The demise of the marine cyanobacterium, *Trichodesmium* spp., via an autocatalyzed cell death pathway

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**Abstract**

We present experimental laboratory evidence and field observations of an autocatalyzed, programmed cell death (PCD) pathway in the nitrogen-fixing cyanobacterium *Trichodesmium* spp., which forms massive blooms in the subtropical and tropical oceans. The PCD pathway was induced in response to phosphorus and iron starvation as well as high irradiance and oxidative stress. Transmission electron microscopy revealed morphological degradation of internal components including thylakoids, carboxysomes, and gas vesicles, whereas the plasma membranes remained intact. Physiologically stressed cells displayed significantly elevated endonuclease activity and terminal d-UTP nick-end labeling. Nuclease degradation was concordant with mortality and was inhibited by the irreversible caspase inhibitor Z-VAD-FMK. A search of the *Trichodesmium erythraeum* genome identified several protein sequences containing a conserved caspase domain structure, including the histidine- and cysteine-containing catalytic diad found in true caspasas, paracaspases, and metacaspases. Induction of PCD by caspase-like proteases in a bacterial photoautotroph with an ancient evolutionary history requires a reassessment about the origins and roles of cell death cascades. This process is a previously unappreciated mortality mechanism that can lead to the termination of natural *Trichodesmium* blooms and that can influence the fluxes of organic matter in the ocean.

Planktonic marine cyanobacteria of the genus *Trichodesmium* form extensive blooms in the oligotrophic tropical and subtropical oceans, where they make significant contributions to global nitrogen fixation (Capone et al. 1997). Natural blooms and laboratory cultures of *Trichodesmium* often terminate abruptly, with cell lysis and biomass degradation occurring within 1–2 d (Ohki 1999). The mechanisms controlling the dramatic and abrupt termination of *Trichodesmium* blooms are not well understood, even though this termination drives nutrient flow and biogeochemical cycling of organic and inorganic matter produced by these organisms, including the redistribution of fixed nitrogen in the upper ocean and the flow of organic matter through ecosystem pathways like the grazer food chain, the microbial loop, and vertical sinking flux (Azam 1998).

Grazing by the harpacticoid copepod *Macrosetella gracilis* (O’Neil 1998) and bacteriophage infection (Ohki 1999) are factors that have been implicated in the termination of *Trichodesmium* blooms. At the same time, a variety of microscopic phytoplankton cells also appear to lyse and die via an autocatalytic cell death pathway triggered by nutrient stress (Brussaard et al. 1995; Berges and Falkowski 1998; Vardi et al. 1999; Segovia et al. 2003). This self-destruction is analogous to programmed cell death (PCD) in multicellular organisms and refers to an active, genetically controlled, cellular self-destruction driven by a series of complex biochemical events and specialized cellular machinery—receptors, adapters, signal-kinases, proteases, and nuclear factors (Aravind et al. 1999).

Here we demonstrate that a PCD pathway operates in the cyanobacterium *Trichodesmium* spp. in aging cultures and in response to relevant environmental and physiological stresses, including combined phosphorus and iron depletion, high irradiance, oxidative stress, and cell age. Furthermore, we present evidence that PCD in *Trichodesmium* displays physiological and biochemical characteristics consistent with PCD in metazoa and higher plants. These results, taken together with similar recent findings in yeast (Madeo et al. 2002) and another marine phytoplankter, *Dunaliella tertiolecta* (Segovia et al. 2003), provide mechanistic insight into the control of phytoplankton mortality and intriguing ecological and evolutionary context for PCD.
Materials and methods

Cultures, natural populations, and growth conditions—Trichodesmium IMS101 cultures were grown in YBCII medium at 26°C with a 12 : 12 L : D cycle and constant aeration. Natural Trichodesmium populations, predominantly Trichodesmium erythraeum, were collected with 35-mm crashed. Trichodesmium was hand-picked, placed in a separating flask in filtered seawater (<0.2 μm), irradiated with ~450 μmol quanta m⁻² s⁻¹ and followed with time until the biomass crashed.

Detection of viruses—Presence of viruses was examined via induction of temperate phage with mitomycin C, as described by Ohki (Ohki 1999). Phages were viewed using Epifluorescence microscopy (Zeiss-Axiscope) following staining with SYBR Gold (Molecular Probes), as previously described by Ohki (Ohki 1999). Phages were viewed using Epi-Fluorescence microscopy (Zeiss-Axioscope) following fixation of Trichodesmium, and lead citrate. The stained sections were photographed with a JEM-100CXII electron microscope.

