Coupling between autocatalytic cell death and transparent exopolymeric particle production in the marine cyanobacterium *Trichodesmium*

Ilana Berman-Frank,1* Gad Rosenberg,1 Orly Levitan,1 Liti Haramaty2 and Xavier Mari3

1Mina and Everard Goodman Faculty of Life Sciences, Bar Ilan University, Ramat Gan, 52900, Israel.
2Environmental Biophysics and Molecular Ecology, Institute of Marine and Coastal Sciences, Rutgers University, 71 Dudley Road, New Brunswick, NJ 08901, USA.
3IRD, UR 103, Noumea Center, BP A5, NC-98848 Noumea, New Caledonia.

Summary

Extracellular polysaccharide aggregates, operationally defined as transparent exopolymeric particles (TEP), are recognized as an important conduit for carbon recycling and export in aquatic systems. Yet, the factors controlling the build-up of the TEP pool are not well characterized. Here we show that increased TEP production by *Trichodesmium*, an oceanic bloom-forming nitrogen-fixing (diazotrophic) cyanobacterium, is coupled with autocatalytic programmed cell death (PCD) process. We demonstrate that PCD induction, in both laboratory cultures and natural populations, is characterized by high caspase-like activity, correlates with enhanced TEP production, and occurs under iron and phosphorus starvation, as well as under high irradiance and oxidative stress. Enhanced TEP production was not observed in actively growing populations. We provide further evidence that iron is a key trigger for the induction of PCD. We demonstrate, for the first time, the concomitant enhanced build-up of the TEP pool when *Trichodesmium* is Fe-stressed. These results suggest a functional linkage between activation of caspases and PCD in *Trichodesmium* and regulation of vertical carbon and nitrogen fluxes. We hypothesize that modulation of TEP formation and its qualities by different mortality pathways could regulate the fate of phytoplankton blooms and particulate organic matter in aquatic ecosystems.

Transparent exopolymeric particles (TEP) (Allredge *et al.*, 1993) are increasingly recognized as an important component of carbon recycling and export in aquatic systems (Passow and Allredge, 1994; Mari and Burd, 1998; Mari, 1999; Passow, 2000; 2002; Engel *et al.*, 2004). The physicochemical properties, characteristics of distribution, and dynamics of TEP contribute significantly to trophic web structure and flux processes in the ocean (Passow, 2002). Transparent exopolymeric particle form from dissolved organic carbon released by phytoplankton (Passow, 2000) and, due to their high stickiness (Kiorboe and Hansen, 1993; Dam and Drapeau, 1995; Engel, 2000), can coagulate with other more conventional particles (e.g. bacteria, phytoplankton and detritus) to promote the formation of marine snow aggregates (Passow *et al.*, 2001). Therefore, TEP formation and accumulation in the euphotic zone may directly enhance the downward vertical flux of major elements, such as carbon, and of adsorbed trace elements (Passow, 2002; Engel *et al.*, 2004). Alternatively, when the TEP fraction is high, TEP-mediated aggregation can lead to a reduced settling velocity and even to an upward flux of substances due to the lower density of TEP compared with that of seawater (Passow, 2002; Engel *et al.*, 2004; Mari *et al.*, 2007).

While the role of TEP in regulating the fate of organic matter has been extensively addressed (see above references), the factors controlling the build-up of the TEP pool are not as clearly understood. Cyanobacterial blooms may serve as a significant source for the TEP pool via the production of large amounts of extracellular polymeric substances, consisting mainly of polysaccharides (Bertocchi *et al.*, 1990; Gloaguen *et al.*, 1995), which are known TEP precursors (Passow, 2000). Extracellular release and the production of TEP precursors may also increase under conditions of nutrient limitation coupled with sufficient light and carbon (Passow, 2002), leading to the accumulation of the TEP pool during bloom termination. While phytoplankton bloom formations are well studied, relatively few studies are available on the demise of blooms. Generally, grazers...
and viruses are considered as typical agents of phytoplankton death.

Recently, an autocatalytic programmed cell death (PCD) has been documented in several phytoplankton species, including bloom-forming species (Berges and Falkowski, 1998; Vardi et al., 1999; Ning et al., 2002; Segovia et al., 2003; Berman-Frank et al., 2004; Franklin and Berges, 2004). Well documented in metazoans and higher plants, PCD refers to a genetically controlled cell suicide resulting from a cascade of interacting biochemical processes that include specific receptors, adapters, signal-kinases, proteases and nuclear factors (Leist and Nicotera, 1997; Aravind et al., 1999). Autocatalytic PCD in prokaryotic and eukaryotic phytoplankton displays morphological, physiological, biochemical and genetic characteristics reminiscent of apoptotic pathways in metazoans and higher plants and is induced by oxidative, dark, temperature, nutrient or salt stresses (Berges and Falkowski, 1998; Vardi et al., 1999; Ning et al., 2002; Segovia et al., 2003; Berman-Frank et al., 2004; Franklin and Berges, 2004).

