind positively to a cyanobacteria-specific (CYA762) 16*S* ribosomal RNA–targeted oligonucleotide probe (Fig. 2D) which cross-reacts with a large number of cyanobacteria (*16*, *19*). Additionally, 16*S* ribosomal DNA sequencing using cyanobacteria-specific primers (*16*, *20*) yielded a 556-bp sequence (GenBank accession number AY580333) from genomic DNA preparations that match cyanobacterial sequences ($n = 100$) related to *Synechococcus* sp., *Prochlorococcus* sp., or uncultured cyanobacteria in the Order Chroococcales, a paraphyletic group (*21*), with 93 to 97% sequence homology.

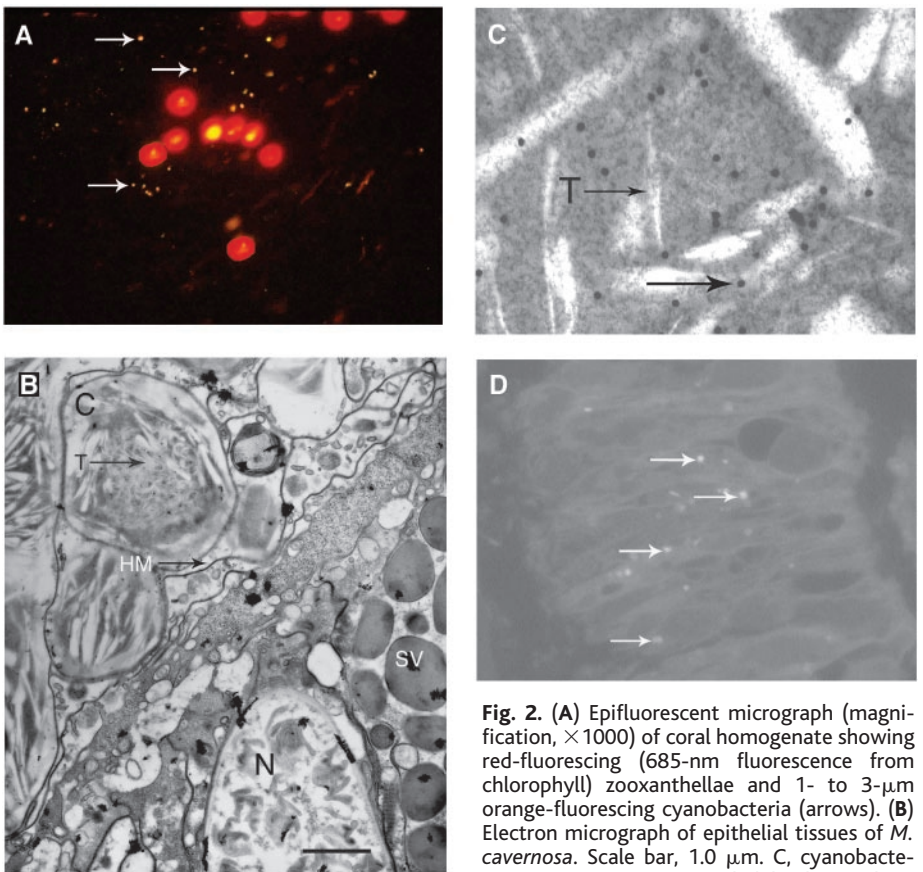

Fig. 2. (**A**) Epifluorescent micrograph (magnification, ×1000) of coral homogenate showing red-fluorescing (685-nm fluorescence from chlorophyll) zooxanthellae and 1- to 3-μm orange-fluorescing cyanobacteria (arrows). (**B**) Electron micrograph of epithelial tissues of *M. cavernosa*. Scale bar, 1.0 μm. C, cyanobacterium; N, nematocyst; T, thylakoid; HM, host membrane; SV, secretory vesicle. (**C**) Immunogold labeling (20-nm gold particles, arrows) of thin sections for phycoerythrin (magnification, ×50,000). (**D**) Fluorescent in situ hybridization micrograph (magnification, ×1000) of *M. cavernosa* epithelial tissues showing positive binding of cyanobacterial-specific probe (arrows).

To assess the potential for nitrogen fixation in this coral, we challenged protein extracts with a polyclonal antibody to the 32-kD Fe protein subunit of nitrogenase (Fig. 3A). The results yielded a single positive cross-reaction, strongly indicating expression of the gene in the coral. Again, using the same antibody to nitrogenase, we used immunogold probing of thin sections (Fig. 3B) and found that the antibody binds significantly (ANOVA, $P = 0.006$) to the cyanobacterial cells [8.8 particles per cell ± 1.3 (SE)] when compared to controls [4.5 particles per cell ± 0.5 (SE)] without the primary antibody. Many members of the Order Chroococcales are capable of fixing nitrogen. Our results clearly suggest that endosymbiotic cyanobacteria capable of fixing nitrogen are present in *M. cavernosa* and form a stable long-term association within host cells. This symbiont could potentially be a source of the limiting element nitrogen for the symbiosis through the release of fixed nitrogen products to the coral host.