Transmission electron microscopy (TEM)—Cells were filtered on 5-μm polycarbonate filters under low vacuum, blocked in 2% agar (in seawater), and preserved in Truppm’s electron microscopy (EM) fixative (4% formaldehyde and 1% glutaraldehyde in phosphate buffer, pH 7.2). The cells were rinsed three times (15 min each time) in Milloning’s phosphate buffer (pH 7.3), postfixed for 2 h in 1% buffered OsO₄, washed three times, and dehydrated through a graded series of EtOH. After replacement of ethanol with propylene oxide, cells were embedded in Epon–Araldite cocktail. Sections were cut using an LKB 2088 ultramicrotome, collected on 200-mesh copper grids, and stained with uranyl acetate and lead citrate. The stained sections were photographed with a JEM-100CXII electron microscope.

Terminal d-UTP nick-end labeling (TUNEL)—Cells were labeled using the APO-BrdU Tunel Assay Kit (Molecular Probes). Fixation of Trichodesmium cells prior to labeling was done by filtration of cells onto 5-μm polycarbonate filters and resuspension in ice-cold 100% ethanol for at least 18 h before labeling with APO-BrdU TUNEL; this process was examined using an epifluorescent Zeiss Axioscope microscope.

Western analysis—Samples for immunochemical analysis were loaded on an equal protein basis, separated on 12% polyacrylamide gels, and transferred onto polyvinylidene fluoride (PVDF) membranes. Membranes were probed with polyclonal antibodies for recombinant human caspase-3 (Stressgen) and detected using a horseradish peroxidase chemiluminescence system (SuperSignal, Pierce).

Nuclease activity assay—Trichodesmium IMS101 cells grown in complete YBCII media were gravity filtered and resuspended in either complete YBCII or YBCII medium minus phosphorus and iron. At designated times, 100–150 ml of each culture type was harvested onto 5-μm-pore size membrane filters. Filters were immediately frozen in liquid nitrogen and stored at −80°C until processing. Cells were resuspended in 300 μl of nuclease activity buffer (10 mmol L⁻¹ Tris-Cl, pH 7.5; 2.5 mmol L⁻¹ MgCl₂; 0.1 mmol L⁻¹ CaCl₂) and probe sonicated (Misonix Microson ultrasonic cell disruptor; Power 2) for 1 min on ice at 20-s intervals. Cellular debris was pelleted by centrifugation (16,000 × g; room temperature; 1 min). Cell lysates were adjusted to equal protein concentrations in nuclease activity buffer (~200 μg ml⁻¹). One microgram of linear plasmid DNA (pBK-CMV) was added to cell lysates and incubated at 26°C for 1–3 h. The reaction was stopped by addition of 1 μl of 0.5 mol L⁻¹ EDTA and stored at −20°C. The entire reaction was loaded onto 0.8% agarose gel run in 1× tris-acetate EDTA (TAE) buffer and visualized with ethidium bromide (0.5 μg ml⁻¹). Control reaction consisted of linear plasmid DNA digested with 1 U DNaseI in nuclease reaction buffer for 1 h.

Photosynthetic efficiency—Fast repetition rate fluorimeter measurements of fluorescence kinetics were used to derive the maximum photochemical quantum yield of photosystem II (Fₚ/Fₚₒ) (Kolber et al. 1998).

Results and discussion

We observed a catastrophic decrease in biomass (>45% in 24 h) in stationary-phase laboratory cultures of Trichodesmium IMS101 (Fig. 1A, days 30–31), independent of viral infection. Neither epifluorescence microscopy nor TEM revealed evidence of an inducible, temperate phage in response to mitomycin C treatment (Ohki 1999) or in physiologically stressed cells. We considered whether PCD was responsible for this abrupt mortality by searching for distinct morphological changes (Kerr et al. 1972). Based on TEM images, cell death was characterized by degradation of thylakoids, carboxysomes, gas vesicles, and cyanophycin granules (Janson et al. 1995), but there was no evidence of plasma membrane rupture (Fig. 1B, right panels). The cells had large areas of electron-transparent, intracellular vacuolar spaces, an increase in the number of electron-dense particles that are likely lipid droplets, and increased gaps between cells along a trichome (Fig. 1B, right panels) that are indicative of cellular shrinkage. Epifluorescence microscopy of cultures stained with SYTOX green verified membrane integrity during the early stages of cell death. In contrast, cells with compromised membrane integrity (e.g., due to boiling)
Programmed cell death in *Trichodesmium*