Autocatalytic PCD also exists and operates in the diazotrophic cyanobacterium Trichodesmium spp. (Berman-Frank et al., 2004). Trichodesmium spp. form extensive blooms in the tropical and subtropical oceans and provide a significant source of ‘new nitrogen’ to these oligotrophic environments (Capone et al., 1997; Capone, 2001). Bloom termination may be due to copepod grazing (O’Neil, 1998), viral lysis (Ohki, 1999), or via an autocatalytic PCD that is induced in response to environmentally relevant stresses such as high irradiance, P and Fe starvation, and oxidative stress (Berman-Frank et al., 2004).

Currently the nature and mechanistic controls of PCD in phytoplankton remain virtually unexplored even though it may have important consequences to ecosystem and biogeochemical dynamics. The objective of this study was to examine potential coupling between autocatalytic PCD in Trichodesmium and the production of TEP that may regulate the fate of organic matter.

Results and discussion

Autocatalytic PCD in Trichodesmium – a physiological response to nutrient and oxidative stress

Ageing, nutrient (P and Fe starvation) or high light (oxidative) stress induce a genetically controlled PCD in Trichodesmium (Berman-Frank et al., 2004). Reduced photosynthetic capacity, measured as a decline in the quantum yield of photosystem II ($F_{v}/F_{m}$), characterizes the early physiological response of Trichodesmium to nutrient stress (P and Fe starvation) with values falling from $0.48 \pm 0.14$ in the control cultures to $0.27 \pm 0.12$ and $0.17 \pm 0.02$ in the [-Fe–P]-depleted cultures respectively. The photophysiological response is eventually mirrored in the chlorophyll a concentrations which dramatically decline after 2 days in the [-Fe] and in the [-Fe–P]-depleted cultures (Fig. 1A). The stressed cultures activate an autocatalytic PCD which is characterized by morphological and biochemical changes such as degradation and increased vacuolization of internal cellular components, with no evidence of plasma membrane rupture as observed under accidental necrotic death (Berman-Frank et al., 2004).

A unique family of cysteine aspartate-specific proteases, caspases, is involved in the initiation and activation of PCD in metazoans ranging from Hydra to humans (Thornberry and Lazebnik, 1998). Caspase orthologues (metacaspases) with similar catalytic properties have been reported in higher plants, yeast, eukaryotic algae, various bacterial species (reviewed in Bidle and Falkowski, 2004), and recently, in Trichodesmium undergoing PCD (Berman-Frank et al., 2004). Although caspase activity may sometimes occur without causing death (Wright et al., 1999), caspase activity, measured by cleavage of the caspase-specific substrates, is routinely used as a hallmark feature of cells undergoing autocatalytic PCD (Kohler et al., 2002). Our previous work showed that in Trichodesmium a caspase-like protein is indeed involved during PCD. This was evidenced by cross-hybridization to caspase-3, -8 and -9 antibodies, increased caspase-like activity with PCD induction, and the lack of activity when applying caspase-specific inhibitors (Berman-Frank et al., 2004). Here, we utilized this caspase-like activity, measured by cleavage of the caspase-specific substrate DEVD, as the cellular indicator of autocatalytic PCD (Figs 1–3). Healthy actively growing cultures show very little DEVD cleavage (Fig. 1B). Depletion of either Fe or both Fe and P in the medium caused a substantial increase in DEVD cleavage (Fig. 1B).

High DEVD cleavage was correlated with a large increase in TEP production again when either Fe or Fe and P were depleted (Fig. 1C). Transparent exopolymeric particle production was low in, nutrient-replete, healthy growing cells (Fig. 1C). One-way ANOVA and Tamahane Post Hoc analyses showed significant differences between treatments (d.f. = 3,9; $F = 25.4$, $P < 0.001$) with significant differences found between the controls and [-Fe] and [-Fe–P] ($P < 0.0001$). Compilation of independent experiments showed a significant positive correlation between TEP volume and DEVD cleavage representing caspase-like activity in PCD-induced cells ($r^2 = 0.66$, $n = 22$, $P < 0.05$) (Fig. 2).
Dynamics of PCD induction and TEP production under oxidative stress

The time scale for enhanced TEP production coincided with an increase in DEVD cleavage (Fig. 3). When natural populations were artificially exposed to high irradiance and, thus, to oxidative stress, both DEVD cleavage and TEP production were induced and increased from ~3 to 12 h after exposure (Fig. 3A), with instantaneous TEP and caspase-like production rates peaking at ~8 h after exposure (Fig. 3B).