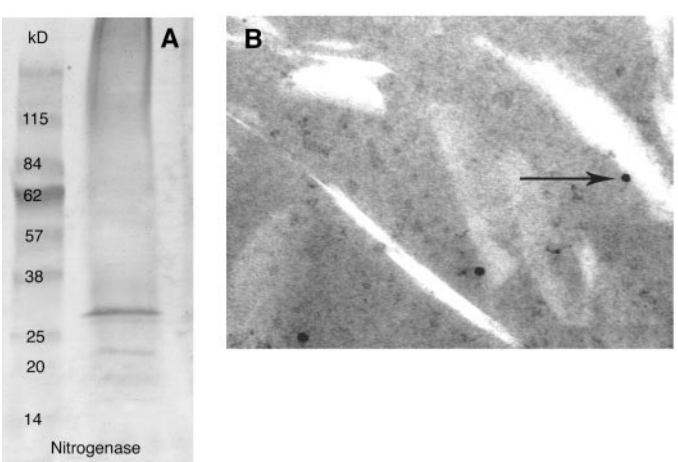

Fig. 3. (**A**) Positive immunoblot for the 32-kD Fe protein of nitrogenase in zooxanthellae-free tissues of *M. cavernosa*. (**B**) Immunogold labeling (20-nm gold particles, arrow) of thin sections for nitrogenase (magnification, ×50,000).

Like the symbiotic cyanobacteria of *M. cavernosa*, free-living cyanobacteria and prochlorophytes that contain phycoerythrin often exhibit strong fluorescence under certain conditions, with a maximum emission between 570 to 580 nm (*22*, *23*). Uncoupled phycoerythrin has been proposed to serve as a storage pool of nitrogen in phycobilin-containing cyanobacteria (*22*) but not in prochlorophytes (*23*). Phycoerythrin detachment from the photosynthetic apparatus in cyanobacteria and prochlorophytes

can be caused by exposure to glycerol and results in strong fluorescence by eliminating the quenching associated with energy transfer from phycoerythrin to the reaction centers (*22*, *23*). The cyanobacterial symbionts of *M. cavernosa* are exposed to high concentrations of glycerol in the coral, because it is the major carbon compound translocated from the symbiotic zooxanthellae to the host tissues (*24*), and this may explain both the unusual ultrastructure and the characteristic orange fluorescence emission of the symbiotic cyanobacteria. Because little or no energy is transferred from phycoerythrin to primary photochemistry, glycerol supplied from the zooxanthellae may serve as an energy source for the cyanobacteria operating heterotrophically and provide a steady supply of reductant and adenosine triphosphate for nitrogen fixation in the symbionts. Nitrogenase is also sensitive to molecular and reactive species of oxygen that accumulate during photosynthesis (*25*, *26*), and the symbiotic cyanobacteria, operating heterotrophically, could quench molecular oxygen via respiration and/or by the Mehler reaction (*27*). Additionally, the coral environment is well suited for the temporal separation of photosynthesis and nitrogen fixation, because coral tissues experience extreme hypoxia at night (*25*). The presence of an additional symbiont in a zooxanthellate coral that is nitrogen-limited (*2*) suggests that nitrogen fixation may be an important supplemental source of the limiting element for the symbiotic association, and it highlights the potential significance of microbial consortia composed of photosynthetic eukaryotes and prokaryotes (*28*). Cyanobacteria are involved in many diverse mutualistic symbioses in both terrestrial and marine environments, and they provide critical ecological services, including important contributions to the global nitrogen cycle (*29*).

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Supporting Online Material

www.sciencemag.org/cgi/content/full/305/5686/997/DC1

Materials and Methods

Fig. S1

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Modulation of Hematopoietic Stem Cell Homing and Engraftment by CD26

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Hematopoietic stem cell homing and engraftment are crucial to transplantation efficiency, and clinical engraftment is severely compromised when donor-cell numbers are limiting. The peptidase CD26 (DPPIV/dipeptidylpeptidase IV) removes dipeptides from the amino terminus of proteins. We present evidence that endogenous CD26 expression on donor cells negatively regulates homing and engraftment. By inhibition or deletion of CD26, it was possible to increase greatly the efficiency of transplantation. These results suggest that hematopoietic stem cell engraftment is not absolute, as previously suggested, and indicate that improvement of bone marrow transplant efficiency may be possible in the clinic.

The efficiency of hematopoietic stem cell (HSC) transplantation is important when donor-cell numbers are limiting. For example, since the first cord blood transplants (*1–3*), the use of cord blood has been mainly restricted to children, not adults, as a result of apprehension about limited cell numbers. Attempts at ex vivo expansion of stem cells for clinical transplantation have not been encouraging (*4*, *5*). An alternative means to enhance engraftment is to increase HSC homing efficiency to bone marrow (BM) niches. Recently it was suggested that HSCs engrafted mice with absolute efficiency (*6–8*). However, if all HSCs homed with absolute efficiency and engraftment, problems of limiting donor cells would not be a concern for clinical transplantation (*3*). Thus, enhancement of homing and engraftment of HSC is needed if advances in transplantation with limiting numbers of HSC are to be realized. On the basis of our work implicating CD26 in granulocyte colony-stimulating factor (G-CSF)–induced mobilization of HSCs and hematopoietic progenitor cells (HPCs) (*9–11*), we investigated the involvement of CD26 in homing and engraftment. Inhibition or deletion of CD26 on donor cells enhanced short-term homing, long-term engraftment, competitive repopulation, secondary transplantation, and mouse survival, which suggests that CD26 is a novel target for increasing transplantation efficiency.

Mouse bone marrow HSCs were defined as cells within the Sca-1$^+$lin$^-$ population

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