Fig. 1. A) Growth and crash of *Trichodesmium* IMS101 during a simulated bloom. Arrows indicate phase from which cultures were sampled for TEM. B) TEM micrographs showing changes in cellular morphology of the aging culture. Left panels are of cells during the early stages of the bloom, when growth rates were positive. Right panels are of cells sampled during biomass decline. Abbreviations: th, thylakoids; gv, gas vesicles; cp, cyanophycin granules; cb, carboxysomes. C–D) Caspases in *Trichodesmium*. C) Western blot illustrating an increase in caspase-3 immunoreactivity during *Trichodesmium* IMS101 bloom progression. Whole-cell protein extracts were challenged with polyclonal antibodies to recombinant human caspase-3. Samples were loaded with equal protein and detected using a horseradish peroxidase chemiluminescence system. Lane numbers identify age of culture in days. Days 1–13 and days 14–36 were run on separate gels. Gels were run, blotted, probed, and exposed to film at the same time under identical conditions. D) Dependence of DEVD cleavage on physiological status of *Trichodesmium* IMS101 (mortality rate, expressed as changes in particulate organic carbon). Different symbols represent two separate bloom simulations. Diamonds represent the samples presented in Western analysis. Correlation coefficients are indicated for linear regression.
Fig. 2. Phosphorus (P) and iron (Fe) limitation leads to reduction in photosynthetic efficiency, activation of DEVD cleavage, and activation of nuclease activity. Upper panel: Time series of the maximum quantum yield of photosynthesis ($F_v/F_m$; circles) and DEVD cleavage (bars) for *Trichodesmium* IMS101 incubating in either complete YBCII growth medium (filled symbols/bars) or YBCII lacking in P and Fe (open symbols/bars). DEVD cleavage was not determined on days 3–4. Error bars are included and indicate standard deviation from triplicate measurements. Lower panel: 0.8% Agarose gel showing elevated nuclease activity in cell lysates of P- and Fe-stressed cells (S) compared to control cells incubating in complete YBCII (C). Data are for cells harvested on days 6 and 7, as indicated by arrow and lane number. One microgram of linear plasmid DNA (pBK-CMV; ~6 kb) was added to cell lysates and incubated at 26°C for 1–3 h. Cell lysates were adjusted to equal protein concentrations (200 μg ml$^{-1}$) in nuclease activity buffer. Nuclease activity is represented by degradation of linear plasmid DNA. Reactions with linear plasmid DNA incubating for 1 h in nuclease buffer with (D) or without (P) DNase I (Ambion) are included. Cell lysates were also incubated in nuclease buffer without addition of plasmid DNA in order to depict background DNA (“no plasmid”). Abbreviation: m, molecular weight markers.
Programmed cell death in *Trichodesmium*

Fig. 3. Oxidative stress induces cell death and elevated caspase-like activity in natural *Trichodesmium* populations collected in the subtropical western Pacific Ocean (off the coast of New Caledonia). Populations were collected from surface waters, cleaned and separated from the majority of other phytoplankton and zooplankton populations, and resuspended in enclosed spheres under high irradiance (~450 μmol quanta m⁻² s⁻¹). A) After 3 h, a majority of colonies and single trichomes are suspended throughout the sphere and are positively buoyant. B) At >7 h, exposure led to massive sinking of *Trichodesmium* populations. C–E) TEM micrographs of trichomes. C) After 3 h gas vesicles (gv) occupied a high percentage of the cells, which are also packed with other cellular components, such as thylakoids, carboxysomes, and cyanophycin granules; D) after 8 h, vacuolization (v) increased and gas vesicle volume decreased; E) sinking trichomes after 24 h, at which point cellular interior is highly vacuolated and external membranes have begun to lyse. F) A time course of caspase-like activity (DEVD cleavage) in the suspended population during exposure to high irradiance. G–I) Visualization of *Trichodesmium* DNA exposed to ROS (G) DNA of healthy, uninduced (no peroxide added) *Trichodesmium* IMS101 with a general DNA stain, Hoechst. Being a prokaryotic organism, DNA is distributed throughout the cells and trichomes rather than packaged in a nucleus. H) Low-level, diffuse TUNEL staining of uninduced “healthy” cells. We observed a similar low-level background staining from healthy human lymphoma cells supplied with the TUNEL kit. I) Cells under enhanced ROS (3 h after induction with 10 μmol L⁻¹ H₂O₂) displayed intense, localized incorporation of the TUNEL fluorescent label indicative of DNA fragmentation and a large population of 3’-hydroxyl ends.
desmum cell lysates inhibited DEVD cleavage by 87% at a 50 μmol L⁻¹ final inhibitor concentration.