Exposure to stress also resulted in modified characteristics of the TEP pool, as shown by the alteration of the spectral slope, \( \delta \). The observed increase of \( \delta \) from ~4 to 17 h (Fig. 3A) derived from an increase of the large TEP fraction [i.e. > 20 \( \mu \)m equivalent spherical diameter (ESD)]. Microscopic observations revealed that most large TEP originated from the detachment of the polysaccharide capsule from Trichodesmium cells after PCD induction (Figs 3B and 4). This alternative mechanism of TEP production resulted in the massive release of long ‘sock’-shaped TEP in the medium after instantaneous TEP production and caspase-like activity peaked (Fig. 4C). After release of these ‘socks’ TEP can be degraded and smaller TEP particles were observed when biomass mortality increased and caspase-like activity declined (Figs 3 and 4D). As TEP are usually produced by coagulation of TEP precursors of colloidal size (Passow, 2000), the TEP pool is thought to take its roots in the dissolved phase. Our
study showed that under specific circumstances TEP may also be produced directly as large particles (Fig. 4) and corresponds to a study from Lake Kinneret (Israel) showing that much of the TEP originates from bits and pieces of algae and from mucous-like excretions around bacteria and algae (Berman and Viner-Mozzini, 2001).

Contribution of the TEP pool to total particulate organic carbon during bloom demise induced by nutrient stress

In *Trichodesmium*, the contribution of the TEP pool to total particulate organic carbon (POC) varies as a function of nutrient stress (Table 1). When cells were P-limited, TEP comprised ~10% of the POC pool. Under Fe starvation TEP production increased drastically; eventually comprising almost the total pool of POC. A dual P and Fe starvation led to an intermediate state in which TEP constituted ~45% of the POC pool. Despite the uncertainties associated with the TEP size versus carbon content relationship, our experiments demonstrate that, on a similar time scale, varying nutrient stresses influence cellular growth and mortality responses differently. In the nutrient-depletion experiments, P depletion caused an increase in POC compared with the control but this was not mirrored in TEP carbon (TEP-C) or in DEVD cleavage as was the case when Fe was depleted. This may be due to the availability of intracellular stores of phosphorus in the form of phospholipid granules (Janson et al., 1995) reflected in flexible N : P stoichiometry (Krauk et al., 2006; White et al., 2006) or in *Trichodesmium*’s ability to utilize phosphonates (Dyhrman et al., 2006). In contrast, Fe is a crucial limiting nutrient for *Trichodesmium* with Fe starvation leading to both PCD induction and massive TEP production (Fig. 1). This is consistent with the high intracellular quotas of Fe required for both nitrogen-fixation and photosynthetic pathways in diazotrophs and especially for *Trichodesmium* (Reuter, 1988; Berman-Frank et al., 2001; Kustka et al., 2003; Tuit et al., 2004). Thus, we would expect that in oceanic areas with limited bioavailability of Fe, Fe would regulate not only N2 fixation, primary production, and growth of *Trichodesmium* (Reuter, 1988; Falkowski, 1997; Berman-Frank et al.,

© 2007 The Authors
but also the mortality pathway and TEP production.

Implications for Trichodesmium and for the fate of organic matter produced during Trichodesmium blooms

In the oceans, Trichodesmium blooms are frequently observed on the surface layer and regulate their position in the water column by modification of their gas vesicles (Walsby, 1978). One of the morphological characteristics associated with autocatalytic PCD in Trichodesmium is loss of gas vesicles required for flotation, with the population dividing into a large sinking fraction of cells and a smaller fraction of trichomes that remain suspended in the water column (Berman-Frank et al., 2004). These natural populations from New Caledonia are described here in the sphere experiment (Figs 2 and 3). In these populations, sinking trichomes had elevated (by a factor of 24–400) DEVD cleavage (17.1 RFU mg protein⁻¹) compared with buoyant trichomes (0.30 ± 0.22 RFU µg protein⁻¹; range 0.04–0.70) and lysed after 24 h. Transparent exopolymeric particle production was associated with high DEVD cleavage (i.e. cells undergoing PCD) and thus sinking cells (Figs 2 and 3).

Positive coupling between PCD and TEP production may have important implications for the fate of organic matter produced during Trichodesmium blooms. The build-up of the TEP pool enhances particle aggregation by increasing both the apparent stickiness of particles and the collision frequency, thus, modifying the vertical flux of substances (Passow et al., 2001; Engel et al., 2004). The TEP-mediated formation of large aggregates may either enhance or retard the downward flux of matter depending on the density of aggregates compared with seawater. Whereas TEP-mediated aggregates are assumed to increase rates of sedimentation from the surface, a relative increase of TEP concentration within aggregates may prolong their residence time in the euphotic layer (All-dredge and Crocker, 1995; Mari et al., 2007), and even create an upward flux of substances (Azetsu-Scott and Niven, 2005). In our sphere experiments, using natural populations, the positive coupling between cells undergo-

Table 1. Contribution of the TEP-C pool to total POC during nutrient starvation experiment, in the mother culture (T0) and in the four treatments after 1 week incubation (T1).

<table>
<thead>
<tr>
<th>Treatments</th>
<th>T0</th>
<th>Control</th>
<th>[-P]</th>
<th>[-Fe]</th>
<th>[-P–Fe]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured POC (µg ml⁻¹)</td>
<td>9.4</td>
<td>6.43 ± 1.19</td>
<td>13.83 ± 3.45</td>
<td>4.03 ± 1.54</td>
<td>8.21 ± 1.31</td>
</tr>
<tr>
<td>Estimated TEP-C (µg ml⁻¹)</td>
<td>0.44</td>
<td>2.54</td>
<td>1.64</td>
<td>4.88</td>
<td>3.74</td>
</tr>
<tr>
<td>Contribution of TEP to total POC (%)</td>
<td>4.7</td>
<td>39.5</td>
<td>11.9</td>
<td>121.1</td>
<td>45.6</td>
</tr>
</tbody>
</table>

Fₕ/Fm and POC concentrations are average values (± standard deviations, n = 3). Calculation of TEP-C concentrations from the TEP size spectra does not permit calculation of error estimates, as they are individually derived from a single size spectrum.

© 2007 The Authors
Journal compilation © 2007 Society for Applied Microbiology and Blackwell Publishing Ltd, Environmental Microbiology, 9, 1415–1422
ing PCD and TEP production (Fig. 3) was observed predominantly in the sinking fraction of cells (Berman-Frank et al., 2004) suggesting higher sedimentation rates for cells producing high TEP.

Our study provides further evidence that iron is a key trigger for the induction of PCD and, demonstrates for the first time, the concomitant enhanced build-up of the TEP pool when *Trichodesmium* is starved for Fe. We suggest that bloom demise catalysed by Fe depletion would be characterized by a TEP-dominated standing stock of POC, resulting in an enhanced downward flux of organic matter. Therefore, the global contribution of *Trichodesmium* blooms to vertical export of material may be to increase the downward flux when Fe flux to the oceans, and subsequent Fe availability to *Trichodesmium*, is low (via the PCD-high TEP production pathway). Alternatively, high Fe flux to the oceans may promote the formation of high-biomass, low-TEP forming *Trichodesmium* blooms (more likely terminating via necrotic low-TEP pathways such as grazing or viral lysis). These may promote recycling in the surface oceans. We do not currently know the chemical and buoyancy characteristics of *Trichodesmium*-produced TEP and whether these features change in populations undergoing PCD or necrotic death. Moreover, we do not know how the myriad associated populations found within *Trichodesmium* colonies and blooms (Nausch, 1996; Sheridan et al., 2002) utilize and/or influence the formed TEP or the process of PCD. Extensive research is required to test these hypotheses on *Trichodesmium* especially for natural populations under bloom conditions.

**Experimental procedures**

*Trichodesmium* source and stress conditions

*Trichodesmium* IMS101 cultures in YBC II medium (Chen et al., 1996) were grown for three independent experiments (different mother cultures) in two laboratories at 25°C to 26°C with a 12:12 light/dark cycle at ~85 μmol quanta m⁻² s⁻¹ and constant aeration. Experimental treatments were initial stock inoculates (identified as T0); control (5 × 10⁻⁶ M KH₂PO₄; 4 × 10⁻⁷ M FeCl₃); P-depleted conditions (YBC II with no addition of phosphate); Fe-depleted conditions (YBC II with no addition of iron) and in P and Fe-depleted conditions (YBC II with no addition of either phosphate or iron). Phosphorus, iron and dual starvations are identified as [–P], [–Fe] and [–P–Fe]. Stock cultures were transferred to two replicate bottles of each treatment by gravity filtration and several washes in the sterilized medium of the respective treatments. Biomass was followed in the respective treatments until it crashed (several days and up to a week in each treatment).