In the contemporary open ocean, phosphorus and iron are often drawn down to extremely low concentrations during late-phase phytoplankton blooms, leading to physiological limitation of metabolic functions. Incubation of Trichodesmium IMS101 in phosphorus- and iron-free media resulted in elevated DEVD cleavage after 5 d, concomitant with physiological stress, as indicated by the dramatic decline in the maximum quantum yield of photosynthetic energy conversion efficiency (Fv/Fm) (Kolber et al. 1998) (Fig. 2A). DEVD cleavage remained very low under nutrient-replete conditions (Fig. 2A). Significant reductions in Fv/Fm also coincided with a dramatic increase in nuclease activity in stressed cultures compared to healthy control cells. One hallmark of PCD is a commitment to degradation of the dying cells’ genome by the activation of endonucleases. Cell lysates (containing in situ endonucleases) from nutrient-stressed cells collected after 6 d demonstrated enhanced degradation of linear pBK-CMV plasmid DNA. Little to no degradation was detected in nutrient-stressed cells in earlier time points or in control cells over the complete time course of the experiment (Fig. 2B).

We also examined whether natural populations of Trichodesmium activated caspase-like proteases in response to environmental stress. A time course of DEVD cleavage was measured in natural populations of Trichodesmium, which were collected in the subtropical western Pacific Ocean (off the coast of New Caledonia) and exposed to the high irradiance (HL) (≈450 μmol quanta m⁻² s⁻¹) commonly experienced by surface blooms of Trichodesmium. HL enhances the production of ROS in oxygenic photoautotrophs (Berman-Frank et al. 2001), which can induce PCD (Chandra et al. 2000). During early exposure to HL (<3 h), a large proportion of Trichodesmium cells, which were positively buoyant by virtue of intracellular gas vesicles (Walsby 1978) (indicative of healthy cells), displayed low DEVD cleavage (Fig. 3A,C). Increased exposure (>7 h) to HL resulted in increased DEVD cleavage, enhanced sinking, increased vacuolization (Fig. 3B,D–E), and the selective removal of cells with high caspase activity (Fig. 3F). Sinking trichomes had elevated (by a factor of 24 to 400) caspase-like specific ac-
Table 1. Conserved PCD domains that were detected in *Trichodesmium erythraeum* IMS101 protein sequences.

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<th>Gene</th>
<th>Length (a.a.)</th>
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‡ KEGG; http://www.genome.ad.jp.
tivity (17.1 RFU µg protein−1) compared to buoyant tri-
chomes (0.30 ± 0.22 RFU µg protein−1; range 0.04 to 0.70) and lysed after 24 h (Fig. 3E). Direct exposure of Tricho-
desium to ROS (10 µmol L−1 H2O2) induced an ordered
fragmentation of DNA, as revealed by positive TUNEL, charac-
teristic of PCD (Gavrieli et al. 1992) (Fig. 3I).

Using the Position-Specific Iterative BLAST (PSI-
BLAST) program, we identified several protein sequences in the T. erythraeum genome (http://www.ncbi.nlm.nih.gov) that are
homologous to the family of caspase-like proteases (“me-
tacaspases”). Metacaspase sequences have been identified in
plants, fungi, unicellular protozoa, and multiple bacterial
species, including Streptomyces, Rhizobium, Anabaena, Bor-
detella, Geosulfurococcus, Rhodosphaera, Dehalococcoides,
Xylella, and Synechocystis (Uren et al. 2000). Several de-
duced T. erythraeum protein sequences contained a caspase
domain structure (Domain identifier COG4249) (NCBI Con-
served Domain Database; http://www.ncbi.nlm.nih.gov) and
display sequence similarities to metacaspases in the unicel-
lar protists Saccharomyces cerevisiae (Yca-1) and Try-
panosoma brucei (Fig. 4), including the histidine- and cyste-
tine-containing catalytic diad found in true caspses (p20
subunit), paracaspases, and metacaspases (Uren et al. 2000).

Yeast Yca-1 has been shown to mediate PCD in aging yeast
cells and in response to H2O2 stress (Madeo et al. 2002),
and overexpression of the T. brucei metacaspase causes loss of
respiration competence and clonal death in yeast (Szallies et
al. 2002). Some of the metacaspase-like T. erythraeum se-
quences also contained WD-40 propeller domains (Table 1),
which are regulatory nodules for signal transduction. Or-
thologs were also identified for other key enzymes of the
eukaryotic PCD machinery, including apoptosis activating
factor (APAF-1), apoptotic ATPases (AP-APTases), NACHT
family NTPases, and mitochondrial HtrA-like proteases (Ta-
ble 1).