Natural *Trichodesmium* populations, predominantly *T. erythraeum*, were collected using 35 μm net tows from surface waters off Noumea (New Caledonia) during December 2002. To isolate *Trichodesmium*, tows were size fractionated and zooplankton was separated from *Trichodesmium* by phototaxis. *Trichodesmium* were hand-picked, placed in a separating flask in filtered seawater (~0.2 μm), irradiated with ~450 μmol quanta m⁻² s⁻¹ and populations were followed with time until the biomass crashed. Experimental data presented here augment the experiments and populations described in Berman-Frank and colleagues (2004) with TEP production measured on the same populations.

**Measurement of caspase-like activity**

Cells filtered on 5 μm Nucleopore filters were frozen in a buffer containing 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10% sucrose, 500 μM ethylenediaminetetraacetic acid and 10 mM dithiothreitol. After sonication, the extract was incubated with 50 μM Z-DEVD-APC (Calbiochem) for 24 h at 26°C. Fluorescence was read in a Spectra Max Gemini XS plate reader (excitation 400 nm, emission 505 nm).

**Photosynthetic efficiency**

Fast repetition rate fluorometer measurements of fluorescence kinetics were used to derive the maximum photochemical quantum yield of photosystem II (Fₚ/Fₘ), a measure of photosynthetic efficiency and physiological status of the cells (Kolber et al., 1998).

**Determination of TEP**

Transparent exopolymeric particles were stained with Alcian Blue (Alldredge et al., 1993) and TEP size spectra were determined from 1 to 5 ml samples filtered onto 0.2 μm polycarbonate filters, after transfer of the particles retained on a microscope slide (Passow and Alldredge, 1994). For each slide, TEP size spectra were determined by counting and sizing TEP at successive magnifications using a compound light microscope (Mari and Burd, 1998). Ten images were taken per slide and for each magnification and the TEP size spectra were compiled by combining the size distributions obtained at each magnification. The ESD of each TEP was calculated by measuring of its cross-sectional area with a semiautomatic image-analysis system (ImagePro Plus, MediaCybernetics). Counts were combined and classified according to their ESD. Transparent exopolymeric particle size distributions were described using a power relation of the type dN/d(dₜ) = k dₜ⁻₅, where dₜ is the ESD and dN is the number of particles per unit volume in the size range dₜ to [dₜ + dₜ⁺]. The spectral slope, δ, describes the size distribution and was estimated from regressions of log[dN/d(dₜ)] versus log[dₜ].

**Transparent exopolymeric particle carbon concentration**

Transparent exopolymeric particle size spectra were used along with the well-defined carbon–size relationship TEP-C = 0.25 ²₁⁸ (pg C TEP⁻¹), where TEP-C (pg C) is the carbon content of a given TEP particle with a radius r (μm) (Mari, 1999), to estimate the size of the TEP-C pool. This
approach provides only rough estimates of the size of the TEP-C pool because this relationship was determined for TEP produced from exudates of organisms other than Trichodesmium (i.e., diatoms). Different sources of TEP are likely to result in TEP with varying carbon contents. As the above relationship is the only tool so far available in the literature allowing the estimation of TEP-C concentration from the size spectra, it was used in the present study.

Particulate organic carbon concentration

Samples for POC measurements were immediately filtered onto 25 mm Whatman GF/F filters (nominal pore size = 0.7 μm) pre-combusted at 550°C for 2 h. After filtration the filters were dried at 60°C for 24 h and then frozen for later analysis on a CHN-analyser.

Comparison of TEP production and caspase-like enzymatic activity

In order to compare enzymatic activity detected from DEVD cleavage, with TEP-C concentration at a given time we compare production rates. During the time-series oxidative stress experiment, such estimates of TEP-C and caspase-like production rates can be directly obtained by calculating the derivatives of the TEP-C and DEVD cleavage versus time plots (Fig. 3). The derivatives obtained have an informative value and provide a conceptual representation of the correlation between TEP-C and caspase-like production rates.

Acknowledgements

We thank A. Le Bouteiller and C. Dupouy for providing assistance during the set-up of the experiments in New Caledonia. This research was supported by IRD, by the PROOF-DIAPAZON program (JGOFS-France). Support for L.H. was provided by the National Science Foundation in a Biocomplexity Grant to P.G. Falkowski.

References


Kiorboe, T., and Hansen, J.L.S. (1993) Phytoplankton aggregation – observations of patterns and mechanisms...