The demise of Trichodesmium via PCD with caspase-like
components and its retention in modern eukaryotic algae
(i.e., D. tertiolecta) indicates that it is an integral mortality
pathway in aquatic photoautotrophs with early origins. It also
provides another dimension to the current debate on the
 evolutionary origins and the role of PCD in unicellular and
prokaryotic organisms (Aravind et al. 1999; Ameisen 2002).
As these organisms have very different evolutionary histo-
dories (Delwich 2000), PCD components either evolved inde-
pendently in host cells prior to the appropriation of a plastid
or were derived from plastid inheritance of a cyanobacteri-
ial ancestor early in the endosymbiotic association. Trichodesmium
displays biochemical similarities to components of metazoan-
like PCD execution, but it evolved well before the first iden-
tifiable metazoans appear in the fossil record (Lipps 1993).
The origin of cyanobacteria is traced to ca. 3 Ga (Holland
and Rye 1997; Summons et al. 1999; Kasting and Siefert
2002), with clades closely related to Trichodesmium branch-
ing early in the diazotroph phylogenetic NifH tree (Zehr et
al. 1997). Furthermore, execution of PCD in Trichodesmium
appears to be distinct from controls of PCD in other bacteria
involving “addiction modules” (Ameisen 2002), providing
intriguing evolutionary context on the involvement of caspase-like proteases in PCD. Indeed, caspase-like cysteine
proteases may represent the initial, ancestral core of execu-
tioners that allowed the emergence of PCD and essential
effectors of the cell death machinery (Ameisen 2002).

In other bacteria, PCD is known to play an important role
in the developmental and differentiation processes as part of
an adaptation to environmental stress. Examples include
sporulation in Bacillus and Streptomyces, swarm cell
formation and differentiation in Caulobacter cresus, the
formation of multicellular fruiting bodies in Myxobacteria, het-
erocyst formation in Anabaena, the development of
bacteroids in Rhizobium, and transition to the viable but non-
cultural state in various Gram-negative bacteria (Hochman
1997; Lewis 2000). It is noteworthy that in some of these
bacteria, PCD pathways are independent of metacaspases.

An additional function of the PCD pathway in diazotroph-
ic cyanobacteria may be the formation of hormogonia, small
groups of cells that are released from a parent filament after
death and serve as dispersal and infective units in plant-
cyanobacterial symbioses. Hormogonia occur in several dia-
zotrophs (e.g., Nostoc) under unfavorable growth conditions.
In Trichodesmium populations, hormogonia are observed,
typically deep in the euphotic zone around or below the 1% light level after a large fraction of the trichomes has
decomposed (E. Carpenter pers. comm.). Thus, it is tempting to
speculate that PCD pathways in Trichodesmium may regu-
late differentiation of selected cells into hormogonia, pro-
viding an innocula for a new bloom upon improved envi-
ronmental conditions. In the dinoflagellate P. gatunense,
PCD leads to spore formation under limited CO2 availability
and oxidative stress (Vardi et al. 1999).

Induction of PCD in response to nutrient, light, salt, or
oxidative stress (Berges and Falkowski 1998; Vardi et al.
1999; Ning et al. 2002; Segovia et al. 2003) further indicates
that this mortality pathway operates in the modern ocean and
may facilitate biogeochemical cycling through transfer of or-
ganic and inorganic matter to heterotrophic microbial com-
unities. Although retention of a PCD pathway would ap-
ppear to exert negative selection pressure, there must be a
selective advantage based on phenotypic expression. Tricho-
desmium sp. IMS101 (this study), D. tertiolecta (Segovia et
al. 2003), and Emiliania huxleyi (Bidle un publish. data) dis-
play immunoreactivity to caspase-3 antisera in both healthy
and physiologically stressed cells, but immunoreactivity in-
creases under elevated physiological stress. This pattern of
expression indicates that these proteins may have additional
cellular roles, such as housekeeping or regulatory functions.
Under physiological stress, however, overexpression and
posttranslational processing may initiate catastrophic cell
death that appears to be maladaptive. Examination of the
activation pathways and ensuing processes in cyanobacteria
may provide critical insight into the evolution of PCD in
eukaryotic organisms.

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